UNDERSTANDING THE EFFECTS OF β-CASEIN PHENOTYPE ON THE COMPOSITION AND QUALITY OF MILK AND DAIRY PRODUCTS

Davor Daniloski

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PHD THESIS 2024 · Davor Daniloski

Abstract

Bovine milk is composed of milk proteins, with two major groups, caseins and whey proteins. One type of casein, β-casein, comes predominately in two genetic forms, known as β-caseins A2 and A1. The difference between these two variants appeared due to a single nucleotide polymorphism on the sixth chromosome of the CSN2 gene (essential for coding of β-casein) with the inclusion of either proline in β-casein A2 or histidine in β-casein A1 at position 67 in the peptide chain. There has been significant attention on the health implications of consuming milk containing β-casein A1, everything from links to detrimental health issues, none of which have been fully substantiated, but nevertheless, it has created a drive in some markets towards promoting A2/A2 milk. However, the fact that milk is now segregated for certain markets based on β-casein phenotype means that from a milk functionality perspective, there may be implications on product functionality.

While the aim of the study is not focused on answering the health question, it is intended to address the issue of diversifying of β-casein as a function of cow genotypes and various processing conditions commonly applied in the dairy industry. The outcomes shed new light on discovering the effects of protein phenotype and gene polymorphism on protein structure and chemical composition, and consequently on the conformational, functional, and *in vitro* digestion properties of milk and dairy products with known β-casein phenotype. Therefore, the aim of this study is to assist and provide knowledge to the dairy industry on the possible impacts of changing national milk pools to the β-casein A2 variant. To improve our understanding of the structural and functional properties of casein, milk, and dairy products and how they are influenced by various processing parameters, A1/A1, A1/A2 and A2/A2 samples were studied simultaneously by utilising *in-situ* spectroscopic techniques, such as Fourier Transform Infrared, Nuclear Magnetic Resonance and Raman spectroscopy, supported by numerous physicochemical, imaging, chromatographic techniques and analysis and *in vitro* digestion patterns.

The developed method successfully distinguished between temperature and pH in unheated and heat-treated β-casein A2 and A1 milk groups, revealing that a minimum of 50 % of all structural variation between the milk samples could be attributed to the β-casein variant. From a technological perspective, it was found that A1/A1 and A1/A2 milks had significantly different heat coagulation properties to A2/A2 milk, which was less heat stable. Differences were also observed between β-casein variants in acid- and rennet-induced gels, as well as, their

counterpart products, i.e., yoghurt and cheese, respectively. It was found that the onset of gelation was faster in A1/A1 and A1/A2 milks compared to those from A2/A2 milk. Several reasons may account for the differences in these milks processability, including greater level of polyproline II helixes, larger casein micelle size, and lower total calcium, κ-casein amounts in A2/A2 samples compared to A1/A1 and A1/A2 samples that were comprised mainly of α-helical motifs.

Although the greater gel strength observed in A1/A1 and A1/A2 milks may positively affect the techno-functional characteristics of yoghurt or cheese, it may also alter the curd formation properties during the gastric phase of digestion. Interestingly, the gastric digestion of these milks and dairy products showed significant differences with faster gastric digestion occurring in A1/A1 and A1/A2 samples compared to A2/A2 milk. This work suggests that the gastric transit of dairy products carrying β-casein A1 is more rapid, compared to A2/A2 samples and may have impacts in terms of product digestibility.

This study has clearly identified that milk and dairy products with β-casein A2 and A1 variants are quite different, based on their structure, functionality, and behaviour to environmental factors. Overall, the findings from this project to date, have revealed significant implications if there was an initiative to change the national dairy herds to the A2/A2 phenotype, specifically impacting dairy processors and their products. However, there are both advantages and disadvantages to using A2/A2 milk only and must be assessed on an individual basis by the dairy companies.

Student Declaration

I, Davor Daniloski, declare that the PhD thesis entitled **"Understanding the effects of β-casein phenotype on the composition and quality of milk and dairy products"** is no more than 100.000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references, and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

"I have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University's Higher Degree by Research Policy and Procedures".

Signature: [redacted]

Date: **17 March 2024**

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Audaces Fortuna Juvat

Details of included papers: Thesis with publication

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This table must be incorporated in the thesis before the Table of Contents.

Chapter No.	**Publication Title**	**Publication Status** • Published • Accepted for publication • In revised and resubmit stage • Under review • Manuscript ready for submission	**Publication Details** • Citation, if published • Title, Journal, Date of acceptance letter and Corresponding editor's email address • Title, Journal, Date of submission
1	**Introduction (Mandatory part of the Thesis)**		
Literature Review			
2	What is the impact of amino acid mutations in the primary structure of caseins on the composition and functionality of milk and dairy products?	**Published**	https://doi.org/10.1016/j.crfs.2022.09.026
Main findings: Part 1			
3	Casein micelle with different β-casein phenotypes: Fingerprinting pH-induced structural changes using FTIR and NMR spectroscopies.	**Published**	https://doi.org/10.1016/j.foodhyd.2023.108881
4	Conformational and physicochemical characteristics of bovine skim milk obtained from cows with different genetic variants of β-casein.	**Published**	https://doi.org/10.1016/j.foodhyd.2021.107186

5	Impact of heating on the properties of A1/A1, A1/A2, and A2/A2 β-casein milk phenotypes.	**Published**	https://doi.org/10.1016/j.foodhyd.2022.107604
6	Authentication of β-casein milk phenotypes using FTIR spectroscopy.	**Published**	https://doi.org/10.1016/j.idairyj.2022.105350
7	Properties of sodium caseinate as affected by the β-casein phenotypes**.**	**Published**	https://doi.org/10.1016/j.jcis.2022.07.021
8	Rheological and structural properties of acid-induced milk gels as a function of β-casein phenotype.	**Published**	https://doi.org/10.1016/j.foodhyd.2022.107846
Main findings: Part 2			
9	Health-related outcomes of genetic polymorphism of bovine β-casein variants: A systematic review of randomised controlled trials.	**Published**	https://doi.org/10.1016/j.tifs.2021.02.073
10	Impact of β-casein phenotype on the physical properties of skim milk powders and their subsequent digestion characteristics.	**Published**	https://doi.org/10.1016/j.foodhyd.2024.109918
11	Physicochemical and simulated gastric digestion properties of A1/A1, A1/A2 and A2/A2 yoghurts.	**Under review**	Submitted and under review in one of the Food Science journals (Q1)
12	Cheddar cheese matrix and *in vitro* semi-dynamic gastric digestion: The role of β-casein phenotype.	**Under review**	Submitted and under review in one of the Food Science journals (Q1)
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Figure 4. SDS-PAGE patterns under reducing conditions of oral and GE 1 - 4 phases obtained during the gastric digestion at different times. BSA (Bovine Serum Albumin); Igs (Immunoglobulins); β-Lg (β-lactoglobulin), α-lactalbumin (α-La). A1/A1 (blue star), A1/A2 (green square), and A2/A2 (purple triangle).

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Figure 1. A) RP-HPLC chromatographic profiles used for identification of different milk samples (1 = κ-casein A/A; 2 = αs_2-casein A/A; 3 = αs_1-casein B/B; 4 = β-casein [A1/A1, A1/A2, and A2/A2]; 5 = α-lactalbumin; 6 = β-lactoglobulin A/B). B) Elastic modulus (*G'*) as a function of time during coagulation of cheese milks. C) Loss tangent (*tan δ*) as a function of time during coagulation of cheese milks. Control (orange hexagon), A1/A1 (blue star), A1/A2 (green square), and A2/A2 (purple triangle) cheese milks. Data shown are average values of data from three collections. Error bars represent standard deviation.

Figure 2. Cheese texture: A) hardness; B) fractuability; C) springiness; D) chewiness; E) cohesiveness at different ripening times. Control Cheddar cheese (orange Hexagon); A1/A1 Cheddar cheese (blue star); A1/A2 Cheddar cheese (green square); A2/A2 Cheddar cheese (purple triangle). Error bars represent standard deviation.

Figure 3. A) SEM and B) CLSM micrographs of Cheddar-type cheeses during ripening times. Control Cheddar cheese (orange Hexagon); A1/A1 Cheddar cheese (blue star); A1/A2 Cheddar cheese (green square); A2/A2 Cheddar cheese (purple triangle). Scale bar for SEM is 100 μm (magnification = 200 X) and for CLSM is 75 μm.

Figure 4. A) Change in pH of the Cheddar cheese during gastric digestion. Error bars represent standard deviation. B) CLMS micrographs of the clots obtained during the gastric digestion of cheeses at different times, scale bars represent 250 μm. Control Cheddar cheese (orange Hexagon); A1/A1 Cheddar cheese (blue star); A1/A2 Cheddar cheese (green square); A2/A2 Cheddar cheese (purple triangle).

Figure 5. SDS-PAGE patterns under reducing conditions of oral and GE 1 - 4 phases obtained during the gastric digestion of Cheddar cheeses at different times. BSA (Bovine Serum Albumin); Igs (Immunoglobulins); β-Lg (β-lactoglobulin), α-lactalbumin (α-La). Control Cheddar cheese (orange Hexagon); A1/A1 Cheddar cheese (blue star); A1/A2 Cheddar cheese (green square); A2/A2 Cheddar cheese (purple triangle).

Chapter 13.

Figure 1. A flow chart outlining the article search and inclusion process.

Figure 2. The chemical structure of the bovine β-casomorphins (BCMs) included in this study.

Figure 3. Overview and flow diagram of the static and semi-dynamic *in vitro* and *ex vivo* digestion procedures proposed by COST INFOGEST. SSF (Simulated Salivary Fluid), SGF (Simulated Gastric Fluid), and SIF (Simulated Intestinal Fluid). Adapted in part from Asledottir et al. (2017); Asledottir et al. (2018); Brodkorb et al. (2019); Minekus et al. (2014); Mulet-Cabero et al. (2020a).

Figure 4. A schematic model of possible scenarios in which gut microbiota-produced DPP-4, membrane-bound DPP-4 (enterocytes) and serum DPP-4 (endothelial cells) might influence human health during proteolysis of β-casomorphins.

Figure 5. Transport and bioactivity β-casomorphins. The blood-gut-brain axis and β-casomorphins (BCMs) liberation and transport. Transient receptor potential cation channel subfamily V member 1 (TRPV1); Dipeptydil peptidase - 4 (DPP-4); Myeloperoxidase (MPO); Potassium (K^+), Calcium (Ca^{2+}), Sodium (Na^{2+}) channels; Adenylyl cyclase (AC); Adenosine triphosphate (ATP); Cyclic adenosine monophosphate (cAMP); Nuclear Factor NF-κB (NF-kB); Protein kinase A (PKA); G-protein coupled receptors (GPCRs: α, β, and γ subunits); Peptide transporters (PEPT1). Adapted in part from Kodukula and Zeng (2018); Listos et al. (2019); Tyagi et al. (2020).

Chapter 14. Not applicable

List of Abbreviations

AA = Amino acid

α = Alpha

α-La = α-Lactalbumin

β = Beta

β-CN = β-casein

β-Lg = β-Lactoglobulin

BCM = β-casomorphin

˚C = Degree Celsius

CCP = Colloidal calcium phosphate

CN = Casein

CN micelle = Casein micelle

CLSM = Confocal Laser Scanning Microscopy

FAA = Free amino acids

FTIR = Fourier Transform Infrared Spectroscopy

GI = Gastrointestinal

GIT = Gastrointestinal tract

GE = Gastric empting

h = Hour

His = Histidine

κ = kappa

MCP = Micellar calcium phosphate

N = Nitrogen

NaCN = sodium caseinate

nM = Nanometre

NPN = Non protein nitrogen

NMR = Nuclear Magnetic Resonance Spectroscopy

PAGE = Polyacrylamide Gel Electrophoresis

Pro = Proline

SA = Serum albumin

SSF = Simulated saliva fluid

SGF = Simulated gastric fluid

SDS = Sodium Dodecyl Sulphate

SEM = Scanning Electron Microscopy

TEM = Transmission Electron Microscopy

min = Minute

mL = Millilitre

mM = Millimolar

MPa = Mega pascal

MW = Molecular weight

V = Volts

v/v = Volume per volume

w/w = Weight per weight

W = Watt

Introduction to the thesis. Chapter 1.

1.1. Background

Bovine milk is an excellent source of nutrients, containing relatively high levels of proteins, lipids, carbohydrates, and minor components, such as minerals and vitamins, making it widely used in human nutrition (McSweeney & Fox, 2013). Milk protein can be mainly divided in two fractions, caseins, and whey proteins, present at a ratio of approximately 80:20 in mature bovine milk. The casein group of proteins can be further classified in four different types, namely αs_1-, αs_2-, β-, and κ-caseins, in a ratio of 4:1:4:1, respectively (Bijl, Holland, & Boland, 2020). Approximately 40 % of the total casein and one third of the total protein content in bovine milk is β-casein. However, β-casein can be further sub-divided according to the changes in its amino acid composition, which is encoded by the CSN2 gene on the chromosome 6 of the polypeptide chain of β-casein (Aschaffenburg, 1961). Based on the amino acid substitution arising from the cleavage site in the polypeptide chain, twelve to seventeen different genetic variants of β-casein exist, with variants A1 (histidine^{67}) and A2 (proline^{67}) being the most abundant in modern cattle, which frequency depends on cow genetics (Daniloski, McCarthy, Huppertz, & Vasiljevic, 2022). The distinction between these two forms is a mutation in the amino acid polypeptide chain at position 67 (Daniloski, Markoska, McCarthy, & Vasiljevic, 2023). Despite the mixture of many other major and minor proteins, macro- and micro-molecules, when the milk carries a homozygous β-casein A1, it is called A1/A1 milk; A2/A2 milk is comprised of β-casein A2; and the mixture of both proteins, normally refers to A1/A2 or conventional milk (Daniloski et al., 2022).

Although nearly identical proteins, the difference of one amino acid in their structure, has opened a scientific debate, questioning if conventional bulk milk is "Friend" or "Foe". In the early years of the 21st century, an agricultural scientist, Professor Keith Woodford, postulated that the production methods had little to do with the health outcome of milk and dairy products (Woodford, 2009). Nevertheless, the study claimed that the presence of β-casein A1 in conventional milk is the issue of many non-communicable diseases, including cardiovascular, neurological disorders, diabetes, to name a few (Woodford, 2009). Therefore, during the last decade, there has been significant interest and research on the health implications of consuming conventional versus A2/A2 milks (Deth, Clarke, Ni, & Trivedi, 2015; Ho, Woodford, Kukuljan, & Pal, 2014; Jianqin et al., 2016; Milan et al., 2020). In this regard, a recent systematic review, concluded that a link between β-casein (A1 or A2) and human health could not be established, but did not rule out either (Daniloski et al., 2021); in agreement with past reports from the European and the New Zealand Food Safety Authorities (De Noni et al., 2009;

Swinburn, 2004). Since the above-mentioned studies have focused on the effects of β-casein A1 and A2 on animal and human health using *in vitro, ex vivo,* and *in vivo* trials, less focus was directed towards structural and techno-functional properties of β-casein phenotypes on dairy products. Therefore, the main purpose of this thesis is to address and establish the dairy matrix affected by the casein genotype that will directly or indirectly influence the composition and quality of milk and dairy commodities and products.

1.2. Research aims and objectives

The primary aim of this study was to address a gap in knowledge by creating the links between a type of milk carrying either β-caseins A1/A1, A1/A2 or A2/A2, their structural and techno-functional properties, and consequently *in vitro* gastric digestion patterns on these commodities.

The specific objectives were:

- Determination of conformational and functional differences among casein micelles with specific β-caseins. Particularly, spectral and nuclear magnetic methods were employed to provide a quantitative assessment and differentiate between phenotypes that were consequently linked to their physicochemical functionalities.

- Developing a novel and rapid method to identify milk samples based on their β-casein genetic profile. Structural, physical, and chemical properties of A1/A1, A1/A2, and A2/A2 milks treated under different conditions (pH and temperature) were established, milk ingredients were produced, and the properties of acid and rennet gels manufactured from the above-mentioned milks were evaluated.

- Quantification and determination of the effects of β-casein phenotype on dairy product manufacture. Three different types of products were assessed including liquid milk, fermented dairy products (yoghurt and cheese) and milk powders.

- The fate of A1/A1, A1/A2, and A2/A2 dairy products upon semi-dynamic *in vitro* gastric digestion was established.

1.3. Thesis outline

The thesis is organised in fourteen chapters sub-divided into four sections: Review of the literature; Main findings: Part I; Main findings: Part II; and Future aspects of the study. Chapter 1 presents the introduction of the thesis, including, study background, aims, objectives, and outline of the thesis. Chapter 2 outlines the critical review of the literature that relates to the study with focus on the core research findings and fundamental concepts. Chapters 3 to 8 provide information of the structural transition and physicochemical properties of the casein micelle, milk, milk ingredients, and milk gels examined and obtained under defined conditions. In Chapters 9 to 12, the impact of β-casein phenotype on the physical properties of skim milk powders, yoghurts, and cheeses was examined, including their subsequent gastric digestion characteristics. The penultimate Chapter 13, for the first time in the literature, reveals the gastrointestinal and the blood serum pathway of a liberated group of peptides from A1/A1, A1/A2, or A2/A2 dairy products, known as β-casomorphins, which gives an indication of our future work in the field. Notably, Chapters 9 and 13 are systematic and comprehensive review papers, respectively. The final Chapter 14 provides conclusions of the entire work performed in this project and scope for future research.

Let me now explain the reason behind the decision to choose these important objectives to be part of the thesis. Milk and milk goods are manufactured under various conditions (temperature and pH control or introduction of additives) in the dairy industry in order to produce safe and nutritious food with a long shelf life. Moreover, these processing conditions are important not only for the reduction of bacterial growth in milk but also for the stabilisation of milk proteins (Deeth, 2022; McMahon & Sharma, 2022; McSweeney & Fox, 2013; Singh & Li, 2022). The current research helped uncover critical areas in milk quality that research had not yet investigated. Those areas established the differences in traits of importance to milk processing, including the production of dairy commodities from bovine milk with different β-casein phenotypes. Consequently, a new theory on the structure-functionality of caseins, milk and dairy products was revealed. Finally, none of the previous studies have linked the effect of the dairy matrices of milk and dairy products carrying various β-casein phenotypes on their gastric digestion under *in vitro* digestion conditions. It was essential to be clarified, to what extent the structure of the dairy products, but also the processing technologies mentioned here, contributed to the altered gastric digestibility of either A1/A1, A1/A2, or A2/A2 milks and dairy commodities. Also, it provided information to primary milk producers, and the

subsequent consequences to dairy processors, if a change to A2/A2 milk ever occurred in the national dairy herd.

The expected benefits and disadvantages were adequately and realistically described in terms of scientific and industry impact.

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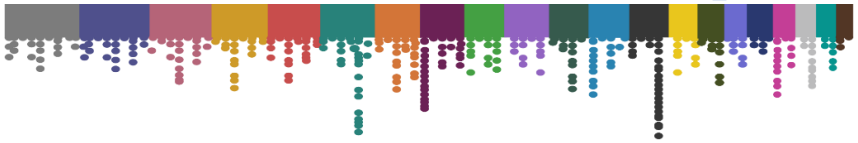
Literature Review: Part I. Chapter 2.



Bovine milk: General insights

1. Bovine milk composition

Bovine milk, commonly known as cow's milk, is a nutritious fluid produced by the mammary glands. It is composed of water, fat, protein, lactose, and mineral compounds, but also some minor components, such as organic acids, enzymes, and a range of vitamins, making milk a complete source of highly valuable nutrients for the calf (McSweeney & Fox, 2013). The composition of milk varies between individual animals, breeds, seasons, feed, stages of lactation and animal's health (O'Mahony & Fox, 2014). The composition of cows' milk is important for the dairy industry, affecting its nutritive value as well as its processability. Milk structure is particularly dependent on the existence of colloidal lipids in the form of fat globules (fat droplets stabilised by mixture of lipoproteins and bilayer structures), protein in the form of casein micelles, sterically stabilised by κ-casein, and whey proteins as oligomers (Fox, 2008).

Table 1. General bovine milk composition (Goulding, Fox, & O'Mahony, 2020).

Component	%
Total solids	12.7
Fat	3.7
Protein	3.5
Casein	2.8
Whey proteins	0.7
Lactose	4.8
Ash	0.7

1.1. Water

Most of the milk's composition is water (~ 87 %) and in milk it can be free and bound. Free water is also a solvent of organic and inorganic substances in milk, minerals, acids, and milk sugars. In contrast, the water by form, strength and durability of bonding to the milk components may be bonded in three ways, such as chemical bond, physicochemical bond, and physical bond. The water is also a major component in milk to meet the hydration needs of the neonate. It also controls the speed of many reactions, including lipid oxidation, enzyme activity and microbial growth. These reactions affect the stability of milk and dairy products (Goulding et al., 2020).

1.2. Lactose and other sugars

Bovine milk is as an excellent source of lactose which is renowned as a basic carbohydrate in the milk. It is a disaccharide composed of galactose and glucose linked to a β-1-4 glycoside bond and its concentration proportionally depends on the concentration of lipids and casein in the milk. The main role of lactose along with lipids in milk is an energy source. Additionally, lactose is known as the main determinant of the osmotic pressure of milk and drives the milk yield of cows (Fox, Mcsweeney, & Paul, 1998). The process of milking above 100 °C changes the colour of the milk to a tan (due to the formed melanoidins), and at a temperature above 160 °C the lactose caramelizes and browns. Being the principal carbon source for microorganisms in milk, it is indispensable for manufacture of fermented dairy products like cheese and yoghurt (Jenness & Holt, 1987). In addition to lactose, a large number of free saccharides mainly oligosaccharides and small amount of monosaccharides and glycosylated proteins (κ-casein), mucins and glycoproteins in fat globule membrane are also present (Oliveira, Wilbey, Grandison, & Roseiro, 2015).

1.3. Fats

Bovine milk contains an average of 3.8 % total fat, though considerable variation may be present between breeds and indeed between individual cows. Fat plays an important role in the growth and development of the calf, while also providing essential fatty acids and fat-soluble vitamins (Jensen, 2002). The fat fraction of milk is dominated by triglyceride esters with almost 98 % of total milk fat, composed of fatty acids of varying chain length and degree of saturation. Namely, their constituent fatty acid distribution determines the melting and rheological behaviour of milk fat (Gresti, Bugaut, Maniongui, & Bezard, 1993) and, hence, the texture and mouthfeel of milk fat-based products, with butter being the most popular. The remaining proportion of total milk fat is composed of phospholipids, cholesterol, free fatty acids, and diacylglycerol, with 1, 0.5, 0.1, and 2 %, respectively (Moate, Chalupa, Boston, & Lean, 2007).

1.4. Minerals

Milk minerals occur in one or more chemical forms, including ions and salts (organic and inorganic), complexes or constituents of organic molecules like proteins. They constitute only a small portion of bovine milk and includes cations (calcium, magnesium, sodium and potassium) and anions (inorganic phosphate, citrate and chloride) (Gaucheron, 2005). These

cations and anions are partitioned between the colloidal and aqueous phase of milk. About one third of calcium, two thirds of magnesium, half of the inorganic phosphate and over 90 % citrate is in aqueous phase whereas the rest is associated with casein molecules and are an integral part of the casein micelle (Huppertz, Fox, & Kelly, 2018). Most importantly, concentrations of calcium and phosphorous tend to be too high to be maintained in the soluble phase at native milk pH and consequently, associate with the casein micelle to form insoluble colloidal calcium phosphate (Walstra, 1990).

1.5. Vitamins

Bovine milk is a source of fat soluble (A, D and E) and water-soluble vitamins (C, B1, B2, B6, B12, pantothenic acid, niacin, biotin and folic acid). The concentrations of fat-soluble vitamins are more variable in different dairy products, as increasing fat concentration will consequently increase the fat-soluble vitamin content of products such as cream or butter, although this would therefore necessitate supplementation of these vitamins in low-fat products. Vitamin A (particularly in the form of β-carotene) and Vitamin D are most abundant in milk, and concentrations of Vitamin E and Vitamin K are substantially lower (Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015).

1.6. Protein system in bovine milk

The protein composition of bovine milk is complex, and it consists of 80 % caseins (flexible and unordered proteins), and the remaining 20 % are classified as whey proteins (globular in nature), including some minor proteins (Horne, 2020). Two centuries ago, the works of Berzelius (1814) and Braconnot (1830) were some of the first scientific publications describing milk proteins, and particularly caseins. Both publications investigated the physical differences between caseins and whey proteins and established that acidification of milk resulted in formation of a curd (casein) that can be separated from a clear solution (serum or whey proteins) (Berzelius, 1814; Braconnot, 1830).

1.6.1. Whey proteins

The whey protein fraction is generated from milk as a by-product of the cheese-making process or as a by-product of acid casein production. In the cheese-making process, after treatment of

milk with rennet and the removal of the caseins, the majority of the whey proteins remains in the milk serum (Lucey, 2002). The whey protein fraction is comprised of several proteins of high to medium abundancy, including α-Lactalbumin, β-Lactoglobulin, serum albumin, immunoglobulins, and some other biologically important proteins, such as lactoferrin and lactoperoxidase, that are generally of a globular nature and are somewhat heat labile (Boland, 2011; Kilara & Vaghela, 2018). Table 2, adopted from Gazi, Johansen, and Huppertz (2022) shows the general characteristics of whey proteins in bovine milk. It is worth mentioning that the process of heat-induced modification of whey proteins can be either reversible or irreversible. The reversible modifications include partial unfolding of proteins with loss of helical structure, while irreversible changes lead an aggregation process involving thiol (–SH)/disulphide (S–S) interchange reactions and other intermolecular interactions including hydrophobic and electrostatic interactions (Anema, 2000). Apart from the interactions between whey proteins, they can also interact with cysteine-containing caseins, especially κ-casein. Moreover, the evidence points out that the thermal denaturation behaviour of individual whey proteins is distinctly different (Anema, 2020) and the reaction might strongly influence the technological properties of dairy products (McKenzie, Norton, & Sawyer, 1971), but also digestibility of milk or dairy commodities (Huppertz & Chia, 2021).

Table 2. General properties of whey proteins in bovine milk

Protein	**β-Lactoglobulin**	**α-Lactalbumin**	**Bovine serum albumin**	**Lactoferrin**
Gene name	LGB	LALBA	ALBU	LTF
Concentration in milk (mg/mL)	2 - 4	0.6 - 1.7	0.4	0.02 - 0.1
Phosphorylation	n/a	n/a	Yes	n/a
Number of glycans	n/a	0 - 1	0 - 1	4 - 5
Disulphide bridges	2	4	17	17
Reference proteform	β-Lactoglobulin B	α-Lactalbumin B	n/a	n/a
Number of amino acids within the protein	162	123	583	689
Average mass (Da)	18281.01	14185.92	66432.27	76143.08
pI of the proteins	4.80	4.84	5.64	8.07

1.6.2. Caseins and casein micelle

Caseins are distinguished from whey proteins for their ability to be heat stable and their propensity to precipitate at pH 4.6, while individually they can be differentiated by electrophoretic mobility or primary sequencing (Walstra, 1990). This group of milk proteins originates from the family of phosphoproteins, encoded with a specific genetic code of the bovine chromosome 6, and are classified mainly in four different types, including αs_1-, α_2-, β-, and κ-caseins with an approximate ratio of 4:1:4:1 of whole bovine casein (Bijl, Holland, & Boland, 2020). The general properties and primary structure of these four caseins are well established and presented in Table 3 (Farrell Jr et al., 2004; Gazi et al., 2022; Huppertz et al., 2018; Huppertz & Gazi, 2022).

Table 3. General properties of caseins in bovine milk

Protein	**αs_1-casein**	**α_2-casein**	**β-casein**	**κ-casein**
Gene name	CSN1S1	CSN1S2	CSN2	CSN3
Concentration in milk (mg/mL)	12 - 15	3 - 4	9 - 11	2 - 4
Phosphorylation range	7 - 9	9 - 15	4 - 5	0 - 3
Glycosylation sites	n/a	n/a	n/a	0 - 6
Reference proteform	αs_1-casein B - 8P	αs_2-casein A - 11P	β-casein A2 - 5P	κ-casein A - 1P
Number of amino acids within the protein	199	207	209	169
Proline residues within the protein	17	10	35	20
Positively charged residues	25	33	20	17
Negatively charged residues	40	39	28	28
Aromatic residues	20	20	14	14
Average mass (Da)	23614.43	25228.03	23982.89	19037.14
pI of the proteins	4.43	4.95	4.66	5.62

Most, but not all, of the caseins exist in a colloidal particle known as the casein micelle. Casein micelles have an essential function in the production of milk, milk processing, and its conversion to different dairy products (Huppertz & Gazi, 2022). Namely, its purpose is the

transport of proteins, calcium, and phosphate at elevated amounts that otherwise would be insoluble in water (Horne, 2020). The size of casein micelles in bovine milk range considerably from around 150 to 230 nm, however, the variability in casein micelle size might be associated with age, milk output (lactation of cows), fat or protein content of milk (De Kruif, Huppertz, Urban, & Petukhov, 2012; Huppertz et al., 2018). To form a casein micelle, the four individual caseins interact with each other via predominantly non-covalent, but also some covalent, interactions, as well as via ionic interactions with calcium phosphate nanoclusters (Lucey & Horne, 2018). The nature of the interactions between caseins has been extensively discussed, with some suggesting that hydrophobic interactions are essential (Horne, 2020), while others argue that backbone interactions are the most important (Carver & Holt, 2019). De Kruif et al. (2012) outlined that interactions between caseins involve collective hydrophobic and hydrogen bonding, electrostatic interactions, and van der Waals attractions. Different models for casein micelle assembly and structure have been proposed, e.g., the sub-micelle model (Slattery, 1976; Slattery & Evard, 1973), the dual-binding model (Horne, 1998, 2017, 2020), the nano-cluster model (Holt, 1992, 2004, 2016; Holt & Carver, 2022), the water channel model (Dalgleish, 2011; Dalgleish & Corredig, 2012; Dalgleish, Spagnuolo, & Douglas Goff, 2004), and the network model (Huppertz et al., 2017). Nevertheless, despite its ubiquity and numerous investigations, there is also no general agreement either on the interactions, or on the fine structure of the casein micelle assembly since the structure of the casein micelle cannot be simply visualised (Day, Williams, Otter, & Augustin, 2015; De Kruif et al., 2012; Huppertz & Gazi, 2022).

For all caseins at least 39 genetic variants have been identified. Namely, 8 variants were assigned to αs_1-casein (A, B, C, D, E, F, G, and H), 4 for αs_2-casein (A, B, C, and D), 15 for β-casein (A1, A2, A3, A4, B, C, D, E, F, G, H1, H2, I, J, K), and 12 for κ-casein (A, B, B2, C, E, F1, F2, G1, G2, H, I, J). These caseins' genetic variants were found to maintain the isoelectric point and protein charge, and ultimately can impact the physicochemical and the functional properties of milk proteins, liquid milk, and dairy products (Daniloski, McCarthy, Huppertz, & Vasiljevic, 2022; Day et al., 2015; Gai, Uniacke-Lowe, O'Regan, Faulkner, & Kelly, 2021). Nevertheless, to what extent this genetic polymorphism might affect the casein micelle structure-functionality properties and the interaction among caseins within the casein micelle, received less attention. This is because, the natural variation in casein composition affected by the casein genotype is hard to study as it requires sample sets with different variants, some of which are hard to find in populations.

1.7. A1-free milk

Bovine β-casein and its phenotypes have gained significant recognition within the dairy industry, as they have been related to affecting the "structure" of the casein micelle (Daniloski, Markoska, McCarthy, & Vasiljevic, 2023), techno-functionality of dairy products (Daniloski et al., 2022), and even been linked to having an impact on human health (Daniloski et al., 2021). Comprised of 209 amino acids (17 % are proline residues), β-casein is considered the most hydrophobic of the caseins. This protein is coded by the CSN2 gene mapped to chromosome 6 (119 Mb in size) (Ferretti, Leone, & Sgaramella, 1990).

While a number of β-casein variants have been identified, because of a point mutation in its polypeptide chain, the most common are β-caseins A1 and A2 (Aschaffenburg, 1961). In the context of the present research on β-casein variants, a point mutation in the gene encoding the β-casein could result in a change in the amino acid sequence of the protein. More specifically, a point mutation is a change in a single nucleotide base in the DNA sequence. This change can lead to the expression of a protein variant if it occurs in the coding region of a gene (Berry et al., 2020), but it can also alter the structure and function of the protein, thus leading to the expression of a different β-casein variant (Daniloski et al., 2022). Hence, due to the location of the mutation (single nucleotide polymorphism) on exon VII and the sixth chromosome of the CSN2 gene (Caroli, Chessa, & Erhardt, 2009) the transfer from cytosine (CCT) to adenine (CAT) contributes to the substitution of proline with histidine at position 67 in the β-casein polypeptide chain (Daniloski et al., 2021). Hence, the defining feature of these two types of β-casein is the inclusion of either histidine in β-casein A1 or proline in β-casein A2 (Figure 1), with β-casein A1 being a result of a point mutation from proline to histidine arising in the ancestors of modern European - cattle (Jianqin et al., 2015).

Bovine milk comprised of the homozygous β-casein A1 or the homozygous β-casein A2 is termed A1/A1 milk or A2/A2 milk, respectively, whilst a mixture of both β-casein A1 and A2 leads to the production of conventional or A1/A2 milk. Bovine milk absent of β-casein A1 is now commonly available across a variety of countries, including Australia, the United Kingdom, the United States, New Zealand, and the Netherlands, and is generally advertised as helpful to people suffering from some digestive discomfort (Daniloski et al., 2021). When it comes to non-A1 milk as a commercial product, the companies selling A2/A2 milk have certainly created a breakthrough trend in the dairy consumer market and the farming industry to date. For instance, the A2/A2 market is poised for substantial growth between 9 and 18 % over the next decade according to the analyses performed by Market Research Future in 2021,

Future Market Insights and Market Research Report in 2023 (FMI, 2023; MRFR, 2021; MRR, 2023).

In contrast, the high cost of A2/A2 milk, the limited availability and the lack of sufficient scientific evidence that supports the health claims of consuming this milk remain a challenge to the growth of the unconventional milk market. Namely, the main reason for promoting A2/A2 milk and A2/A2 dairy products was due to the claims that β-casein A1 has been related to some gastro-intestinal discomfort and studies have been performed to increase the frequency of the β-casein A2 allele in dairy cattle breeds (Sebastiani et al., 2020). In contrast, β-casein A2 has been found to be the dominant variant in non-coagulating and poor-coagulating milk samples and it has detrimental effects on milk coagulation processes in comparison to β-casein A1, especially when producing fermented dairy products, with yoghurt and cheese being the most popular (Daniloski et al., 2022). The evidence found within this thesis explained and elaborated, if not completely, many unanswered questions regarding the A2 dairy dilemma.

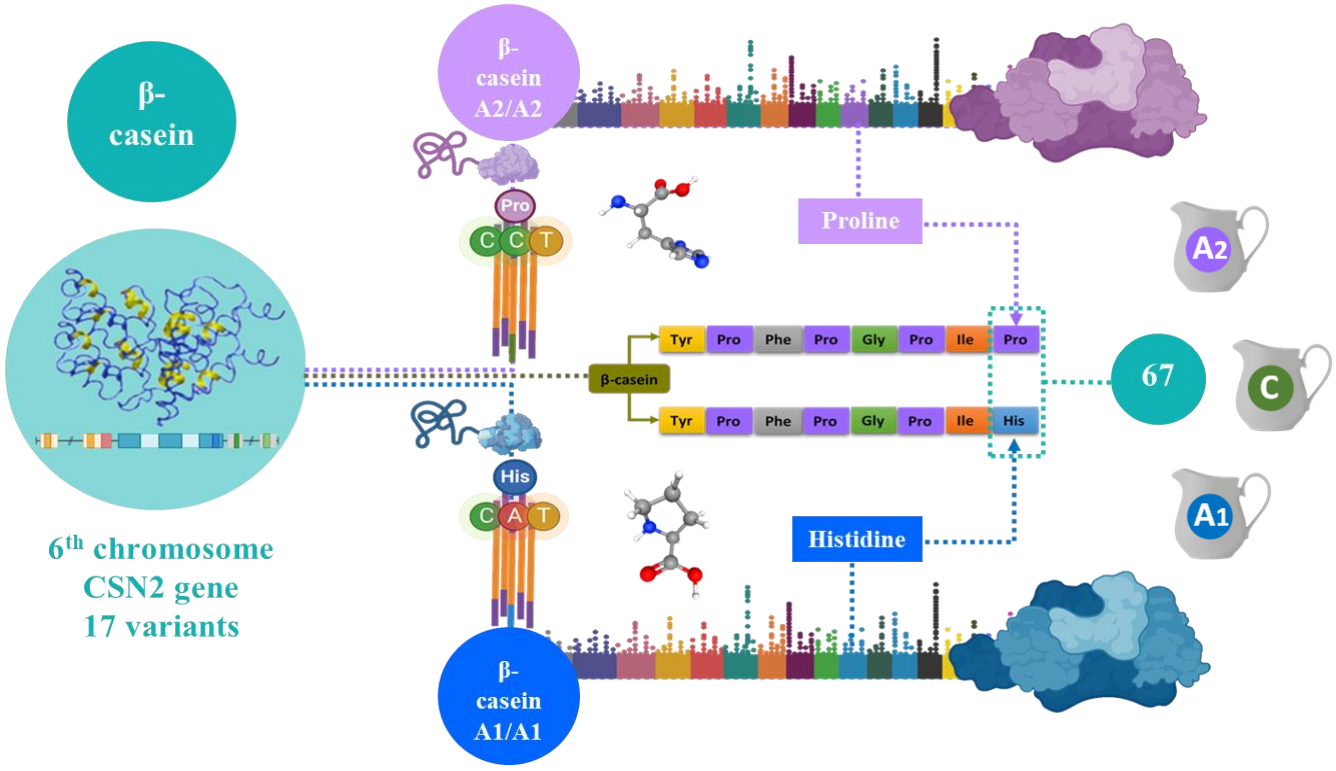


Figure 1. A single nucleotide polymorphism in the structure of bovine β-casein. Visual representation of the point mutation on exon VII in the sixth chromosome of the CSN2 gene. A1 = A1/A1 milk; A2 = A2/A2 milk; and C = conventional or A1/A2 milk.

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Literature Review: Part II. Chapter 2.

What is the impact of amino acid mutations in the primary structure of caseins on the composition and functionality of milk and dairy products?

- Impact of β- and other caseins on the casein micelle structure and functionality
- Proline and histidine in β-caseins play a key role in casein micelle conformation
- Chaperone activity of β-casein A2 towards heat-induced aggregation of whey protein
- Gels prepared of milks with β-casein A1 possess a denser and firmer structure
- Ordered structure of β-casein A2 led to improved emulsion and foam formation

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1. PUBLICATION DETAILS (to be completed by the candidate)

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Noel A. McCarthy	5	Concept development, manuscript editing, revision and supervision		17/03/2024
Thom Huppertz	5	Critical feedback, concept development, manuscript editing and revision		17/03/2024
Todor Vasiljevic	6	Concept development, manuscript editing, revision, submission and supervision		17/03/2024

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What is the impact of amino acid mutations in the primary structure of caseins on the composition and functionality of milk and dairy products?

Davor Daniloski [a,b], Noel A. McCarthy [b], Thom Huppertz [a,c,d], Todor Vasiljevic [a,*]

[a] Advanced Food Systems Research Unit, Institute for Sustainable Industries and Liveable Cities and College of Health and Biomedicine, Victoria University, Melbourne, VIC, 8001, Australia
[b] Food Chemistry and Technology Department, Teagasc Food Research Centre, Moorepark, Fermoy, P61 C996, Cork, Ireland
[c] FrieslandCampina, Amersfoort, the Netherlands
[d] Wageningen University & Research, Wageningen, the Netherlands

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ABSTRACT

The impact of amino acid mutations within the peptide structure of bovine milk protein is important to understand as it can effect processability and subsequently effect its physiological properties. Genetic polymorphisms of bovine caseins can influence the chemical, structural, and technological properties, including casein micelle morphology, calcium distribution, network creation upon gelation, and surface activity. The A1 and A2 genetic variants of β-casein have recently acquired growing attention from both academia and industry, prompting new developments in the area. The difference between these two genetic variants is the inclusion of either proline in β-casein A2 or histidine in β-casein A1 at position 67 in the peptide chain. The aim of this review was to examine the extent to which milk and ingredient functionality is influenced by β-casein phenotype. One of the main findings of this review was although β-casein A1 was found to be the dominant variant in milks with superior acid gelation and rennet coagulation properties, milks comprised of β-casein A2 possessed greater emulsion and foam formation capabilities. The difference in the casein micelle assembly, hydrophobicity, and chaperone activity of caseins may explain the contrast in the functionality of milks containing β-casein from either A1 or A2 families. This review provides new insights into the subtle variations in the physicochemical properties of bovine milks, which could potentially support dairy producers in the development of new dairy products with different functional properties.

1. Introduction

Bovine milk is produced in the mammary gland and comprises a large and diverse collection of proteins, fats, carbohydrates and micronutrients, which vary in abundance across stages of lactation, age, and cow's health (McSweeney and Fox, 2013). The protein composition of milk is complex, which influences its physicochemical, functional, and nutritional properties. There are two main categories of milk proteins, namely caseins and whey proteins, present in milk at a ratio of ~80 : 20 (Gazi, Johansen and Huppertz, 2022). Caseins originate from the family of phosphoproteins found in all mammals (O'Mahony and Fox, 2013) and are categorised into four different types: αs_1-, αs_2-, β- and κ-casein, present at the ratio of ~4.0 : 1.0: 3.5 : 1.5 in bovine milk; all caseins contribute to a colloidal structure known as the casein micelle (Horne, 2020). On dry matter basis, the casein micelle consists of ~93% protein and ~7% salts, the latter commonly referred to as micellar calcium phosphate (MCP) (Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015; Huppertz et al., 2018; Huppertz and Gazi, 2022).

To form a casein micelle, the four individual caseins interact with each other via predominantly non-covalent, but also some covalent, interactions, as well as via ionic interactions with calcium phosphate nanoclusters (Lucey and Horne, 2018). The nature of the interactions between caseins has been extensively discussed, with some suggesting that hydrophobic interactions are essential (Horne, 2020), while others argue that backbone interactions are the most important (Carver and Holt, 2019). De Kruif et al. (2012) outlined that interactions between caseins involve collective hydrophobic and hydrogen bonding, electrostatic interactions, and van der Waals attractions. Different models for casein micelle assembly and structure have been proposed, e.g., the sub-micelle model (Slattery, 1976; Slattery and Evard, 1973), the

* Corresponding author.
E-mail address: todor.vasiljevic@vu.edu.au (T. Vasiljevic).

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dual-binding model (Horne, 1998, 2017a, 2020), the nano-cluster model (Holt, 1992, 2004, 2016), the water channel model (Dalgleish, 2011; Dalgleish and Corredig, 2012; Dalgleish et al., 2004), and the network model (Huppertz et al., 2017). Nevertheless, despite its ubiquity and numerous investigations, there is also no general consensus either on the interactions, or on the fine structure of the casein micelle assembly since the structure of the casein micelle cannot be simply visualised (Day et al., 2015; De Kruif et al., 2012; Huppertz and Gazi, 2022).

Milk protein genes are able to produce a substantial number of polymorphisms. The autosomal genes CSN1S1 (αs_1-casein), CSN1S2 (αs_2-casein), CSN2 (β-casein), and CSN3 (κ-casein) encoding for the caseins are organised as a cluster in a DNA stretch of about 200 kb mapped on chromosome 6 (Gai, Uniacke-Lowe, O'Regan, Faulkner and Kelly, 2021). There are at least 39 genetic bovine casein variants reported (Gazi et al., 2022): 8 for αs_1-casein (A, B, C, D, E, F, G, and H), 4 for αs_2-casein (A, B, C, and D), 15 for β-casein (A1, A2, A3, A4, B, C, D, E, F, G, H1, H2, I, J, K), and 12 for κ-casein (A, B, B2, C, E, F1, F2, G1, G2, H, I, J) (Fig. 1). Important differences in occurrence and frequency of the variants arise among species and breeds (Adamov et al., 2020; Gallinat et al., 2013; Gazi et al., 2022a; Huppertz et al., 2018). Genetic variants of milk proteins differ in amino acid sequence, which can affect the isoelectric point and protein charge, and ultimately can impact the physicochemical and the functional properties of milk proteins, milk and dairy products (Day et al., 2015; Gai, Uniacke-Lowe, O'Regan, Faulkner and Kelly, 2021; Lucey and Horne, 2018). Recently, different genotypes of β-casein in bovine milk have gained significant attention within the dairy industry, as they have been related to affecting the functionality of dairy products, and even been linked to having an impact on human health (Daniloski, McCarthy and Vasiljevic, 2021b; Mendes et al., 2019; Milan et al., 2020; NguyenSchwendel et al., 2018; Sebastiani et al., 2022).

Bovine β-casein is considered an intrinsically disordered protein (IDP) and constitutes between 33 and 45% of the total casein in bovine milk. Due to the location of the mutation (single nucleotide polymorphism) on exon VII and the 6th chromosome of CSN2 gene, the transfer from cytosine (CCT) to adenine (CAT) contributes to the substitution of proline (Pro) with histidine (His) at position 67 in the β-casein polypeptide chain (Caroli, Chessa, & Erhardt, 2009; Jann et al., 2002; Kumar et al., 2018; Sebastiani et al., 2022) (Fig. 2). Namely, β-caseins A1 and A2 are the most commonly secreted variants in milk (Aschaffenburg, 1968; Farrell et al., 2004). It is postulated that the A2 variant of β-casein (β-casein A2-5P; considered as a reference proteoform) carries the original amino acid sequence, before a point mutation caused the appearance of the β-casein A1 variant in some European herds (Daniloski et al., 2021a; De Poi et al., 2020; Gallinat et al., 2013; Gazi et al., 2022). The evolution of the β-casein A1 proteoform led to the grouping of the β-casein variants into two families, i.e., the A2 family with the A2, A3, and I variants of β-casein, and A1 family with the A1, B, and F variants of β-caseins (Table 1) (Caroli et al., 2004; Daniloski, McCarthy, Markoska, Auldist and Vasiljevic, 2022a; Ng-Kwai-Hang & Grosclaude, 2003). Bovine milk possessing β-casein A2/A2 carrying Pro is called A2/A2 milk, whereas milks carrying His in the β-casein A1/A1 or A1/A2 polypeptide chains are known as A1/A1 and A1/A2 milks, respectively (Daniloski et al., 2022a; NguyenSolah et al., 2019).

To date many studies have focused on the effects of β-casein genetic polymorphism on animal and human health using *in vitro*, *ex vivo* and *in vivo* trials (Daniloski et al., 2021a, 2021b; Haq et al., 2014; He et al., 2017; Hockey et al., 2021; Ramakrishnan et al., 2022; Ramakrishnan et al., 2020; Yadav et al., 2020), with limited focus on the technical and functional properties of β-casein phenotype on milk products. Therefore, the main purpose of this review is to address the differences between the physicochemical properties of these β-casein phenotypes, but also the other caseins and their genetic variants. This will include the structure of the casein micelle, chaperone activity, hydrophobicity, emulsion, foaming and rheological properties of milk and the process of acid gelation and rennet coagulation.

2. Polymorphism and protein profiling of β-casein genetic variants

Single-nucleotide polymorphisms are the most abundant form of genetic variation and a resource for mapping complex genetic traits through the amino acid modifications within the structure of milk proteins (Chessa et al., 2007; Gazi et al., 2022a; Martin et al., 2013). Whenever these genetic mutations are correlated with milk protein composition or milk production traits, the data can be useful when choosing for breeding cows that produce milk with improved value, such as higher casein levels or an altered concentration of other proteins

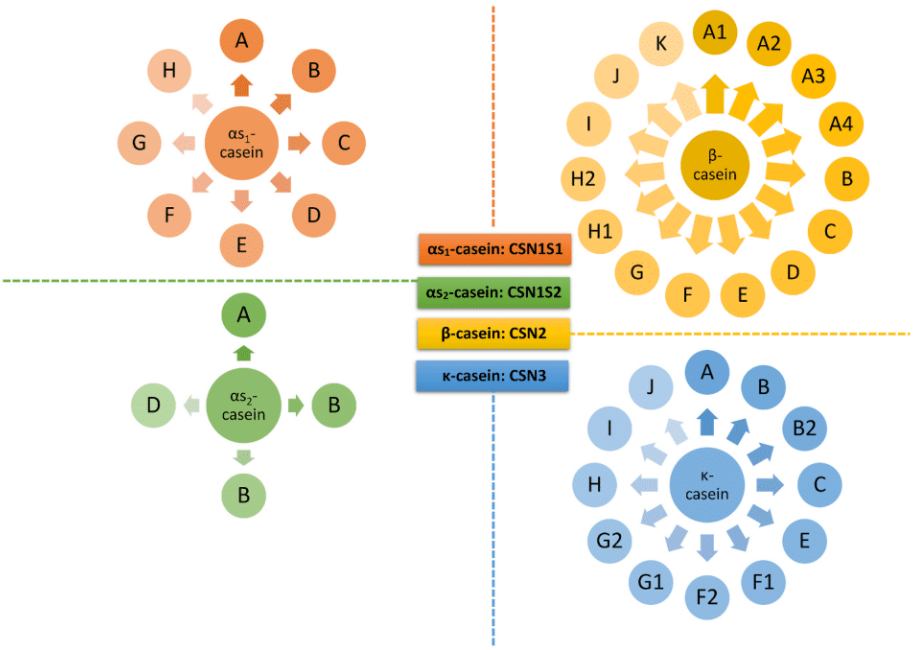

Fig. 1. Different casein forms, their genetic variants and genes.

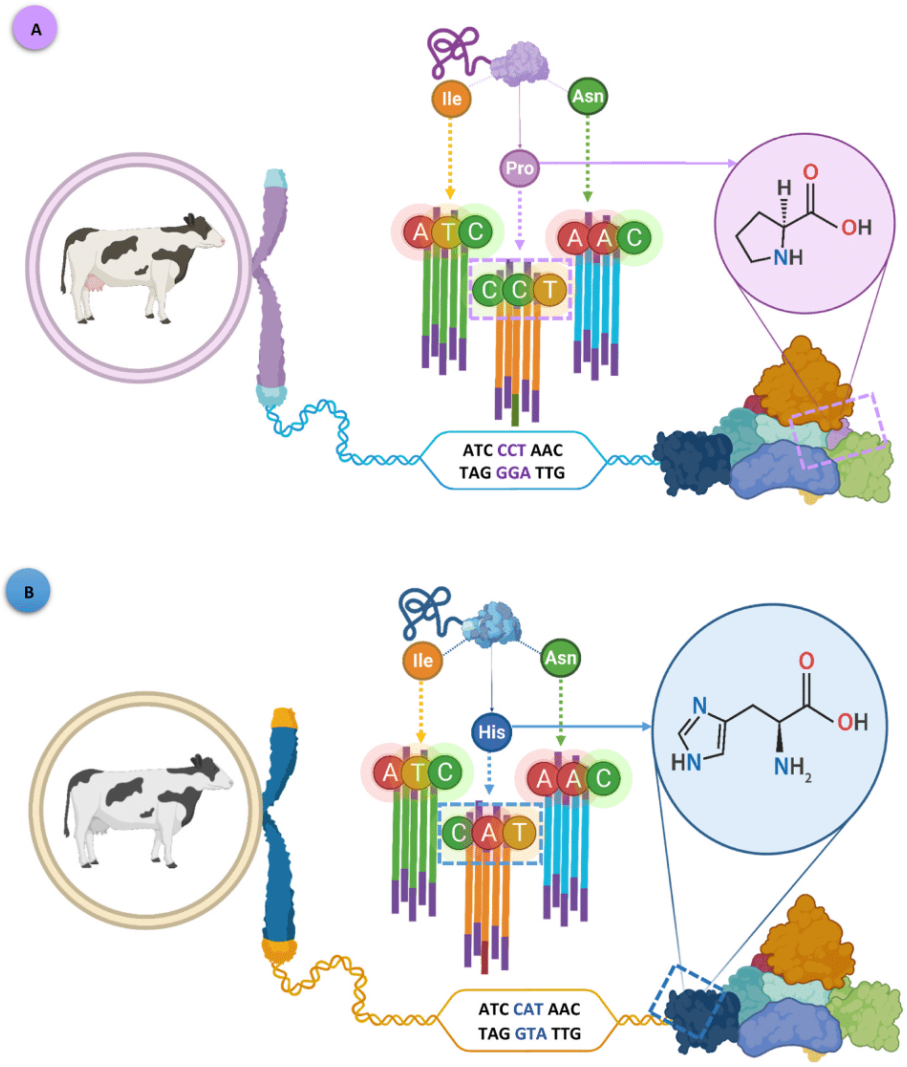

Fig. 2. Bovine genetic polymorphism, variation sequences of A) β-casein A2 genetic variant; and B) β-casein A1 genetic variant. *Iso (Isoleucine), Pro (Proline), His (Histidine), Asn (Asparagine); A (Adenine), T (Thymine), C (Cytosine), G (Guanine).*

Table 1
Differences in the amino acid sequence of the most common genetic variants of β-casein found in the milk of *Bos* genus compared to the reference proteoform β-casein A2-5P.

	β-casein A1 family			β-casein A2 family		
Position			**Proteofroms**			
	β-casein A1-5P	β-casein A2-5P	β-casein A3-5P	β-casein B–5P	β-casein F–5P	β-casein I–5P
			Genetic variants			
	β-casein A1	β-casein B	β-casein F	β-casein A2	β-casein A3	β-casein I
67	His	His	His	Pro	Pro	Pro
93	Met	Met	Met	Met	Met	Leu
106	His	His	His	His	Gln	His
122	Ser	Arg	Ser	Ser	Ser	Ser
152	Pro	Pro	Leu	Pro	Pro	Pro
Average mass (Da)	24022.91	24092.02	24038.96	23982.89	23973.88	23964.85
Isoelectric point (pI)	4.73	4.82	4.73	4.66	4.58	4.66

(Berry et al., 2020). The gene polymorphism in β-casein was first identified in the *Bos* genus, particularly in Jersey and Guernsey cows, by Aschaffenburg in the 20th century and occurred in three genetic variants identified as A, B, and C (Aschaffenburg, 1963; Thompson et al., 1969). Some years later, Peterson and Kopfler (1966) demonstrated, by polyacrylamide gel electrophoresis at acidic pH, that β-casein A variant was not a unique casein but three different variants, denoted as β-caseins A1, A2 and A3. Furthermore, β-casein A4 has also been detected (Chung et al., 1995), however, neither its amino acid sequence or clinical based studies on this genetic sub-type have been reported to date. Despite the significance of the amino acid sequence of these proteins, limited attention was given to the genetic polymorphism of bovine β-casein until the occurrence of the A1/A1, A1/A2, and A2/A2 milk recognition in the 1990s (Elliot et al., 1996).

Over the last two decades, as a result of a greater commercial interest in certain parts of the world, including Australia, Canada, China, New

Zealand and the USA, the selection of cows producing A2/A2 milk has increased (Milan et al., 2020). Therefore, since various β-casein phenotypes can appear in bovine milk and are found to affect the dairy product manufacturing (Section 4), and possibly human health, there is an increased need for reliable analytical methods capable of authenticating a milk labelled as A1/A2 or A2/A2 milk (Daniloski, McCarthy, O'Callaghan and Vasiljevic, 2022c; De Poi et al., 2020). The methods and analytical strategies for the genetic identification and quantification of different β-casein variants in either cows or bovine milks are presented in Table 2. These methods have been primarily based on liquid chromatography, electrophoresis, polymerase chain reaction test, spectroscopy, and microsphere-based immunoassay (Broadbent et al., 2021; Daniloski et al., 2022a; Daniloski et al., 2022c; De Poi et al., 2020; Elferink et al., 2022; Fuerer et al., 2020; Gai et al., 2021; NguyenSolah et al., 2020; Vigolo et al., 2022b).

3. The importance of β-casein phenotypes, other caseins and minerals on the structure and organisation of casein micelle

Bovine β-casein comprises 209 amino acids and is considered the most hydrophobic of the caseins. The hydrophobicity seems to play an important role in the attachment of β-casein within the casein micelle. This protein has a flexible and open conformation, molecular mass of 23.6 kDa (primary structure prior to posttranslational phosphorylation; ~24.0 kDa following phosphorylation), and little tertiary structure (Huppertz et al., 2018). The phosphorylation of all five phosphoserine (SerP) residues (Ser15, Ser17, Ser18, Ser19, and Ser35) in β-casein results in the strong amphipathic nature of this protein (McCarthy, Kelly, O'Mahony and Fenelon, 2013). The first four SerP residues form a centre of phosphorylation, which facilitates the association of β-casein with the calcium phosphate nanoclusters (Gazi et al., 2022a; Horne, 2020; Huppertz et al., 2018). At neutral pH, the C-terminal area of β-casein (residues 41–209) is strongly hydrophobic and shows balanced charges, whereas the N-terminal domain (residues 1–40) is polar and strongly negatively charged (McCarthy et al., 2013). Owing to its amphiphilic nature, β-casein shows a strong propensity to self-assemble into the micelles comprising of 15–60 protein molecules (radius of gyration values ranging between 7.3 and 13.5 nm), where hydrophobic interactions between those molecules are the primary attractive forces (Huppertz, 2013). The formation of β-casein micelles is described by the shell model, also referred to as the consecutive (stepwise) micellization model (De Kruif and Grinberg, 2002), and considers a series of sequential additions of primary particles (dimer forms) to a growing micelle (Atamer et al., 2017). The β-casein micelle has a detergent-like micellar structure with a hydrophobic core and a charged hydrophilic surface (McCarthy et al., 2013). While the presence of a hydrophilic domain in the N-terminus enhances the solubility of β-casein in aqueous media, the hydrophobic domains in the C-terminal section facilitate non-covalent associations with other molecules (Liyanaarachchi and Vasiljevic, 2018).

A large proportion of polyproline II (PPII) conformational motifs, mainly within their Pro and glutamine (P,Q)-rich regions, were observed in all β-casein genetic variants (Sanders et al., 2020), but more in β-casein A2 (Syme et al., 2002) and in A2/A2 milk (Daniloski et al., 2022a). Such PPII regions, known as initiators of the hydrophobic environments of β-casein, were active within the β-casein micelle whilst interacting with other proteins (Thorn et al., 2015). Accordingly, the interactions of β-caseins with other proteins, in particular other caseins, would be enhanced, thus leading to tighter packing within the casein micelle or smaller aggregates when undergoing self-association (especially in the case of β-casein A2) (Raynes et al., 2015). Thorn et al. (2005) revealed that β-casein may manipulate the inherently unstable monomers of native κ-casein by binding to and shielding their hydrophobic surfaces, thus prohibiting interactions with other κ-casein molecules that would otherwise facilitate self-assembly into fibrillar structures. Moreover, due to the presence of one additional Pro in its

Table 2
Identification of β-casein genetic variants and phenotypes in bovine milk or cows by using different methods.

Genetic variant(s) and phenotype(s)	Methodology	Reference
Electrophoresis		
A, B, and C (A varinat does not exist anymore in common nomenclature)	Paper electrophoresis	Aschaffenburg (1963); Thompson et al. (1964)
A1, A2, and A3	Polyacrylamide gel electrophoresis	Peterson and Kopfler (1966)
A4	Urea gel electrophoresis	Chung et al. (1995)
H1	Agarose gel electrophores; Polymerase chain reaction	Han et al. (2000)
A1 and A2	Capillary zone electrophoresis	de Jong et al. (1993)
A1, A2, B, and A1/A2	Capilary electrophoresis	Noni (2008)
A1 and A2	Urea Polyacrylamide gel electrophoresis	Raynes et al. (2015)
A1 and A2		Duarte-Vázquez et al. (2018)
DNA sequencining and polymerase chain reaction		
D	Amino acid composition	Thompson et al. (1969)
A1 and A2	Duplex artificially created restriction site-polymerase chain reaction	Şahin and Boztepe (2022)
A1, A2, A3, B, C, D, E, F, G, H1, H2, I, J, and K	DNA sequencing	Gallinat et al. (2013)
A1, A2, B, I; A2/A2, A1/A2, A2/B, and A2/I	DNA sequencing	Sebastiani et al. (2022)
A1 and A2	Allele Specific Polymerase Chain Reaction	Ristanić et al. (2022)
A1, A2, B, and I	DNA sequencing; Reversed Phase-High Performance Liquid Chromatography	Bonfatti et al. (2008)
A1, A2, A1/A2, and B	Polymerase chain reaction-restriction fragment length polymorphism; Amplification refractory mutation system-polymerase chain; Reversed Phase-High Performance Liquid Chromatography	Vigolo et al. (2022b)
I	Polymerase chain reaction; Reversed Phase-High Performance Liquid Chromatography	Daniloski et al. (2022c); Jann et al. (2002)
High Performance-Liquid Chromatography		
F	Reversed Phase-High Performance Liquid Chromatography	Poulsen et al. (2017); Visser et al. (1995)
G		Visser et al. (1995)
A1/A2, A1/A1, A2/A2, B/A1, B/A2, and I/H2		Bisutti et al. (2022)
A1, A2, and B		Vigolo et al. (2022a)
A1/A1, A1/A2, and A2/A2		Daniloski et al. (2022a); Daniloski et al. (2022b)
A1 and A2	High Performance-Liquid Chromatography Tandem Mass Spectrometry	Guo et al. (2022)
A1, A2, and B	Liquid Chromatography-Mass Spectrometry	De Poi et al. (2020)
A1, A2, A3, B		Miranda et al. (2020)
H2		Senocq et al. (2002)
A1, A2, B, and C	High Performance-Liquid Chromatography-Mass Spectrometry	Givens et al. (2013)
A1/A1, A1/A2, A2/A2, A1/A3, A2/A3, A1/B, A2/B, I/I, A1/I, A2/I, B/I, A1/F, and A2/F	Liquid Chromatography-Electrospray Ionization-Mass Spectrometry	Poulsen et al. (2017)
A1, A2, B, and I	Liquid Chromatography-Electrospray Ionization-Quadrupole-Time of Flight-Mass Spectrometry	Vincent et al. (2016)
A1/A2 and A2	Ultraperformance Liquid Chromatography-High-Resolution Mass Spectrometry	Fuerer et al. (2020)

(continued on next page)

Table 2 (*continued*)

Genetic variant(s) and phenotype(s)	Methodology	Reference
A1/A1, A1/I, A1/A2, A2/A2, and A2/I	Ultra-High-Performance Liquid Chromatography-High-Resolution Mass Spectrometry (Orbitrap™)	NguyenSolah et al. (2020)
A1 and A2	Quantitative Liquid Chromatography-Mass Spectrometry	Broadbent et al. (2021)
A2 and other A named as Am	Top-down high-resolution mass spectrometry-based metabolomics and lipidomics	Jia et al. (2022)
	Spectroscopy	
A1/A1, A1/A2, and A2/A2	Fourier-Transform Mid-Infrared spectroscopy	Cendron et al. (2021)
A1/A1 and A2/A2	Fourier Transform-Infrared spectroscopy	Joshi et al. (2021)
A1, A1/AI, A1/A2, A2, A2/I, and I		Daniloski et al. (2022c)
A1/A1 and A2/A2	Mid-Infrared spectroscopy	Xiao et al. (2022)
A1/A1, A1/A2, and A2/A2	Nuclear Magnetic Resonance spectroscopy	Daniloski et al. (2022a); Daniloski et al. (2022b)
	Immunoassay	
A1 and A2	Microsphere-Based Immunoassay	Elferink et al. (2022)

structure, β-casein A2, had lower hydrophobicity and appeared more frequently as a monomer relative to β-casein A1 (Raynes et al., 2015). Isolated and purified β-casein A1 solutions created larger β-casein micelles, compared to that of β-casein A2. However, various studies found that the milk carrying β-casein A1 possessed smaller casein micelle sizes compared to A2/A2 milks (Daniloski et al., 2022a; Daniloski, McCarthy and Vasiljevic, 2022b; Day et al., 2015).

For casein micelles, it is generally agreed that the κ-casein content was negatively correlated with size of the casein micelles (Bijl, de Vries, van Valenberg, Huppertz and Van Hooijdonk, 2014a; Dalgleish, 1993; Day et al., 2015). A number of studies have associated κ-casein and its genetic variants with the casein micelle size of individual bovine milk samples (Bijl et al., 2014a; Bonfatti et al., 2014; Day et al., 2015; Hallén et al., 2008; Vallas et al., 2012; Walsh et al., 1998). Bijl et al. (2014); Dalgleish et al. (1989); Daniloski et al. (2022a); Day et al. (2015) confirmed that κ-casein possesses the path-terminating function, and thus it can impact the casein micelle size. Both the A and B variants of κ-casein and glycosylation of κ-casein were correlated significantly with average casein micelle size in milks obtained from individual cows; however, κ-casein B was predominately associated with a smaller casein micelle size (Bijl et al., 2014a; Ketto et al., 2019; Ketto et al., 2017; Walsh et al., 1998). Differences in the casein micelle size may also be influenced by factors other than β- and κ-casein concentration, specifically their genetic variants and post-translational modifications (phosphorylation and glycosylation of casein molecules). This would include cow genetics, protein content, farming practices, environmental factors (feed and season), and the mineral content (Bijl et al., 2020; Day et al., 2015; Devold et al., 2000).

Almost a decade ago, Gustavsson et al. (2014) related small casein micelle size with a greater level of total and ionic calcium in milk. A similar trend was observed in the study of Daniloski et al. (2022a) as the authors postulated that A1/A1 and A1/A2 milks possessed greater amounts of total and ionic calcium and smaller casein micelle sizes compared to A2/A2 milk. In the casein protein fraction of milk, calcium is associated both in the form of calcium phosphate nanoclusters and also with amino acid residues via ionic bonds (Huppertz et al., 2021), e.g., with SerP, glutamic acid (Glu) and asparagine (Asp) residues that are not part of the calcium phosphate nanoclusters (Bijl et al., 2019; Lucey and Horne, 2018). The caseins can readily bind calcium ions to their phosphate cluster residues in order of $\alpha s_2 > \alpha s_1 > \beta > \kappa$-caseins (O'Mahony and Fox, 2013). Hence, Daniloski et al. (2022b) explained that the higher mineral content in milk carrying β-caseins A1/A1 and A1/A2 was due to the higher presence of αs_2-, αs_1-, and β-caseins in both milks compared to A2/A2 milk.

4. The impact of the casein variants on processing and production of dairy products

Rennetability, heat stability, emulsion and foaming characteristics, renowned as technologically essential properties of milk and dairy products, are influenced in part by the genetic polymorphisms of β-casein (Gai et al., 2021; Goulding, Fox, & O'Mahony, 2020; NguyenSchwendel et al., 2018). Table 3 summarises the influence of specific β-casein genetic variants on the technological properties of milk and dairy products, including heat treatment of milks carrying β-casein A1/A1, A1/A2, or A2/A2, behaviour of either A1/A1, A1/A2, or A2/A2 milks during acid gelation and rennet coagulation, including yoghurt and cheese production, and the colloidal and interfacial properties of β-casein variants (Table 3).

4.1. Heat treatment and stability

Heat treatment is one of the most common methods employed in the dairy industry in order to reduce bacterial and enzymatic activity, thus ensuring safety and prolonging the shelf-life of milk and dairy products (Anema, 2019; McCarthy et al., 2022). Nevertheless, some additional effects might take place during or after heat treatment, including gelling or coagulation during processing, thickening during storage, and constituent fouling, hence the exploration of heat stability of milk is essential for the food industry (Dumpler et al., 2020). It is well established that during the heat treatment of milk, whey proteins, especially β-lactoglobulin, can denature and associate with casein micelles via κ-casein linkages or form κ-casein/β-lactoglobulin complexes in the serum phase depending on pH (Anema, 2021). While extensive research has been carried out on the effects of various genetic variants of κ-casein on heat-treated milk and its stability (Choi & Ng-Kwai-Hang, 2002; McLean et al., 1987; Robitaille, 1995), currently, the knowledge of the influence of thermal treatment on milks carrying β-casein phenotypes A1/A1, A1/A2, and A2/A2 is limited. At elevated temperatures (50–145 °C), β-casein may behave as a molecular chaperone (Zhang et al., 2005), whereby it could interact with partially unfolded whey proteins, via hydrophobic domains, preventing their normal thiol-disulphide interchange with other whey proteins and subsequent aggregation (Liyanaarachchi and Vasiljevic, 2018; Yousefi et al., 2009). For instance, β-casein was shown to reduce heat-induced aggregation of β-lactoglobulin, α-lactalbumin, and bovine serum albumin, suggesting some potential chaperone action (Kehoe and Foegeding, 2011). Daniloski et al. (2022b) recently showed that lower levels of soluble β-casein and non-denatured whey proteins were found in heated A1/A1 and A1/A2 milks compared to in A2/A2 milk. This may suggest that β-casein A2 showed a stronger molecular chaperone activity towards heat-induced aggregation of whey proteins than β-casein A1 (Daniloski et al., 2022b).

The phenotypes of κ-casein have been related to functionality and stability of heat-treated bovine milks and manufactured dairy products. In this regard, upon heat treatment of bovine milk, the B variant of κ-casein possessed some ability to stabilise β-lactoglobulin against heat-induced denaturation (Choi & Ng-Kwai-Hang, 2002), but was less effective stabiliser of the casein micelle compared to κ-casein A (Jensen, Holland, Poulsen and Larsen, 2012a). Compared to κ-caseins A/A and A/B, the greater heat stability of milk was correlated with κ-casein B/B at natural milk pH (6.6–6.8) (Robitaille, 1995). Milk containing κ-casein A/B showed a longer maximum in heat coagulation time compared to κ-casein A/A, and the composite B/B-A/B haplotype of κ-casein--β-lactoglobulin, was associated with more heat-stable milk compared to A/A-A/A of the same haplotype (McLean et al., 1987; Robitaille, 1995).

Table 3
The impact of various β-casein genetic variants on the technological properties of milk.

Sample type	Technological trait	Outcome	Reference
	Milk ingredients (caseinate)		
Milk samples (n = 2) - A1/A2 milk - A2/A2 milk Cow's breed: unknown.	Physicochemical properties of sodium caseinates: Visosity, internal structure and particle size of A1/A2 and A2/A2 sodium caseinates.	The study did not find any noticeable differences between the structural and interfacial properties of sodium caseinate obtained from A1/A2 and A2/A2 milks.	Hemar et al. (2021)
Milk samples (n = 3) - Australian Holstein A1/A1 (n = 1) - Australian Holstein A1/A2 (n = 1) - Australian Holstein A2/A2 (n = 1).	Structure of sodium caseinates was assessed with FTIR and NMR spectroscopies. Physicochemical and interfacial properties were evaluated by analysing adsorbed protein content, hydrophobicity, solubility, and emulsion stability of the samples.	The β-casein A2 in both, A1/A2 and A2/A2 sodium caseinates, appeared to be able to more rapidly reach the oil droplet surface. Sodium caseinates carrying β-casein A2 were more efficient as emulsifying agent, compared to sodium caseinates with β-casein A1.	Daniloski et al. (2022e)
	Milk coagulation and gelation		
Milk samples (n = 892) - Jersey (coagulation): good, n = 27; poor, n = 25; - Holstein-Friesian (coagulation): good, n = 26; poor, n = 18; none, n = 6).	Rennet (chymosin)-induced coagulation	Significantly lower contents of total protein, total casein, minerals (Ca, P, Mg), and κ-casein were identified in A2/A2 as part of poorly coagulating milks.	Jensen et al. (2012b)
Milk samples (n = 892) - Jersey (n = 24); - Holstein-Friesian (n = 24).		The high prevalence of the β-casein B in milk was related to good coagulation ability, whereas poorly coagulating milk was associated with β-casein A2 variant.	Jensen et al. (2012a)
Milk samples (n = 121) - Swedish Red breed (n = 75); - Swedish Holstein breed (n = 46).		The A2/A2 phenotype in milk was associated with poor and the A1/A2 genotype with good coagulating properties and higher firmness.	Hallén et al. (2007)
Milk samples (n = 1299) - Danish Holstein (n = 456); - Danish Jersey (n = 436); Swedish Red (n = 407).		Most pronounced effect was the negative influence of A2 and I β-caseins on milk coagulation compared with β-casein A1, which was essential for curd firming rate and rennet coagulation time.	Poulsen et al. (2013)
Milk samples (n = 888) - Danish Holstein (n = 455); - Danish Jersey (n = 433).		The possible association between β-casein F and noncoagulation milk still remains to be elucidated as it was not directly related to the relative β-casein content.	Poulsen et al. (2017)
Milk samples (n = 299) - Italian Holstein Friesian mixed; milk samples contained different amont of either A1, A2, and B varinats.		The β-casein A1 family, but especially β-casein B varinat showed shorter rennet coaulation time, curd-firming time, and firmer gel compared to β-casein A2.	Vigolo et al. (2022a)
Milk samples (n = 1133; 50 mL from each cow) - Italian Holstein Friesian mixed; milk samples contained different amont of either: 1. A1/A1, A1/A2 or A2/A2 β-caseins; 2. A/A, A/B, or B/B κ-caseins; 3. A/A and B/B β-lactoglobulins.		The β-casein A1/B presented the best performace with a lowest rennet coagulation time and higher curd firmness at 30 min, followed by β-casein A1/A1. The worst cheese-making ability was attributed to β-casein A2/A2.	Bisutti et al. (2022)
Milk samples (n = unknown; 20 L per each milk type) - Brazilian A1/A1 (n = unknown) - Brazilian A2/A2 (n = unknown).	Rennet (chymosin)-induced coagulation; *Petit Suisse* and *Minas Fresca* cheeses manufacturing	A2/A2 compared to A1/A1 cheeses were characterised as a softer and creamier cheesess, but it did not compromised its sensory acceptance.	Mendes et al. (2019)
Milk samples (n = 2; 30 L per each milk type) - Kiwi Cross A1/A1 (n = 1) - Kiwi Cross A2/A2 (n = 1).	Acid-induced gelation Yoghurt manufactoring	Gels produced from A2/A2 milk were more porous, contained thinner protein strands, and had lower gel strength compared to gels from A1/A1 milk.	NguyenSchwendel et al. (2018)
Milk samples (n = 114) - Australian Holstein A1/A1 (n = 5) - Australian Holstein A1/A2 (n = 5) - Australian Holstein A2/A2 (n = 5).	Acid-induced gelation	The associated findings of the more porous A2/A2 milk gel compared to A1/A1 and A1/A2 gels might be related to the increased content of random/PPII structures due to the fact that Pro possesses a tendency to create these conformations.	Daniloski et al. (2022d)
Milk samples (n = 2) - Ultra-high temperature A2 milk - Normal bovine milk (Purchase: JDcom direct-sale store of "Ren Yang Yi Tou Niu" brand)	Acid-induced gelation - Commercial fermentation bacteria (*Lactobacillus delbrueckii* subsp. *bulgaricus* 6047 and *Streptococcus thermophilus* 6038. Mixed with: - *Lactiplantibacillus plantarum* (MWLp-12) and *Limosilactobacillus fermentum* (MWLf-4) isolated from human milk.	Fermented A2 milk possessed smoother microstructure, better texture and rheological properties than the fermented normal milk. Supplementation with MWLp-12 and MWLf-4 would bring in various advantages on firmness, consistency, water holding capacity, and acidity of fermented milk compared with only using commercial fermentation bacteria.	Wang et al. (2022)
	Heat stability		
Milk samples (n = 114) - Australian Holstein A1/A1 (n = 5) - Australian Holstein A1/A2 (n = 5) - Australian Holstein A2/A2 (n = 5).	Heating treatment 1. 72 °C for 15 s; 2. 121 °C for 2.6 min; 3. 140 °C for 3 s	A1/A1 and A1/A2 milks were characterised with greater amounts of calcium and phosphorus, and a higher net negative zeta potential than A2/A2 milk. Histidine present in A1/A1 milk govern the formation of dehydroalanine. Intramolecular β-sheet, β-turn, and random coil were found in A1/A1, A1/A2, and A2/A2	Daniloski et al. (2022b)

(*continued on next page*)

Table 3 (*continued*)

Sample type	Technological trait	Outcome	Reference
		milks; increasing the temperature decreased the intramolecular β-sheets in all three milk types.	
		Emulsion and foam	
Milk samples (n = 2; 30 L per each milk type) - Kiwi Cross A1/A1 (n = 1) - Kiwi Cross A2/A2 (n = 1).	Foam formation and stability	The reconstituted A2/A2 milk showed significantly better foam formation but minimal differences were observed between foam stabilities compared to A1/A1 milk; A2/A2 milk might be a good natural ingredient for dairy products where milk foam is important.	NguyenSchwendel et al. (2018)
Milk samples (n = unknown) Crude casein: protein prepartion β-casein A1 and A2 genetic variants.		The β-casein A1 exhibited the best foaming properties; It would thus appear that the β-casein A1 spread more extensively at the interface and facilitated a faster build up of a coherent interfacial layer. That corresponded in a foam that was both more voluminous and had increased stability compared to the β-casein A2.	Ipsen and Otte (2004)
Milk samples (n = unknown) - Holstein - Jersey - Geman black - German white Whole casein extracted.	Emulsion formation and stability	Both B and A1 variants of β-casein had a higher surface load and higher content of ordered structure in the absorbed state than the β-casein A2, which postulated a correlation with the emulsion-stabilising properties. However, A2 variant of β-casein was able to more rapidly reach the oil droplet surface; consequently more efficient as emulsion forming agent.	Darewicz and Dziuba (2007)
Bovine β-casein containing mainly the genetic variants A1 and A2.		The hydrophobic teflon surface layer favored the transformation of the loop fragments of β-casein and into α-helix. Moreover, dephosphorylation increased the helix-forming propensity. Suggested relationship between surface load and emulsions stabilising properties.	Darewicz et al. (2000)

4.2. Acid gelation and rennet coagulation

The gel strength, curd formation, water holding capacity, and syneresis of milk during acid gelation and rennet coagulation are important functional attributes essential for the end-product functionality for a variety of products, including yogurt and cheese (Lucey, 2020). In the last decade, several studies showed that β-casein genetic variants in milk can be correlated to milk gelation and coagulation properties (Bisutti et al., 2022; Gustavsson et al., 2014; NguyenSchwendel et al., 2018; Poulsen et al., 2013; Poulsen et al., 2017; Vigolo, Franzoi, Penasa and De Marchi, 2022a). During acid-induced milk gelation of Kiwi breeds and rennet-induced milk coagulation of Scandinavian and Italian breeds, bovine β-casein A2/A2 as part of the casein haplotype was found to be the dominant in non-coagulating and poor-coagulating milk samples (Bisutti et al., 2022; NguyenSchwendel et al., 2018; Poulsen et al., 2013).

Very recently, Bisutti et al. (2022) and Vigolo et al. (2022a) found during rennet-induced coagulation that milk containing β-casein A2/A2 showed extended rennet coagulation time and lowered curd firmness compared with the other genetic variants, particularly with respect to the milk containing β-casein A1/A1, which was also observed in other studies (Frederiksen et al., 2011; Jensen et al., 2012a, 2012b; Kumar et al., 2018; Poulsen et al., 2013, 2017). Mendes et al. (2019) reported that a longer time was needed for the rennet coagulation of A2/A2 milk, during the *Petit Suisse* cheese processing. The gel used for manufacturing the cheese was also more porous, contained thinner protein strands and showed low strength. As a result of that, the cheese was softer, creamier, and possessed different sensory characteristics. However, those attributes did not result in A2/A2 cheese samples to be unacceptable by the panellists (Mendes et al., 2019).

Moreover, NguyenSchwendel et al. (2018) showed that the storage modulus was significantly lower for acid-induced gels from A2/A2 milk compared to A1/A1 milk, and gels also had a more porous microstructure, thinner protein strands, and lower gel strength. This suggested that the acid-induced gels and yoghurt from A2/A2 milk were more prone to breakage and deformation by external mechanical powers (NguyenSchwendel et al., 2018). Similarly, observing the rheological and the structural characteristics of acid-induced A1/A1, A1/A2, and A2/A2 gels, Daniloski, McCarthy, Gazi, and Vasiljevic (2022d) determined that the firmer gels obtained from A1/A1 and A1/A2 milks possessed a greater storage modulus. On the contrary, Wang et al. (2022) concluded that acidified and fermented A2/A2 milk had smoother microstructure, better texture, and rheological properties than the fermented A1/A2 milk. Nevertheless, the authors neither stated the amount of proteins (ratio of caseins and whey proteins) in both milks, nor they declared the content of milk's minerals, both of which are crucial for firmness and structure of milk gels (Lucey, 2020).

Upon the gel creation, the system relies on re-arrangement of the bonds among individual caseins creating the original casein micelles (Lucey, 2020). Therefore, an improved gel firmness is rather associated with a higher number of such bonds (Lucey, 2002; Lucey et al., 2000; Van Vliet, Van Dijk, Zoon and Walstra, 1991). Namely, the rearrangements of the casein particles into a more compact structure would increase the number of bonds, which could lead to a gradual formation of more protein-protein bonds at each junction between the casein particles, resulting in firmer gels and decreased total free energy of the system (Daniloski et al., 2022d; Lucey et al., 2022; NguyenSchwendel et al., 2018). The reason behind this phenomena can be related to the difference in κ-casein contents in A1/A1, A1/A2, and A2/A2 gel types (Daniloski et al., 2022d; NguyenSchwendel et al., 2018; Poulsen et al., 2013). On that account, lower amount of κ-casein indeed translates into fewer interactions, at least at the surface of the casein micelles during coagulation (Lucey, 2020); milks comprised of β-casein A1 contained more κ-casein, that theoretically led to a much larger number of particles and higher surface area (Daniloski et al., 2022d; NguyenSchwendel et al., 2018). Therefore, in A2/A2 milk a lower number of interactions were created, compared to A1/A1 and A1/A2 milks and therefore a softer gel (Daniloski et al., 2022d).

The total and ionic calcium contents were found to greatly influence the acid-induced gelation and rennet-induced coagulation of bovine milk (Hallén et al., 2007; Poulsen et al., 2013; Poulsen and Larsen, 2021). In this regard, milk samples carrying β-casein A1 contained higher calcium amount, especially ionic calcium in A1/A2 milk compared to A2/A2 milk and smaller casein micelle sizes (Daniloski et al., 2022d; Day et al., 2015; Jensen et al., 2012a; Poulsen et al., 2013). Almost a decade ago, Gustavsson et al. (2014) revealed that higher calcium content in milk was related to smaller casein micelle size and improved rennet-induced gelation properties.

Compared to other caseins, the genetic variants of κ-casein have been most discussed and related to acid gelation and rennet coagulation of bovine milk (Bijl et al., 2014a; Bonfatti et al., 2010; Poulsen and Larsen, 2021). Different studies have found that the genetic polymorphisms of κ-casein might substantially affect the rennet-induced coagulation characteristics; milks containing κ-casein B variant were represented with a higher amount of κ-casein and smaller casein micelles, decreased curd firming time, and increased whey protein expulsion compared with milks carrying κ-casein A (Bisutti et al., 2022; Gambra et al., 2013). Similarly, Poulsen et al. (2013) and Day et al. (2015) suggested that lower levels of total κ-casein and the presence of κ-casein A, were associated with poor rennet-induced coagulation of milk and greater casein micelle size. In contrast, Ketto et al. (2017) found that the highest levels of micellar κ-casein and a high prevalence of κ-casein A variant were connected with milks possessing good acid-induced coagulation ability, firmer gels, and smaller casein micelles, also identified in the study of Daniloski et al. (2022d).

An important role during and upon milk processing is played by αs_1-casein and its genetic variants and phenotypes. When comparing the genetic variants of this protein, αs_1-casein C possesses a smaller net charge compared to αs_1-casein B. As a consequence, C variant of αs_1-casein has greater association constants and ultimately stronger self-association, which contributes to a firmer curd in cheese making (Fox et al., 2015; Sadler et al., 1968; Schmidt, 1970). Frederiksen et al. (2011) found that an increased content of αs_1-casein B in milk was considered as a main differentiating feature for the occurrence of the non-coagulating milks. Additionally, Poulsen et al. (2013) determined that αs_1-casein C improved milk coagulation. Even though, B and C are generally most discussed αs_1-casein variants, variant A is the most different compared to other variants. Its residues f14 - 26 are deleted, thus it is less hydrophobic, thus the curd formed during cheese making from milk with αs_1-casein A variant was found to be softer (Creamer et al., 1982; Sadler et al., 1968). On the contrary, the genetic variants of αs_2-casein did not show a substantial effect during and upon milk processing (Cipolat-Gotet et al. 2018), simply as a matter that it is hardly to see any genetic variation in this protein. A number of years ago, Ketto et al. (2017) found that the content of αs_2-casein was negatively correlated with properties of acid-induced gels. Furthermore, these authors also observed that due to the increased concentration of αs_2-casein in milk, and its correlation to κ-casein B, was a reason for the poor acid gelation of milk (Ketto et al., 2019).

The composite genotype of αs_1-β-κ-casein was found to have a stronger relationship with acid gelation and rennet coagulation properties than only a single protein phenotype (Gai et al., 2021). In this regard, an improved acid gel firming rate and firmness at 30 and 60 min, and shorter gelation times were correlated to B/B-A2/A2-A/A haplotypes compared to the other proteins' genotypes (Ketto et al., 2017). In contrast, Jensen et al. (2012a) stated that the same composite haplotype was predominant in poorly coagulating milks. The B/B-A2/A2-A/A (αs_1-β-κ-casein) haplotype was positively associated with percentages of fat and protein in Holstein cows, Brown Swiss cows (Boettcher et al., 2004), Finnish Ayrshire cows (Ikonen et al., 2001), and Italian Reggiana cows (Caroli et al., 2004), but negatively associated with milk yield (Boettcher et al., 2004). Interestingly, the composite β-κ-casein genotype, namely A1/A1-A/B, A1/A2-A/B, and A2/A2-A/B were associated with better firmness and shorter coagulation time (Comin et al., 2008). Frederiksen et al. (2011) actually related the composite A/B-A1/A2 (β-κ-casein) haplotype, considered as a sufficient factor for good milk coagulation properties, with a higher content of κ-casein in the gels. Thus, genetic selection of dairy cows for milk with good acid gelation or rennet coagulation abilities, should be highly considered, since they can potentially lead to an improvement in yoghurt and cheese production (Poulsen and Larsen, 2021).

4.3. Interfacial properties

The formation and stability of emulsions and foams are strongly dependent on the interactions between air and liquid (interfacial layer), and the surfactants adsorbed to this surface (Darewicz et al., 2000). The faster adsorption of surfactants on the interfacial layer and their greater capacity to minimise surface tension are crucial for the development of emulsions and foams (NguyenSchwendel et al., 2018). The fact that β-casein is a major constituent of casein micelles and is also commonly used as a foaming or emulsifying agent means that its association behaviour is of importance in the food industry (Chen et al., 2018; Neill and Jingsi, 2021). When comparing emulsion and foam formation and stabilisation, A2/A2 milk showed better foam formation than A1/A1 milk, however both milk types indicated similar foam stability (NguyenSchwendel et al., 2018). In addition, Darewicz and Dziuba (2007) revealed that the superior emulsion properties of β-casein A2 compared to β-casein A1 could be attributed to improved solubility of this protein, its faster migration and adsorption to the interfacial layer, driven mainly by hydrophobic interactions between its C-terminal tail and the surface, and less ordered structure (Darewicz and Dziuba, 2007; Raynes et al., 2015). On the contrary, almost two decades ago, Ipsen and Otte (2004) found that β-casein A1 possessed greater foaming properties (more voluminous foam with an increased stability) than β-casein A2, which appeared in accordance with their results from the measurements of surface pressure and interfacial rheology. Namely, the authors explained that β-casein A1 spread more extensively at the interface and facilitated a faster build up of a coherent interfacial layer (Ipsen and Otte, 2004). Thus, the additional Pro^{67} (found to form a hinge between the polar C-terminal and the primarily hydrophobic N-terminal region) provided for a less extensive part of the hydrophobic domain of β-casein A2 to be adsorbed on the interfacial layer, thus explaining why the β-casein A2 was less space filling compared to β-casein A1 (Ipsen and Otte, 2004).

Hemar et al. (2021) reported no noticeable differences between the physicochemical and interfacial properties of sodium caseinate dispersions obtained from A1/A2 and A2/A2 milks. However, Daniloski, McCarthy, Auldist, and Vasiljevic (2022e) observed that the sodium caseinates carrying β-casein A2 were more efficient as emulsifying agents than the sodium caseinate with β-casein A1. The authors explained that the presence of α-helixes was the main driver for the different protein structure of A1/A1, A1/A2, and A2/A2 sodium caseinates (Daniloski et al., 2022e). The α-helical conformational motifs were found predominately in A1/A1, A1/A2 milks (Daniloski et al., 2022c) and β-casein A1 (Darewicz and Dziuba, 2007). These conformations display a tight structure with no cavities, which may play a role in driving different functionalities. The superior emulsion and foam forming capabilities indicate that A2/A2 milk can potentially be a good natural ingredient for dairy products where milk foam is essential, such as ice cream, whipped cream, mousses and cappuccino's milk with better alternative quality and enhanced sensory attributes.

Although the impact of the genetic variants of all 4 caseins on physicochemical and functional properties of dairy products have been extensively studied and reviewed (Gai et al., 2021; Mendes et al., 2019; NguyenSchwendel et al., 2018; Poulsen and Larsen, 2021), there is no consensus on the structure of casein micelle governed by the major milk proteins and their genetic variants. Therefore, greater scale and extensive data studies that would contain different levels of αs_1-, αs_2-, β-, and κ-caseins including their polymorphic variants, degree of κ-casein glycosylation, composite αs_1-αs_2-β-κ-casein variants are needed to further elaborate on the impact of these genetic variants on both milk and micellar casein. These genetic variants, as stated above, may influence various interactions in the casein micelle and its size, mineral levels, protein conformation, and most importantly, the functionality of milk and dairy products (Bijl et al., 2014a, 2020; Daniloski et al., 2022a; Ketto et al., 2017; Vallas et al., 2012).

5. Conclusion

It was hypothesised in this review that differences in β-casein genetic variants can have important implications on certain milk product characteristics. Based on the studies performed over the last number of years, it is obvious that β-casein A1 and A2 phenotypes are quite different, not only by their composition but based on their functionality and behaviour to environmental factors. Despite the importance of these two β-casein variants, most studies have focused on the association between κ-casein genetic variants and the physio-chemical and functional properties of bovine milk. Milk samples carrying β-casein A2 possess a larger average micelle size than samples carrying β-casein A1, simply as a result of less κ-casein present on the micelle surface. In contrast, limited research has been performed on αs_2-casein protein fractions because it is difficult to identify genetic variation in this protein. As far as technological traits are concerned, milk comprised of β-casein A2 has usually been associated with poorer acid gelation and rennet coagulation properties, but superior emulsion and foam formation capabilities. For instance, milk with β-casein A2 is less suitable for cheese- or yoghurt making, however, the weak gel it produces could potentially be responsible for its proposed easier digestibility (Milan et al., 2020), which might be advantageous for certain applications. On the other hand, isolated and purified β-casein A2 produces smaller β-casein micelles and creates poorer and less stable foams compared to β-casein A1, which explains the complexity of the milk system and the importance to define the factors that influence these variations. Therefore, the mechanism of producing dairy products with the same properties using either β-casein A1 or A2, or the factors, which influence gelation, emulsion and foam stability, are not yet fully understood. Given the significant role that milk composition plays in functional properties, further functionality testing correlated to β-casein phenotype is required to fully identify how a single amino acid substitution can have such a significant impact on milk functionality.

CRediT authorship contribution statement

Davor Daniloski: conceived the study and research question, designed, Writing – original draft, Conceptualization, reviewed, edited the manuscript, designed the tables and the figures, Methodology, Formal analysis, Investigation. **Noel A. McCarthy:** Formal analysis, provided critical feedback and analysis, Funding acquisition, reviewed and edited the manuscript, supervised the study. **Thom Huppertz:** provided critical feedback, reviewed and edited the manuscript, All authors have contributed to the manuscript and reviewed the final version. **Todor Vasiljevic:** Formal analysis, provided critical feedback and analysis, Funding acquisition, reviewed and edited the manuscript, supervised the study.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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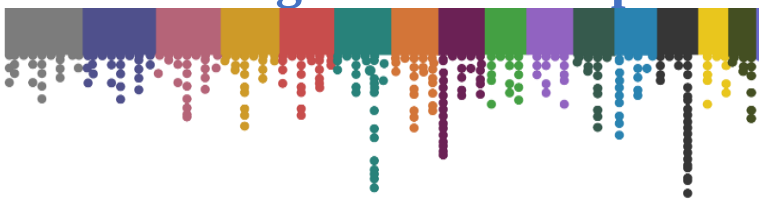

Casein micelle with different β-casein phenotypes: Fingerprinting pH-induced structural changes using FTIR and NMR spectroscopies

- FTIR, 1D- and 2D-NMRs identified conformational differences in casein particles
- Structurally at pH 5.7, A1/A1, A1/A2 and A2/A2 casein micelles were similar
- Acidification increased PPII structures in A2/A2 casein
- Aggregated β-sheets in the samples appear related to κ-casein phenotypes
- In A2/A2 sample, β-casein A2 showed stronger connection to other caseins

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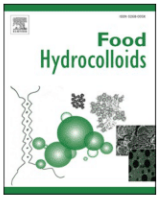

Casein micelle with different β-casein phenotypes: Fingerprinting pH-induced structural changes using FTIR and NMR spectroscopies

Davor Daniloski [a,b], Tatijana Markoska [a], Noel A. McCarthy [b], Todor Vasiljevic [a,*]

[a] Victoria University, Advanced Food Systems Research Unit, Institute for Sustainable Industries and Liveable Cities and College of Health and Biomedicine, Melbourne, Victoria, 8001, Australia

[b] Teagasc Food Research Centre, Food Chemistry and Technology Department, Moorepark, Fermoy, P61 C996, Cork, Ireland

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ABSTRACT

The aim of this study was to investigate the structural characteristics of casein micelles and casein particles at pH 6.7, 5.7, and 2.3 containing β-caseins A1/A1, A1/A2, and A2/A2 at 37 °C using Nuclear Magnetic Resonance, Fourier-Transform Infrared spectroscopies and chemometrics. The amount of all released caseins from the casein micelle at pH 6.7 was significantly different after the pH had been reduced to 5.7 and subsequently decreased to 2.3. Results showed variation in the structure of all three samples mainly dependent of caseins content, caseins' phenotypes, and pH modulation. During the pH modulation, higher levels of α-helixes and intramolecular β-sheets were found in A1/A1 casein, whilst aggregated β-sheets, β-turns, and polyproline II helixes dominated in A1/A2 and A2/A2 samples. Principal Component Analysis was used to characterise and distinguish among the structure of the three caseins based on spectral data. While samples containing β-casein A2 possessed structural properties different to A1/A1 samples at pH 6.7 and 2.3, at pH 5.7 all casein micelles behaved in a similar manner. This study brings significant insights in the importance of the observed casein phenotypes on the structural arrangement of A1/A1, A1/A2, and A2/A2 casein micelles, hence defining their main conformational differences essential for the dairy industry.

1. Introduction

The bovine caseins are categorised into four different types, α_{s1}-, α_{s2}-, β- and κ-caseins, all of which contribute to a spherical structure known as the casein micelle. Generally speaking, to form a casein micelle, caseins interact with each other via predominantly non-covalent, but also some covalent interactions, as well as via ionic interactions with calcium phosphate nanoclusters (Lucey & Horne, 2018). The main purpose of the casein micelle is the transport of proteins, calcium, and phosphate at elevated amounts that would otherwise be insoluble in water (Horne, 2020; Huppertz et al., 2017). The casein micelles have an essential function in the production of milk, milk processing, and conversions to different dairy products. In this regard, it is of great interest to shed light on compositional changes and understanding the alternation of the casein micelle structure caused by some environmental factors (Corredig, Nair, Li, Eshpari, & Zhao, 2019; Huppertz, Fox, & Kelly, 2018).

According to Farrell Jr, Malin, Brown, and Qi (2006), the structure of casein micelle could only come about through functional interactions and absence or limited influence of dysfunctional interactions. Different models of casein micelle assembly and structure have been proposed (Dalgleish, 2011; Dalgleish & Corredig, 2012; Dalgleish, Spagnuolo, & Douglas Goff, 2004; De Kruif, Huppertz, Urban, & Petukhov, 2012; Holt, 1992, 2004, 2016; Holt & Carver, 2022; Horne, 1998, 2017, 2020; Huppertz et al., 2017; Slattery, 1976; Slattery & Evard, 1973). Nevertheless, despite its ubiquity and numerous investigations, there is also no general consensus on the fine structure of the casein micelle assembly since its conformation cannot be simply visualised (Huppertz & Gazi, 2022).

Bovine milk casein consists of approximately 35% β-casein with β-casein A2-5P as a reference proteoform. This protein contains 209 amino acids, including 5 phosphorylated serine residues (Huppertz et al., 2018). There are as many as 12–17 genetic variants of β-casein with A2 and A1 as the most prominent ones (Caroli, Chessa, & Erhardt, 2009; Farrell et al., 2004; Nadugala, Pagel, Raynes, Ranadheera, & Logan, 2022). It is postulated that A2 variant of β-casein carries the original amino acid sequence, before a point mutation caused the appearance of β-casein A1 in some European herds (Ng-Kwai-Hang & Grosclaude, 2003). Namely, as a result of a single nucleotide

* Corresponding author.
E-mail address: todor.vasiljevic@vu.edu.au (T. Vasiljevic).

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polymorphism on exon VII and the 6th chromosome of CSN2 gene, the transfer from cytosine to adenine contributed to the substitution of proline (Pro) in β-casein A2 with histidine (His) in β-casein A1 at position 67 in their polypeptide chains (Caroli et al., 2009). It was found that both genetic variants of β-casein might influence the internal structure of casein micelle (Day, Williams, Otter, & Augustin, 2015) by investigating the protein expression (Raynes, Day, Augustin, & Carver, 2015), but especially the techno-functional properties of milk and dairy products (Daniloski, McCarthy, Gazi, & Vasiljevic, 2022; Daniloski, McCarthy, & Vasiljevic, 2022; Nguyen, Schwendel, Harland, & Day, 2018; Poulsen et al., 2017).

Considering the constraints surrounding sample preparation for the investigation of the structural behaviour of the casein micelle, Fourier Transform Infrared (FTIR) and Nuclear Magnetic Resonance (NMR) spectroscopies were classified as accessible spectroscopy techniques that do not require sample modification (Boiani, Fenelon, FitzGerald, & Kelly, 2018; Daniloski, McCarthy, O'Callaghan, & Vasiljevic, 2022). They have been used to examine some features of casein micelle; for example, secondary structure of caseins (Byler & Farrell, 1989; Holt & Sawyer, 1993), behaviour of casein micelle and its particles during membrane separation and diafiltration of skim milk (Boiani, McLoughlin, Auty, FitzGerald, & Kelly, 2017), and interaction between minerals and casein micelle (Boiani et al., 2018). Although, the presence of some structural elements in the casein micelle have already been confirmed (Curley, Kumosinski, Unruh, & Farrell, 1998; Farrell Jr, Wickham, Unruh, Qi, & Hoagland, 2001; Farrell, Qi, Wickham, & Unruh, 2002; Nasser et al., 2018; Rasmussen et al., 1999; Rollema & Brinkhuis, 1989), limited focus was allocated to observe the effect of the single amino acid mutation in the polypeptide chain of β-casein on the structure of casein micelles.

The current work involved structural analysis of casein micelles (casein particles) at three pH values (6.7, 5.7 and 2.3) and one temperature (37 °C) in order to establish their conformational transitions. These pH conditions are highly appreciated for the formation of various types of interactions, including electrostatic bonds, calcium bonds, hydrophobic interactions, van der Waals, and hydrogen bonds (McSweeney & Fox, 2013). The diversity of these interactions is found to be dominant factor in formation of a homogeneous, three-dimensional protein network with an improved firmness, known as significant trait in yoghurt and cheese manufacturing (Marchesseau, Gastaldi, Lagaude, & Cuq, 1997). For instance, typically, the native milk pH is 6.7 and is subsequently adjusted between 5.8 and 5.6 prior to rennet addition essential for some conventional chesses using a starter culture (Guinee, Harrington, Corcoran, Mulholland, & Mujllins, 2000; McSweeney & Fox, 2013). From a digestion point of view, pH 6.7 and 5.7 were used to observe how the casein micelle structure changes in pH environment close to native intraluminal pH and for the curd modifications during intestinal digestion. Further, pH 2.3 provides insight into the behaviour of casein particles at gastric conditions. The selected temperature was used to observe the structural motifs of casein micelles and its particles mimicking human body temperature (Brodkorb et al., 2019; Fallingborg, 1999).

Therefore, the aim of the present research was to identify the structural components of casein micelles containing β-casein A1/A1, A1/A2 or A2/A2, using FTIR and NMR spectroscopies. The findings will assist in understanding the conformational changes of casein micelle and its particles under certain environmental conditions that might give some indication regarding the different behaviour of β-casein A2 and A1 during milk processing and subsequently the digestion of milk and dairy products carrying various β-casein phenotypes.

2. Materials and methods

2.1. Milk sampling

Milk samples from nine Holstein-Friesian cows with different β-casein phenotypes (A1/A1 cow: n = 3; A1/A2 cow: n = 3; and A2/A2 cow: n = 3) but same genetic variants of κ- and αs-caseins were gifted by the Agriculture Victoria Research Centre in Ellinbank (Victoria, Australia). Using one milk sample per β-casein phenotype daily, allowed triplicate procedure measurements to be performed over period of three consecutive days. In particular, immediately after milking, milk samples from each phenotype were pooled into individual containers and chilled at refrigerator temperature. To determine an approximate milk composition for all samples, a Lactoscan milk analyser (Lactoscan LS-60, Milkotronic Ltd., Nova Zagora, Bulgaria) was used (data not shown). All milk samples were defatted by centrifugation (Avanti J-26XP, Beckman instrument Australia Pty. Ltd, Gladesville, NSW, Australia) at 3225×*g* for 20 min at 20 °C, and the subsequent cream layer was removed. In order to minimise microbial changes, the skimmed milk samples were preserved by adding sodium azide (0.01%: Sigma-Aldrich, St. Louis, MO, USA).

2.2. Preparation of casein samples

Aliquots of skim milk samples (n = 9) were ultracentrifuged at 20 °C for 1 h at 100000×*g* (Ultra L - 70 Centrifuge, Beckman Coulter, Indianapolis, IN, USA) where the micellar phase (pellet) and serum phase were separated (Daniloski, McCarthy, Markoska, Auldist, & Vasiljevic, 2022). Subsequently, the pellets were washed three additional times with simulated milk ultrafiltrate (SMUF, at 20 °C) (Jenness, 1962) to remove residual whey protein and lactose, and after each wash the samples were re-centrifuged at 20 °C for 1 h at 100000×*g* (Ultra L - 70 Centrifuge, Beckman Coulter, Indianapolis, IN, USA). The washed casein micelle samples were then frozen and lyophilised (model FD-300, Airvac Engineering Pty. Ltd., Dandenong, Australia), using a primary and secondary drying step of - 80 °C for 66 and 8 h, respectively. The casein micelle powders were vacuum packed in double-sealed plastic bags at ambient temperature and stored at - 80 °C until further use.

After that, all casein micelle samples (containing 94.21–96.34% protein dry-basis) were rehydrated to the same protein content (2.60%, w/w) in a SMUF solution at 4 °C. To ensure complete solubilisation, the reconstituted dispersions were gently mixed using a magnetic stirrer bar and left to hydrate overnight at 4 °C. Prior to analysis, the casein particle dispersions were equilibrated at 37 °C in a water bath and had a pH of 6.7. Sub-aliquots of each dispersion containing either β-caseins A1/A1, A1/A2, or A2/A2 were acidified to pH 5.7 and 2.3, respectively using 2 M HCL (Sigma-Aldrich, St. Louis, MO, USA). The pH of all dispersions was measured with a calibrated pH meter equipped with a combined pH electrode, temperature sensor, and fixed cable (Metrohm AG, Oberdorfatrasse, Herisau, Switzerland).

In order to check the amount of individual caseins that have dissociated from the casein micelle after acidification, the samples were ultracentrifuged at 20 °C for 1 h at 100000×*g* (Ultra L - 70). The temperature for the FTIR analysis was measured manually using a temperature probe and for the NMR analysis was automated by the instrument. The different casein micelle dispersions at pH 6.7 and 5.7 were named A1/A1, A1/A2 and A2/A2 casein micelles, when obtained from A1/A1, A1/A2, and A2/A2 milks, respectively. As for the samples at pH 2.3, the samples were named as: A1/A1, A1/A2, or A2/A2 casein particles.

2.3. Properties of casein samples

2.3.1. Protein profiling and analysis

Total nitrogen and protein content were determined using the Kjeldahl method (ISO, 2014, pp. 1–18) and a nitrogen to protein conversion factor of 6.38. The protein profile of all dispersions was analysed using the Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) (Daniloski, McCarthy, Gazi, & Vasiljevic, 2022). The RP-HPLC was performed for protein separation, identification and quantification using a Jupiter C4 column (Phenomenex Aeris WIDEPORE, 150 mm × 4.6 mm, 3.6 μm particle size, 300 Å porosity,

Torrance, USA). The samples (0.8 mL) were reduced using a denaturing urea solution (3.2 mL: 8 M urea, 165 mM Tris, 44 mM sodium citrate, and 0.3 mL v/v β-mercaptoethanol, Sigma-Aldrich, St. Louis, MO, USA). Elution was attained with Milli-Q water as Eluent A, and acetonitrile (ACN: Sigma-Aldrich, MO, USA) as Eluent B, both containing 0.1% trifluoroacetic acid (TFA; Sigma-Aldrich, MO, USA). The identification of the genetic variants of milk proteins was performed by using a reversed-phase analytical column C8 (Zorbax 300SB-C8 RP, Agilent Technologies, Santa Clara, SA, USA) with a silica-based packing (150 mm × 4.6 mm, 3.5 μm particle size, 300 Å) (Vigolo, Franzoi, Penasa, & De Marchi, 2022). The samples were prepared by denaturing, reducing, and acidifying in an aqueous solution of guanidine (Gdn) HCL (6 M GdnHCL, 0.1 M bisTris buffer, 5.37 mM sodium citrate, and 19.5 mM DTT, Sigma-Aldrich, St. Louis, MO, USA) in a 1:1 ratio (v/v). Each sample was vortexed for 10 s, incubated at room temperature for 1 h to promote protein solubilisation, and diluted in the proportion 1:3 (v/v) with a solution containing 4.5 M GdnHCl in Milli-Q water, ACN, and TFA (100:900:1, Sigma-Aldrich, St. Louis, MO, USA). Both columns operated at 37 °C, UV detection at 214 nm, and a gradient flow rate of 500 μL/min. Protein analysis was carried out in triplicate to quantify total and individual casein (α-, β-, and κ-caseins) and the possible presence of whey protein (β-lactoglobulin and α-lactalbumin) fractions.

2.3.2. Infrared analysis

Fourier Transform Infrared (FTIR) spectra were collected on an Attenuated Total Reflectance (ATR)-FTIR spectrometer (Frontier 1, PerkinElmer, Boston, MA, USA) between the ranges of 4000 to 400 cm^{-1}. The range resolution of the spectra was 4 cm^{-1} and 16 scans per spectrum were recorded, corresponding to a sampling rate of one spectrum per 1 min. All analyses were performed at 37 °C. The spectra were obtained in the absorbance mode. In addition, the background (SMUF) was collected before every sample and was measured with a blank Diamond ATR cell utilising the same instrumental conditions as for the sample spectra acquisition. The FTIR spectra were derived upon baseline corrections allowing the results to be focused on the caseins and only on a specific region (Daniloski, McCarthy, O'Callaghan, & Vasiljevic, 2022).

2.3.3. Magnetic resonance analysis

Both one (1D NMR) and two (2D NMR) dimensional H-NMRs were used to determine the chemical shifts (δ ppm) of the proton signals of the components in the casein particles following the method by Markoska, Daniloski, Vasiljevic, and Huppertz (2021) with slight modifications. The spectra were calibrated using the water peak as an external reference and its chemical shift was 4.65 ppm (Hu, Furihata, Ito-Ishida, Kaminogawa, & Tanokura, 2004). The spectra of the samples were analysed by referring to the published data of chemical shifts for most compounds of milk (Garwolińska, Hewelt-Belka, Kot-Wasik, & Sundekilde, 2020; Rollema & Brinkhuis, 1989; Sundekilde, Larsen, & Bertram, 2013). To lock the NMR signal, an internal chemical shift reference was made by combining the samples (10 mg/mL protein) with 1 mL of a dispersion of ultra-pure water and deuterium oxide (99.8 atom percent excess, Sigma-Aldrich, St. Louis, MO, USA) in a ratio of 9:1, followed by 30 s mixing in a 1.5 mL Eppendorf tube (Daniloski, McCarthy, Auldist, & Vasiljevic, 2022). Approximately 0.6 mL of each solution was transferred using a glass pipette into a clean 5 mm standard NMR tube (Sigma-Aldrich, St. Louis, MO, USA). The 1D NMR measurements were acquired on a Bruker Avance spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) at 37 °C, operating at a 600 MHz transmitter frequency using 5 mm TXI probe with Z- and XYZ-gradients, using 16 scans and spectra width of 9615 Hz. The bins corresponding to both, water (4.63–4.99 ppm) and lactose (2.85–3.10 ppm) regions were eliminated from the qualitative and quantitative analysis (Daniloski, McCarthy, Auldist, & Vasiljevic, 2022). Utilising the same spectrometer operating at 37 °C, the total correlation (TOCSY) and nuclear overhauser effect (NOESY) spectroscopies were the two-dimensional approaches employed in this investigation. The 2D NMR spectra were obtained with 16 scans and a spectral width of 8196 Hz for the TOCSY spectra and 5882 Hz for the NOESY spectra. The acquisition mode used for the 2D spectra (obtained for the water suppression) was time-proportional phase incrementation (States-TPPI) (Markoska et al., 2021).

2.3.4. Pre-processing of the spectra and chemometric analysis

The FTIR data was initially corrected using a Spectragryph software (v. 1.2.7, Oberstdorf, Germany), followed by a derivatisation (second derivative of the absorbance in the Amide I region between 1700 and 1600 cm^{-1}) originally described by Savitzky and Golay (1964) (9 smoothing points, polynomial older 3). Within the Amide I band, several regions were analysed, including intermolecular/aggregated β-sheet (1700 - 1682 cm^{-1}); β-turn (1681 - 1665 cm^{-1}); α-helix (1664 - 1646 cm^{-1}); random coil (1645 - 1638 cm^{-1}); intramolecular β-sheet (1637 - 1615 cm^{-1}); and side chain (1614 - 1600 cm^{-1}). The area of each component representing secondary protein structures was estimated using the method of Daniloski, McCarthy, O'Callaghan, and Vasiljevic (2022) with Origin software (Origin Pro 2021, v. 95E, OriginLab Corporation, Northampton, MA, USA).

All NMR spectral data were processed using TopSpin (version 4.1.1) software (Bruker BioSpin, Billerica, Massachusetts, USA). The phase correction was performed manually by either 0^{th} or 1st order correction for pk or the baseline and the noise correction was adjusted using qfil mode to 0.1 ppm filter width and symmetrical noise correction for the homonuclear spectra. The NMR spectra were analysed using four NMR regions e.g., Amino (Aromatic and NH regions: 9.00–5.60 ppm), Amide (5.50–3.60), Aliphatic (3.50–1.60 ppm) and Methyl (1.50–0.00 ppm) regions where the area of each component was analysed and estimated (Origin software, Origin Pro 2021, v. 95E, OriginLab Corporation, Northampton, MA, USA) (Markoska et al., 2021).

Calculation of averages and multivariate Principal Component Analyses (PCA) for both FTIR and NMR data were obtained with the software Origin (Origin Pro 2021, v. 95E, OriginLab Corporation, Northampton, MA, USA). Although the NMR spectra were acquired at the same instrument setting for all samples (Sundekilde, Frederiksen, Clausen, Larsen, & Bertram, 2011), the normalisation of the spectra was performed. Specifically, the integral bins were created in such a manner as to ensure that each resonance was in the same bin throughout all spectra. Custom bin sizes were created for each resonance over all spectra, with overlapping resonances being considered together in one bin. The spectra were identical to those used for quantification using the targeted profiling approach. All bins were normalised to the total signal intensity to provide a measure of the absolute contributions of resonances to the spectrum. For this, each spectrum was normalised to its total intensity (sum of all buckets) (Ehlers et al., 2022; Webb-Robertson et al., 2005). The PCA analyses were conducted on the second derivatives of the absorbance within the Amide I region (1700 - 1600 cm^{-1}) and the chemical shifts (9.00–0.00 ppm) for FTIR and NMR data, respectively.

2.3.5. Statistical analysis

The data obtained were analysed using Analysis of variance (two-way ANOVA with temperature as fixed factor; pH and β-casein phenotype as random factors) and the level of significance set at $p < 0.05$ (Minitab statistical software Version 20; Minitab, Pennsylvania, USA). The mean comparison was made using Tukey - HSD's post hoc test. Production and analysis of all samples was conducted in triplicate.

3. Results

3.1. Casein micelle composition analysed by RP-HPLC

The chromatography analysis allowed for acquisition of detailed protein composition and estimated concentrations of the casein proteins is presented in Table 1. As it can be observed, between pH 6.7 and 2.3

Table 1
Protein composition of A1/A1, A1/A2, and A2/A2 casein micelles and casein particles as determined by the Reversed Phase - High Performance Liquid Chromatography.

		Protein content (mg/mL)						
Sample	pH	κ-casein A	κ-casein B	αs_2-casein A	αs_1-casein B	β-casein A2	β-casein A1	Protein content in the pellet after ultracentrifugation
A1/A1	6.7	1.72 ± 0.04^{ab}	1.25 ± 0.01^{ab}	2.52 ± 0.37^{a}	9.45 ± 0.12^{ab}	n/a	10.79 ± 0.23^{a}	25.89 ± 1.34^{a}
A1/A2		1.74 ± 0.01^{ab}	1.34 ± 0.00^{a}	2.35 ± 0.02^{ab}	9.46 ± 0.01^{ab}	4.35 ± 0.22^{b}	6.33 ± 0.02^{b}	25.64 ± 0.82^{a}
A2/A2		2.32 ± 0.02^{a}	1.11 ± 0.01^{b}	2.15 ± 0.01^{b}	9.82 ± 0.21^{a}	10.01 ± 0.11^{a}	n/a	25.52 ± 0.65^{a}
A1/A1	5.7	0.76 ± 0.06^{c}	0.64 ± 0.05^{c}	1.51 ± 0.05^{c}	5.38 ± 0.15^{cd}	n/a	5.64 ± 0.01^{bc}	13.95 ± 2.11^{bc}
A1/A2		0.97 ± 0.22^{bc}	0.55 ± 0.01^{cd}	1.22 ± 0.01^{d}	5.54 ± 0.15^{c}	3.20 ± 0.00^{c}	2.81 ± 0.00^{d}	14.37 ± 1.78^{b}
A2/A2		1.28 ± 0.19^{b}	0.11 ± 0.01^{e}	1.23 ± 0.02^{d}	6.32 ± 0.02^{bc}	4.90 ± 0.02^{b}	n/a	13.92 ± 1.46^{bc}
A1/A1	2.3	0.26 ± 0.02^{d}	0.34 ± 0.15^{de}	1.33 ± 0.04^{cd}	5.01 ± 0.00^{d}	n/a	5.14 ± 0.01^{c}	12.81 ± 1.22^{c}
A1/A2		0.63 ± 0.01^{cd}	0.46 ± 0.03^{d}	0.84 ± 0.01^{e}	5.39 ± 0.20^{cd}	2.95 ± 0.21^{c}	2.74 ± 0.05^{d}	13.09 ± 1.74^{c}
A2/A2		1.26 ± 0.00^{b}	0.08 ± 0.01^{e}	1.04 ± 0.00^{de}	5.99 ± 0.01^{bc}	4.62 ± 0.02^{b}	n/a	13.05 ± 2.39^{c}

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$); n/d: not detectable.

and after ultracentrifugation (section 2.2.), the content of all caseins dissociated from the casein micelle was greater for A1/A1, compared to those in A1/A2 and A2/A2 casein micelle ($p < 0.05$, Table 1). It is worth nothing that this casein dissociation might be one of the reasons for A1/A1, A1/A2, and A2/A2 samples to possess different conformational motifs. In spite some casein proteins were lost during the washing period (section 2.2.; data not shown), a highly significant proportions of both αs_1-and β-caseins were still present in all casein micelle dispersions (approximately 80% of the total casein content [$p < 0.05$, Table 1]). Namely, these proteins were probably still located in the casein micelle and thereby is expected that they both maintained the integrity of the casein micelle.

Even though the three casein dispersions were reconstituted to the same protein content (section 2.2.), the amounts of both, total κ- and αs_1-caseins at pH 6.7 were moderately greater in the samples carrying β-casein A2 ($p < 0.05$). Regarding the genetic variants of κ-casein, A1/A1 and A1/A2 casein micelles at pH 6.7 contained ≈25–32% more κ-casein A than κ-casein B; a similar ratio was maintained during acidification at pH 5.7 and 2.3 ($p < 0.05$, Table 1). Interestingly, in A2/A2 sample, the amount of κ-casein A was greater by 71% compared to the amount of κ-casein B; the difference in the content between these two genetic variants tremendously increased to ≈176% after acidification at pH 5.7 and 2.3 ($p < 0.05$). The amount of αs_1-casein upon the pH alternation was moderately lower in the samples carrying β-casein A1 (Table 1); specifically, between 4 and 18% lower in A1/A1 and A1/A2 samples, respectively in comparison with the amount of αs_1-casein in A2/A2 sample (Table 1).

Furthermore, A1/A2 but especially A1/A1 samples, were comprised of higher amounts of β- and αs_2-caseins ($p < 0.05$) compared to A2/A2 sample (Table 1). The same trend was observed even after their acidification ($p < 0.05$, Table 1). Hence, the concentration of β-casein in the casein micelle decreased by 5.39 mg/mL between pH 6.7 and 2.3 in A2/A2 sample and its content was lower by almost 11 and 21% than that in A1/A1 and A1/A2 caseins at pH 2.3, respectively ($p < 0.05$, Table 1). As for αs_2-casein, during acidification, A1/A1 sample counted almost 7–45% higher amount of αs_2-casein compared to the samples carrying β-casein A2 ($p < 0.05$). Unsurprisingly, no peak corresponding to either β-Lactoglobulin or α-Lactalbumin was detected in the casein dispersions, thus confirming that the samples contained only purified casein micelles (data not shown).

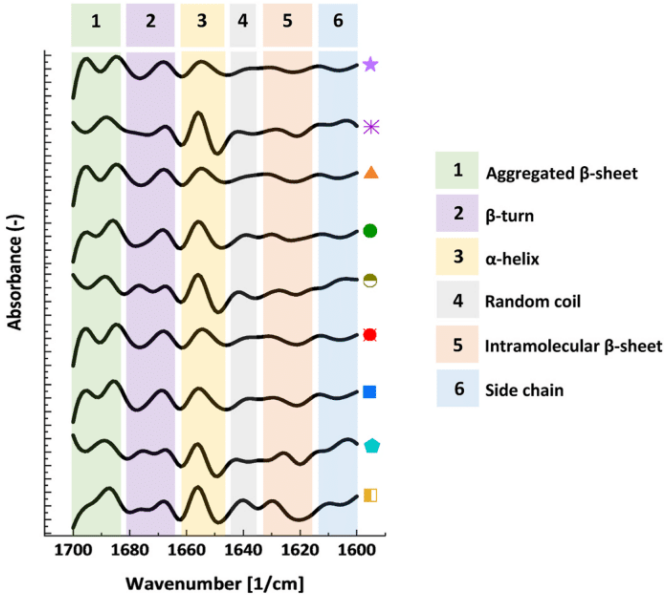

Fig. 1. Second derivative spectra of Amide I region of caseins.

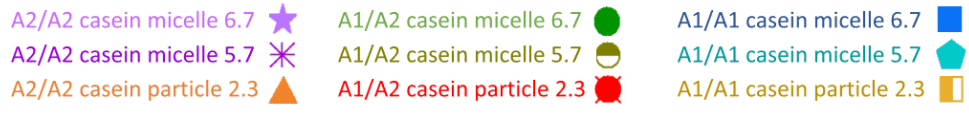

Table 2
Total percentage areas of different secondary structures in Amide I region in all three phenotypes of casein micelles and casein particles.

Sample	pH	Side chain	Intramolecular β-sheet	Random coil	α-helix	β-turn	Aggregated β-sheet
A1/A1	6.7	6.82 ± 1.60^{a}	14.83 ± 0.69^{b}	9.74 ± 1.45^{bcd}	26.81 ± 0.88^{bc}	33.06 ± 3.31^{b}	8.74 ± 1.26^{c}
A1/A2		6.73 ± 0.84^{a}	10.84 ± 0.86^{b}	n/a	40.47 ± 1.17^{a}	28.69 ± 0.69^{b}	13.27 ± 1.08^{b}
A2/A2		7.45 ± 0.38^{a}	11.48 ± 2.62^{b}	9.29 ± 3.32^{bcd}	n/a	49.82 ± 1.81^{a}	21.95 ± 4.10^{a}
A1/A1	5.7	4.73 ± 0.98^{b}	24.30 ± 2.99^{a}	14.55 ± 2.81^{bc}	33.69 ± 2.05^{abc}	15.13 ± 0.67^{de}	7.59 ± 0.04^{c}
A1/A2		3.99 ± 0.83^{b}	n/a	42.18 ± 6.19^{a}	34.81 ± 8.33^{ab}	7.60 ± 1.90^{e}	11.41 ± 1.23^{b}
A2/A2		3.36 ± 0.25^{b}	16.69 ± 3.18^{b}	16.63 ± 1.46^{b}	36.11 ± 0.22^{ab}	18.90 ± 1.56^{cd}	8.31 ± 1.78^{c}
A1/A1	2.3	5.48 ± 0.71^{a}	24.40 ± 4.69^{a}	6.87 ± 0.32^{de}	36.01 ± 4.11^{ab}	18.73 ± 7.76^{cd}	8.52 ± 2.01^{c}
A1/A2		5.79 ± 0.43^{a}	13.11 ± 0.57^{b}	n/a	33.43 ± 1.89^{abc}	25.58 ± 1.86^{bc}	22.10 ± 0.40^{a}
A2/A2		5.96 ± 0.57^{a}	12.93 ± 1.03^{b}	8.18 ± 1.03^{cd}	24.70 ± 1.75^{c}	26.50 ± 1.40^{bc}	21.73 ± 1.57^{a}
Band frequency (cm^{-1})		1600–1614	1615–1637	1638–1645	1646–1664	1665–1681	1682–1700

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$); n/a - not applicable.

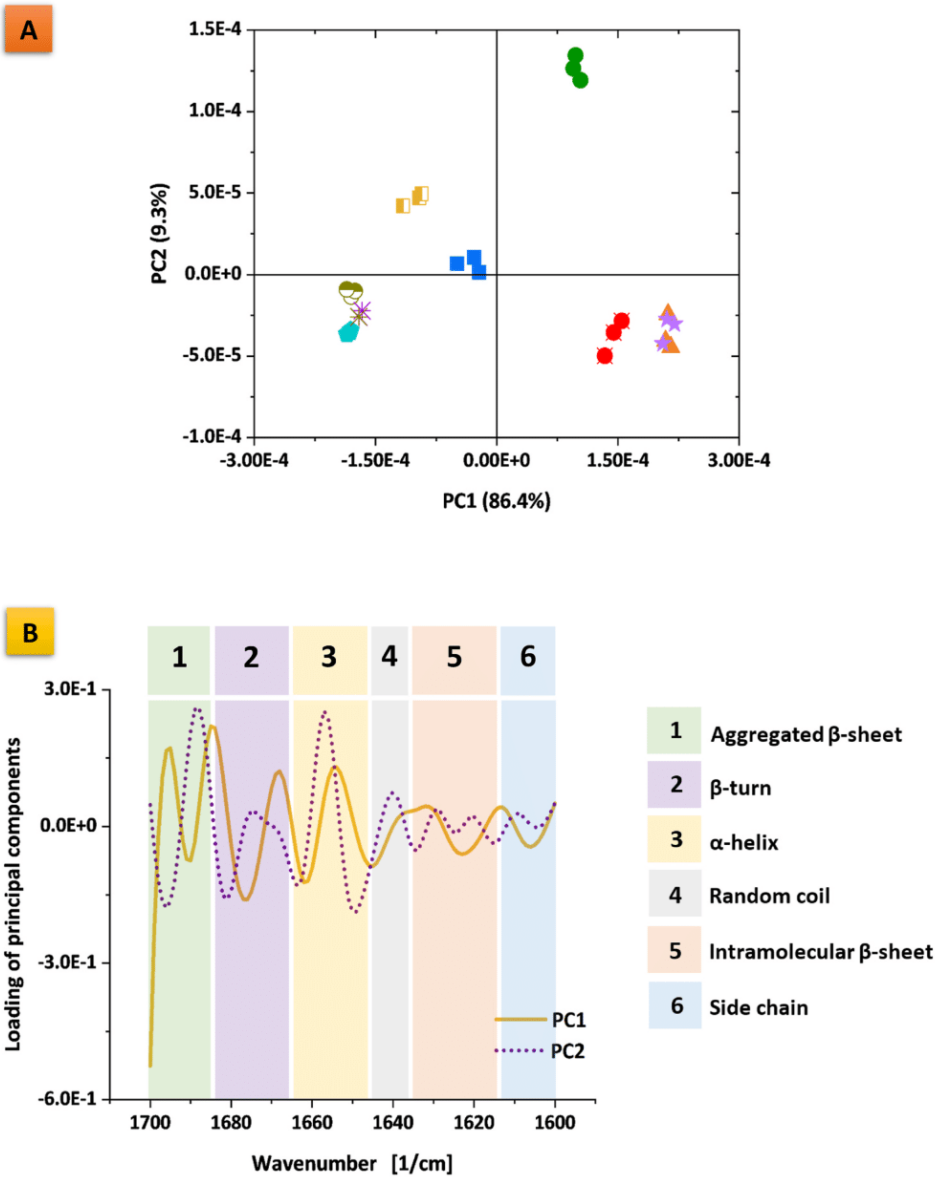

Fig. 2. A) Scatter plot of the PCA scores of FTIR spectra of caseins. **B)** The plot of the PCA loadings of FTIR spectra of casein dispersions. A2/A2 casein micelle 6.7, A1/A2 casein micelle 6.7, A1/A1 casein micelle 6.7, A2/A2 casein micelle 5.7, A1/A2 casein micelle 5.7, A1/A1 casein micelle 5.7, A2/A2 casein particle 2.3, A1/A2 casein particle 2.3, A1/A1 casein particle 2.3

3.2. Spectral evolution profiles by FTIR analysis

The ATR-FTIR spectra of the three samples (A1/A1, A2/A2, and A2/A2 caseins) across all pH measurements, are shown in Fig. 1. The mean spectra of the classes presented visual differences, particularly in 1700–1600 cm^{-1} region that is mainly related to protein composition (secondary structure). Hence, the Amide I region was attributed mainly to C═O stretching vibration of the protein backbone. Additionally, the quantitative estimation of the protein secondary structure obtained from the Amide I spectral profile was calculated in order to visualise otherwise hidden peaks (Table 2).

With a decrease of pH, variations in the intensity of the peaks at 1697 cm^{-1} and 1685 cm^{-1} (aggregated β-sheets), 1668 cm^{-1} (β-turns), 1655 cm^{-1} (α-helixes), 1641 cm^{-1} (random/polyproline II [PPII] helixes), 1620 cm^{-1} (intramolecular β-sheets) were observed (Fig. 1). The peaks' intensity, as expected, has been predominantly steady with lowering the pH from 6.7 to 5.7 and 2.3. Notably, the spectrum of the samples with pH 5.7 and 2.3 in comparison to the samples with pH 6.7, were characterised by lessen intensity of almost every peak proportionally with the acidification step (Fig. 1). Nevertheless, there were some noticeable deviations. For instance, compared to the other peaks, the peak intensity at 1656 cm^{-1} attributed to α-helixes was greater in A1/A1 (pH 2.3, 36.01%), A1/A2 (pH 6.7, 40.47%), and A2/A2 (pH 5.7, 36.11%) casein particles (Fig. 1 and Table 2). Further, A1/A1 sample at pH 6.7 and during acidification produced even greater variations in the peak distribution; a decrease of peaks at 1695 cm^{-1} representing aggregated β-sheets was accompanied with a remarkable increase in the amount of intramolecular β-sheets (1621 cm^{-1}, Fig. 1 and Table 2).

The A2/A2 casein micelle was depicted by a great amount of β-turns (1668 cm^{-1}). Hence, at pH 6.7 the amount of these structures was by 40% and 50% greater in A2/A2 casein micelle compared to that in A1/A1 and A1/A2 samples. The similar tendency was followed across acidification. However, at pH 5.7 the amount of β-turns was almost complementary between A1/A1 and A2/A2 casein micelles. In addition, at pH 2.3 the content of this conformational motif was almost similar (only 4% different) between both casein particles carrying β-casein A2 (Fig. 1 and Table 2). Interestingly, the differences in peaks centred between 1638 and 1645 cm^{-1} (random/PPII helixes, Fig. 1) varied the most in A1/A2 casein micelle. In this regard, at pH 6.7 these conformational motifs were not present; at pH 5.7 their amount in A1/A2 sample was greater by 97% and 87% than that in A1/A1 and A2/A2 casein micelles, respectively; and finally, at pH 2.3 they were again absent (Table 2). Although, the amount of random coils was not statistically different between A1/A1 and A2/A2 samples at pH 6.7, as pH decreased, these structures were more present in A2/A2 casein (Fig. 1).

As it can be observed from the results, the pH effect depicted the expected structural reorganisation. Accordingly, A1/A2 and A2/A2 caseins compared to A1/A1 sample behaved similarly at pH 6.7 and 2.3. This apparently happened due to absence of β-casein A2 in the structure of A1/A1 casein micelle; thus, leading to a possible different functioning. However, it was not the case for the samples at pH 5.7. The samples assessed at this pH, were clearly separated from the other caseins at pH 6.7 and 2.3 along the direction of both Principal Components 1 and 2 (PC1 [86.4%] and PC2 [9.3%]) implying significant changes in the protein secondary structure in each group and at this particular pH (Fig. 2A). The A1/A1 samples across all pH measurements were separated along the direction of PC1 (except the ones at pH 5.7, explained above). Loading plot demonstrated high loadings for regions 1695 cm^{-1} and 1685 cm^{-1} in A1/A2 (pH 5.7) and A2/A2 (pH 6.7) casein micelles, respectively indicating presence of more aggregated β-sheets (Fig. 2B). On the contrary, higher loading for 1654 cm^{-1}, attributed to α-helixes, was greater for A1/A1 sample at pH 2.3, which was in accordance with observations noted in the second derivative spectra (Figs. 1 and 2B).

The second derivatives shift the direction of the principal components, hence a peak in the positive direction on each loading plot was interpreted as a smaller absorbance at the respective wavenumber, and vice versa for peaks in negative direction. Particularly, the largest peaks on the loadings of the PC2 component, associated with both, pH effect and β-casein phenotype in all three samples were: 1688, 1657, and 1641 cm^{-1} peaks on the negative side, and 1697 and 1650 cm^{-1} peaks on the positive side (Fig. 2B). Therefore, it can be interpreted that acidification mainly led to increase in aggregated β-sheets and random coils in A2/A2 sample, whilst α-helixes were mainly present in the samples comprised of β-casein A1. There is also a tendency for scores to be clustered in two groups based on the β-casein phenotypes at the PCA analyses within the remaining pH groups (6.7, 5.7, and 2.3), but the separations are not attributed to only one component (Fig. 2A and B).

3.3. Spectral evolution profiles by NMR analysis

The expectation that the NMR spectrum of the caseins in D_2O would be complex was realised. It was possible to recognise general regions of interest (Table 3 and Figs. 3 and 5) despite the fact that remaining lactose might cause, in general, masking of the resonances that can be of value vis-à-vis structural differences in the caseins arising from the β-casein phenotypes and the specified pH. In fact, these factors of an aliquot of the dispersion yielded a spectrum compromised by the multicomponent nature of the system and resulted in various conformational motifs of the casein micelle. The average ^1H NMR spectra of A1/A1, A1/A2, and A2/A2 caseins is shown in Fig. 3. Generally, a substantial difference between caseins was observed due to the change in pH. For all samples, before and after acidification, Methyl and Aliphatic regions were greater than Amide and Amino spectral profiles.

Interestingly, a constant up-field shifting of peaks was noted when the samples were acidified at pH 2.3 regardless of β-casein phenotype. The pH-dependant proton shifting was initiated as a result of the changes in hydrogen bonding, pH sensitive changes in the structure, and exchange between distinct protein conformations. More specifically, at pH 6.7, A1/A2 and A2/A2 casein micelles were characterised with broader signals between 0.88 and 2.42 ppm, as well as intense and strong signal at 1.93 ppm that was non-existent in A1/A1 casein micelle (Fig. 3). Thus, it may have resulted in slightly lower Methyl and

Table 3
Total percentage areas in different regions of casein micelles and casein particles carrying different β-casein phenotypes.

Sample	pH	Methyl	Aliphatic	Amide	Amino
A1/A1	6.7	35.26 ± 0.07^{a}	43.11 ± 0.15^{b}	11.43 ± 0.07^{bc}	10.19 ± 0.16^{de}
A1/A2		36.99 ± 0.42^{a}	42.18 ± 0.89^{b}	10.82 ± 0.36^{bcd}	10.01 ± 0.83^{de}
A2/A2		35.95 ± 1.64^{a}	45.93 ± 0.39^{a}	9.23 ± 0.69^{d}	8.90 ± 1.3^{e}
A1/A1	5.7	35.32 ± 0.74^{a}	38.90 ± 0.14^{cd}	11.20 ± 0.39^{bc}	14.58 ± 0.97^{bc}
A1/A2		34.26 ± 0.42^{a}	38.67 ± 0.65^{d}	13.74 ± 0.29^{a}	13.40 ± 0.05^{cd}
A2/A2		34.22 ± 0.82^{a}	42.31 ± 1.51^{b}	10.48 ± 0.31^{bcd}	12.99 ± 0.69^{cde}
A1/A1	2.3	33.90 ± 1.14^{a}	41.59 ± 1.75^{bc}	6.62 ± 1.35^{e}	17.89 ± 1.68^{ab}
A1/A2		35.43 ± 2.81^{a}	33.35 ± 0.72^{e}	12.00 ± 0.56^{ab}	19.22 ± 2.65^{a}
A2/A2		37.29 ± 1.10^{a}	35.19 ± 0.95^{e}	9.69 ± 0.62^{d}	17.83 ± 2.63^{ab}
Band frequency (ppm)		0.0–1.5	1.6–3.5	3.6–5.5	5.6–9.0

Mean values within a row that do not share a common superscript letter are significantly different ($p \leq 0.05$); n/d = not detectable.

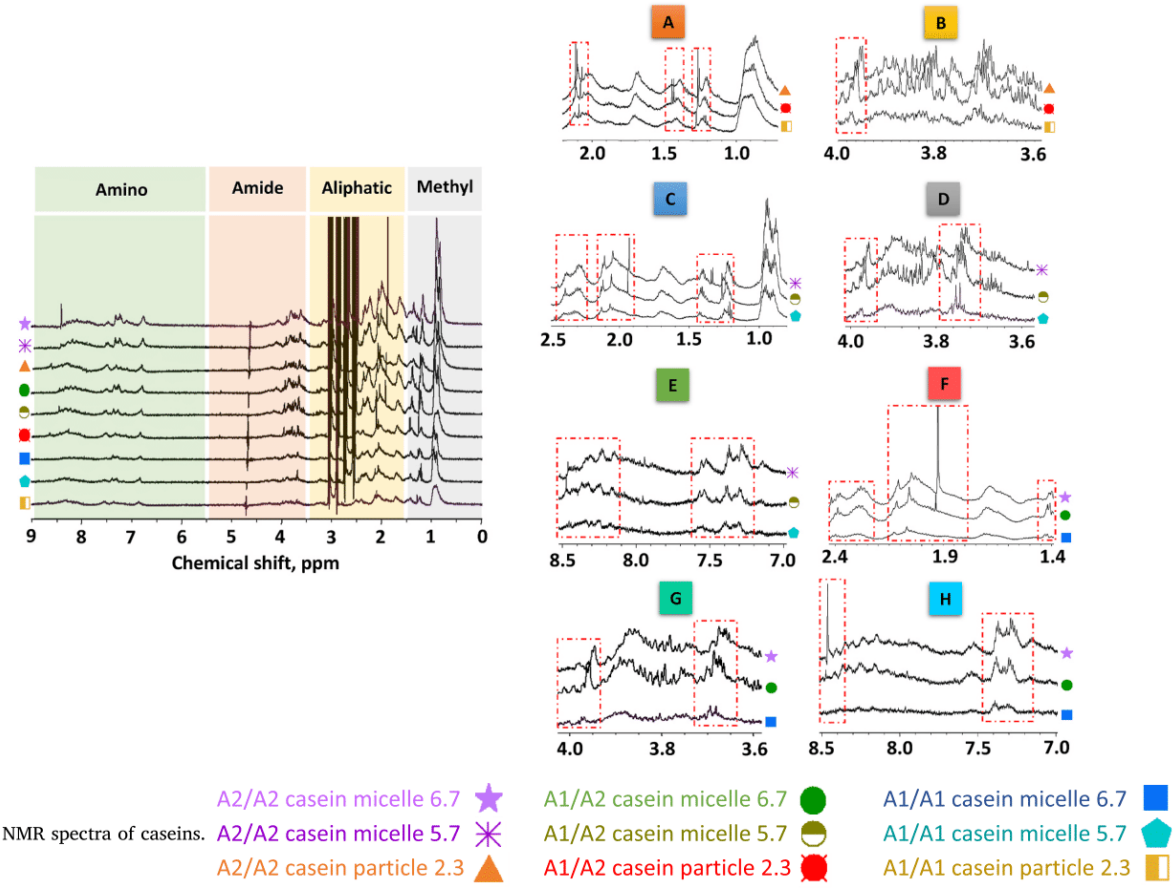

Fig. 3. ^{1}H NMR spectra of caseins.

Aliphatic regions in A1/A1 sample at this particular pH, although there was not a statistical difference among the samples, particularly for the Methyl region ($p > 0.05$, Table 3). In the same fashion, at pH 5.7 both samples carrying β-casein A2 showed some traits different than A1/A1 casein micelle. For example, in A1/A2 and A2/A2 samples, the peaks between 1.10 and 1.25 ppm appeared as multiplets, as opposed to those in A1/A1 casein micelle, which peak was triplet. The sharp singlet peaks (1.27 and 1.93 ppm) and an intense doublet peak (1.34 ppm) seem to appear only in A1/A2 and A2/A2 casein micelles, respectively; hence, specified a moderately higher integrated Aliphatic region in A2/A2 sample (45.93%) compared to the same region of A1/A1 sample (38.90%) ($p < 0.05$, Table 3). Across acidification some changes at Methyl and Aliphatic regions appeared in the sample. More specifically, at pH 2.3, a sharp singlet peaks at 1.27 ppm were present for all three phenotypes of casein particles, yet a doublet peak at 1.44 ppm and 2.11 ppm were only present in A1/A2 and A2/A2 casein particles.

In the Amide region, however, a two-protons quadruple peaks at 3.80 ppm and the number of proton increasing from 3.60 to 4.00 ppm in A1/A2 and A2/A2 caseins across all pH measurements were depicted (Figs. 1 and 3). Further, between 3.55 and 3.98 ppm, for all samples and during acidification an up-field shielding of the peaks was observed (≈0.02 ppm). Despite this difference, upon acidification, the Amide region of all three caseins (only A1/A1 casein particle differ at pH 2.3, $p < 0.05$) was ranging between 9 and 14%. Another difference between the samples was in the Amino region, but only at pH 6.7 and 5.7. These variations between A1/A1, A1/A2, and A2/A2 caseins in Amino region were not observed at pH 2.3 (Fig. 3). The chemical shifts from the up-field to the downfield region of the spectrum revealed the appearance of strong and intense signal peaks at 8.46 ppm in both casein micelles containing β-casein A2 in their structures (particularly A2/A2 casein micelle) that was absent in A1/A1 sample (Fig. 3). Noticeably, the characteristic Amino signals in the interval from 7.00 to 8.50 ppm resulted in an increase of the area percentage values for all three samples across the pH, thus, revealing the possibility for the tremendous impact of the samples' acidification regardless of the β-casein phenotypes. Namely, the content of Amide region, but especially Amino region appeared greatly altered ($p < 0.05$); hence, across acidification the interval between 5.60 and 9.00 ppm increased by ≈ 67% in all three caseins (Table 3).

Unsupervised PCA was primarily carried out; scores and loadings are presented in Fig. 4A and B. From the multivariate analysis, PC1 was responsible for 49.2% and PC2 for 20.2% of the total variance. Fig. 4A shows all three casein particles at pH 2.3 to the left, while the others appeared at more positive PC1 values. More specifically, PC1 possessed the ability to even distinct all samples at pH 6.7 (upper right corner) from all the samples at pH 5.7 (lower right corner), thus stating that PC1 differentiate the samples based on the pH, regardless of the β-casein phenotype. Differently, no overall clear distinction is seen for the samples along PC2 (Fig. 4A). However, the relationship between β-casein phenotype and pH is obvious (Fig. 2b). Clustering was observed for A2/A2 (pH 2.3) casein particle and A2/A2, A1/A2, and A1/A1 (pH 6.7) casein micelles on the positive side of PC2, whilst A1/A1 and A1/A2 (pH 2.3) and A1/A1, A1/A2, and A2/A2 (pH 5.7) samples were shown with negative scores along the second component (Fig. 4A). In addition, in the Amino region, broad and intensive positive and negative loadings on PC1 and PC2 were observed at around 7.10 ppm (multiplet peak) and at 6.30 ppm (singlet peak), respectively; these results were complementary to the data in Table 3 which showed a significant rise of the Amino region for all samples during acidification ($p < 0.05$, Table 3).

The signal of TOCSY and NOESY spectra presented a high pH dependence of the caseins. Highest signal-to-noise ratio in the presented 2D spectra were observed for casein particles at pH 2.3 (Fig. 5). When pH was changed from 6.7 to 5.7 and 2.3 the signal become weak due to intense exchange with the solvent and low signal to noise ratio as a result

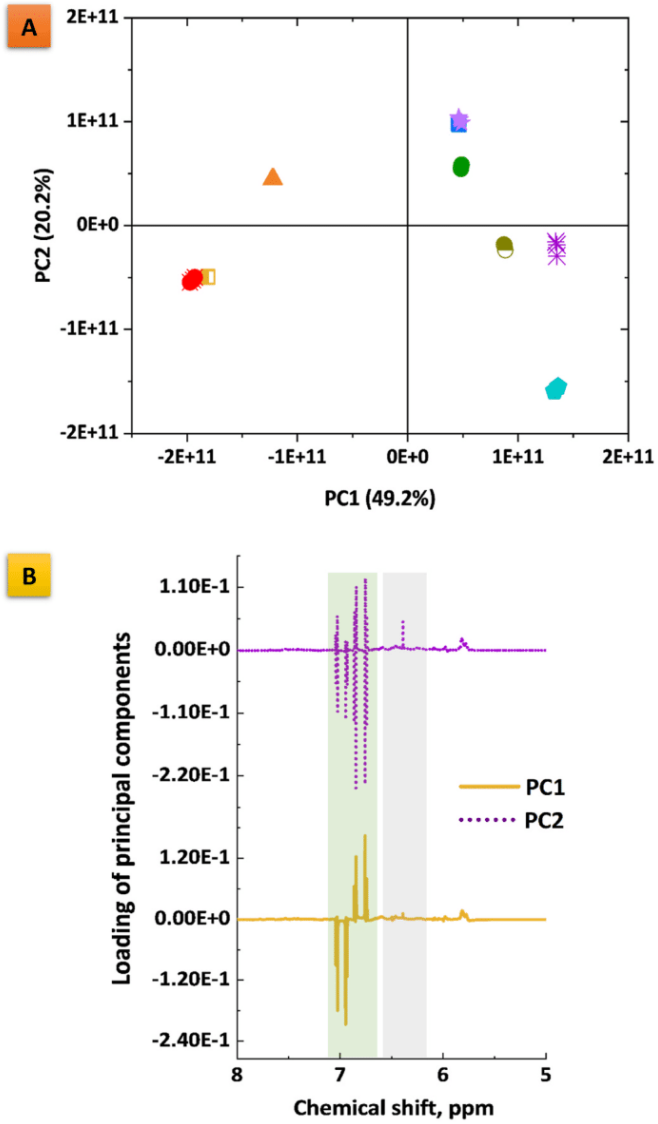

Fig. 4. A) Scatter plot of the PCA scores of ^{1}H NMR spectra of caseins. **B)** The plot of the PCA loadings of ^{1}H NMR spectra of casein dispersions. A2/A2 casein micelle 6.7; A1/A2 casein micelle 6.7; A1/A1 casein micelle 6.7; A2/A2 casein micelle 5.7; A1/A2 casein micelle 5.7; A1/A1 casein micelle 5.7; A2/A2 casein particle 2.3; A1/A2 casein particle 2.3; A1/A1 casein particle 2.3

of the modified conditions. The cross peaks in the NOESY spectra confirm through space coupling of the protons. The lower detection of the cross peaks in the TOCSY and NOESY spectra can relate to buried protons inside the compact casein micelles' structure. At low pH higher solubility of some caseins was expected (mainly β- and αs-caseins, Table 1) in the serum phase; hence, the dissociated monomers can provide better proton signal for the NMR spectra. The pH effect on individual phenotype of the casein particle is observed in NH and Aromatic regions (Amino region) in TOCSY and NOESY spectra (Fig. 5). The Amide and Aliphatic regions had major interference from both water and lactose signals, respectively, and thus did not provide good signal for a correlation analysis. Another interesting signal observed in A1/A2 and A2/A2 samples is the cross-peak for chemical shifts at 8.60 and 7.30 ppm. This correlation was not observed in A1/A1 casein micelle. Due to signal overlapping the correlation cannot be identified to belong to specific amino acid, however it can be considered as a distinguishing point that isolate the casein micelle A1/A1 from the samples carrying either A1/A2 or A2/A2 phenotypes.

4. Discussion

The general properties of casein micelles and their particles are now well established. The mechanism by which the casein micelles in milk are stabilised and destabilised by change of pH have attracted much attention during the last century and there is a vast volume of information in the literature (Anema & Klostermeyer, 1997; Dalgleish & Law, 1988, 1989; Huppertz, 2022; Liu & Guo, 2008; Madadlou, Mousavi, Emam-Djomeh, Sheehan, & Ehsani, 2009; Walstra, 1990; Ye & Harte, 2013). One of the important topics investigated has been the structural fingerprinting of casein micelle influenced by the genetic variants of individual caseins (Day et al., 2015), but relating these conformational motifs to β-caseins A1/A1, A1/A2, or A2/A2 received a trivial attention. During acidification of casein micelle from pH 6.7 to 5.7, Rollema and Brinkhuis (1989) postulated that there were only small insignificant

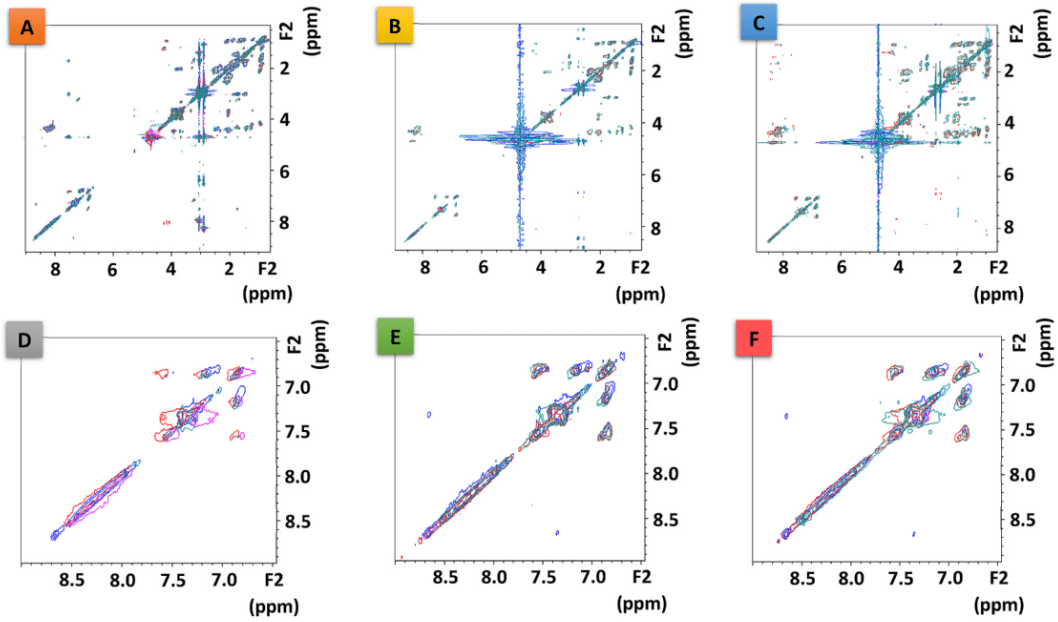

Fig. 5. TOCSY spectra: **A)** Casein particles at pH 2.3 for A1/A1 (blue), A1/A2 (red), and A2/A2 (green); **B)** Casein micelles at pH 5.7 for A1/A1 (blue), A1/A2 (red), and A2/A2 (green); **C)** Casein micelles at pH 6.7 for A1/A1 (blue), A1/A2 (red), and A2/A2 (green); **D)** Amino region for A1/A1 casein particle at pH 2.3 (blue), and casein micelles at pH 5.7 (red), and 6.7 (green); **E)** Amino region for A1/A2 casein particle at pH 2.3 (blue), and casein micelles at pH 5.7 (red), and 6.7 (green); and **F)** Amino region for A2/A2 casein particle at pH 2.3 (blue), and casein micelles at pH 5.7 (red), and 6.7 (green).

differences in both, Aliphatic and Aromatic regions of the casein micelle spectrum. Contrarily, in the present study, alternations in the protein structural motifs among all three types of casein micelles and their primary casein particles in these regions were noticed (Fig. 3 and Table 3).

The intense signals observed at 8.46 ppm (Fig. 3) only in A1/A2 and A2/A2 casein micelles might be initiated by the NH protons of threonine (Thr) and asparagine (Asp) that refers to hydrogen atoms of the residues in a protein with β-sheet conformational motifs (Wishart, Sykes, & Richards, 1991). The indication of more aggregated β-strands in these samples can be related to the higher amount of κ-casein A that contains Thr^{136} and Asp^{148} in its structure (Gazi, Johansen, & Huppertz, 2022). In the past, peptides that contained particularly Thr tended to form β-sheet structures, which may explain high intensity of the peaks in both NMR and FTIR spectra, especially in A2/A2 casein micelles ($p < 0.05$). Comparably, Leslie, Irons, and Chapman (1969) also assigned the difference in the casein micelle due to the higher content of Thr in κ-casein. In particular, the methyl groups of this amino acid absorb at 8.50 ppm, yet the higher content of Thr residues accounted for the signal in the Amino region (Leslie et al., 1969) that might be the case for the present spectra of A1/A2 and A2/A2 samples. Almost a decade ago, Huppertz (2013) revealed that between 20 and 35% of these structures within the casein micelle depended on the conformational state of κ-casein and its association or dissociation with the casein micelle. In this regard, since κ-casein was most present in A1/A2 and A2/A2 samples, the higher amount of aggregated β-sheets might be initiated by the presence of this protein (Table 1). Moreover, as it was expected, the most notable changes happened around pH 5.7, since it is well known that the isoelectric points of κ-caseins A and B are 5.62 and 5.91, respectively (Gazi et al., 2022; Huppertz, 2013).

The aggregated β-sheet predominantly tends to be more stable, but it may undergo a conformational change when lowering the pH to 2 (Johnson Jr, 1988). Therefore, as it can be observed from the data (Fig. 1 and Table 2), the fewer area of aggregated β-sheet strands in all three samples across the pH modulations indicated likely unfolding and loss of the secondary structure of the protein ($p < 0.05$) (Nishinari, Zhang, & Ikeda, 2000). That indeed, can be associated to the conversion of β-sheet structures into random coils (Farrell et al., 2002), particularly in A2/A2 casein particle. Formerly, the random (unordered) structure peaks were specified as short PPII helixes and were specifically found in β-casein A2 (Syme et al., 2002), but also in untreated, heat-treated and acidified A2/A2 milk (Daniloski et al., 2022b, 2022c). Interestingly, PPII motifs have been assigned to β-casein (due to the 17% of Pro residues in its structure) with a secondary structure ranging between 23 and 70% (Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015). The main difference between β-caseins A2 and A1 is the mutation of Pro to His at position 67 that can result in modified secondary structure of the casein micelle (Day et al., 2015; Farrell et al., 2004). Proline possesses the highest tendency to form PPII helixes, and yet β-casein A2 has an additional Pro in its polypeptide chain. Therefore, the formation of PPII structures due to this protein would be enhanced, initiating alternation in the casein micelle's structural dynamics and assembly behaviour (Daniloski, McCarthy, Markoska, et al., 2022; Raynes et al., 2015). It is worth mentioning that the extended PPII helixes as a dominant fundamental secondary structure of caseins confer stability, structural flexibility and mobility, Pro-glutamine rich (P,Q-rich) domains, synergetic motif in protein-protein interactions, and hydrogen bonding with the casein micelle (Adzhubei, Sternberg, & Makarov, 2013). Thorn, Ecroyd, Carver, and Holt (2015) proposed that PPII-mediated interactions are key determinants for the formation of casein micelles via their large tracts of P,Q-rich regions, thus leading to a tighter packing within the casein micelle and a stronger interaction among caseins. In this regard, Thorn et al. (2005) revealed that β-casein may govern and manipulate the inherently unstable monomers of κ-casein by binding to and shielding their hydrophobic surfaces, consequently prohibiting interactions with other κ-casein molecules that would otherwise facilitate self-assembly into fibrillar structures. Moreover, due to the presence of one additional Pro in its structure, β-casein A2 was found to possess a stronger connection to other caseins compared to β-casein A1, that was translated to as a possible and enhanced molecular chaperone-like activity (Raynes et al., 2015). This is in agreement with the present study, as it was shown that the lower levels of soluble β- and κ-caseins were found in A1/A2 and A2/A2 caseins across the pH range (Table 1, $p < 0.05$). This may suggest that β-casein A2 showed a stronger preference

towards fibrillar aggregation of κ-casein than β-casein A1 (Raynes et al., 2015; Thorn et al., 2005). The NMR also showed a presence of random coil conformations in the region from 3.50 to 4.50 ppm (Mielke & Krishnan, 2009), which was mainly related to the alkyl groups of proteins or δ-CH_2 signals (3.46 and 3.70 ppm) and α-protons (Hα, 4.33 ppm) of Pro (Wishart et al., 1991) that corresponds to the present study's results (Fig. 3). Furthermore, the curve fitting of the FTIR spectrum also revealed a presence of PPII helixes in the region between 1636 and 1645 cm^{-1}; the same structures were found in the Amide I region of β-casein (Farrell Jr et al., 2001).

Interestingly, a distinguishing feature among all three casein particles upon acidification was the presence of β-turns mainly in A1/A2 and A2/A2 samples. Huppertz (2013) clarified that caseins create these structures in the following order: $\alpha s_1 > \beta > \kappa$-caseins. The high content of all three caseins likely confirms their involvement in the secondary structure of A1/A2 and A2/A2 casein micelles. Hence, almost 20–35% of the turns in the casein micelle can be initiated individually by the above mentioned caseins owing to the high content of Pro residues - the single most abundant imino acid in the whole casein (Huppertz et al., 2018; Kakalis, Kumosinski, & Farrell, 1990). Specifically, the main key contributor to the formation of β-turns in casein is the cyclic structure of Pro (Daniloski, McCarthy, Markoska, et al., 2022). The unique side chains of this imino acid contributed favourably to conformational stability in certain β-turn positions and it was found that Pro governed the development of β-turn motifs or it was statistically preferred at several β-turn positions (Shapovalov, Vucetic, & Dunbrack, 2019). The presence of turn structures in the polypeptide chain of proteins was related to the intense multiplet peak between 1.10 and 1.25 ppm in A1/A2 and A2/A2 casein micelles. Those peaks might be initiated from the *cis/trans* isomerism of X-Pro bond and can change in the proximity between alkyl fragments (CH_2 - CH_3) of amino acids and Hα protons of Pro (Farahani et al., 2014). Moreover, the strong signal at around 1.90 ppm was only present in the samples comprised of β-casein A2 referring to the γ-CH_2 signals of Pro found in β-turn secondary motif (Wishart et al., 1991). Accordingly, since A2/A2 casein micelle contains an additional Pro, it is expected that it would possess a greater content of β-turns. Similar to NMR, FTIR spectrum confirmed the presence of more turns in both casein micelles carrying β-casein A2 ($p < 0.05$, Fig. 1 and Table 2).

It is well stated in the literature that the loss of β-turns upon acidification may reflect the slow growth of helixes from the neighbouring turns (Daniloski, McCarthy, Gazi, & Vasiljevic, 2022; Prystupa & Donald, 1996), as in A1/A1 sample across the pH range (Table 2). The NMR results confirmed downfield shift of the Hα protons as a result of acidification which is due lower presence of α-helix and greater presence of β-strains or β-turns at low pH (Shen & Bax, 2007b). Expectedly, A1/A2 and A2/A2 caseins were presented with a lower content of α-helical structures (Table 2), likely owing to the presence of the additional Pro^{67} in the polypeptide chain of β-casein A2. Indeed, this imino acid was considered as a potent breaker of α-helixes and it was presumed that the decrease of helical structures in the samples was initiated due to the high rigidity imposed by the Pro cyclic structure which prevents rotations about the N-C^{α} bond (Li, Goto, Williams, & Deber, 1996; Raynes et al., 2015). The presence of α-helixes in A1/A1 sample might be due to the content of both β- and αs_2-caseins (Table 1), since they possess the highest amount of α-helixes, e.g. one-quarter and one-half of the secondary structure of β- and αs_2-caseins, respectively (Fox et al., 2015; Huppertz et al., 2018). It is worth mentioning that the main difference between the casein micelle samples in terms of β-casein is the inclusion of an additional His^{67} within the main structure of β-casein A1 (Farrell et al., 2004). Almost a decade ago, Ghosh, Tucker, and Gai (2014) examined the conformational transitions of His in the Amide I region of the FTIR spectra. Specifically, the protonation of the imidazole ring of His was found in the range from 1642 to 1650 cm^{-1}, that corresponds to α-helical structures in the protein (Ghosh et al., 2014). Even though, His has been weakly correlated with all secondary structure types (Malkov, Živković, Beljanski, Hall, & Zarić, 2008), in many instances, this amino acid possessed a conformational preferences to adopt α-helical motifs (Chou & Fasman, 1974; Huang & Nau, 2003; Levitt, 1978). Further, the most prominent changes within the NMR spectrum of A1/A1 casein micelle happened in the Amino region due to the presence of the His residue. For example, the C4 and C2 protons of His residues are found to be located and subsequently resonated between 8.10 and 6.00 ppm, which also stipulates a greater presence of α-helixes (Mielke & Krishnan, 2009). In the past, Rollema and Brinkhuis (1989) believed that the small changes in the region ranging between 9.00 and 6.00 ppm in the NMR spectra of casein micelle was related to the effect of pH on the chemical shifts of His resonances of the caseins.

Additionally, in the casein particle from A1/A1 variant an up-field shift in the Amino region was observed when pH was altered from 6.7 to 5.7 and 2.3, thus confirming the involvement of H-bonding in the micelles (Daniloski, McCarthy, Markoska, et al., 2022). The sensitivity of proton chemical shifts to structural and conformational effects in proteins have long been known, but it is still difficult to accurately interpret the multiple contributing factors (Mulder & Filatov, 2010). These primarily include sensitivity to protein conformation, but also the hydrogen bonding. For instance, several studies have demonstrated that clear correlations exist between the proton chemical shift and the secondary structure, with up-field shift in α-helical conformations in the presence of hydrogen bonds (Schwarzinger et al., 2001; Shen & Bax, 2007a; Wang & Jardetzky, 2002), similar to this study's results in A1/A1 casein samples upon acidification. Namely, hydrogen bonds often are responsible for the most strongly shifted signals, and display a sensitive dependence on hydrogen bond acceptor-donor distance (Mulder & Filatov, 2010). As mentioned earlier (section 3.2.), the Amide I band in FTIR spectra is mainly composed of C═O stretching (around 80%) and, to a lesser extent, the NH bending vibrations (Arrondo, Young, & Mantsch, 1988). The exact location of these vibrations depends on the hydrogen bonds that C═O form with proximate NH groups. The secondary structure of a protein determines with which NH group the hydrogen bonds will be formed, turning the vibrational spectra into fingerprints of the secondary protein structure (Kong & Yu, 2007).

Further, in A1/A1 casein micelle the peaks between 1681 and 1699 cm^{-1} existed to a lesser extent, thus showing lower intermolecular aggregation of β-sheet, presumably due to the processing at pH lower than 4.5 and lesser hydrophobicity (Daniloski, McCarthy, O'Callaghan, & Vasiljevic, 2022; Dudi & Khatkar, 2023) compared to A1/A2 and A2/A2 samples (Table 1). In the past, this event led to an increased probability of NH bending (Kehoe & Foegeding, 2011). Due to greater bending the remaining groups in A1/A1 samples were free for intermolecular H-bonding (Dudi & Khatkar, 2023), since formerly the NH bending was found to be a candidate involved in hydrogen bonding (Plowman et al., 1994). The observed bending particularly in the samples carrying β-casein A1, led to significant exposure of hydrophilic sites, increasing intermolecular hydrogen bonding, important for the casein micelle internal conformation (Holt, Carver, Ecroyd, & Thorn, 2013; Horne, 2017). In A1/A2 and A2/A2 casein particles, the Amino region had minor shielding, confirming that in these micelles the hydrogen interactions were less present compared to those in A1/A1 sample.

Even though, the content of αs_2-casein was the lowest among the samples (Table 1), its contribution to both NMR and FTIR signals cannot be disregarded. Particularly, Huq, Cross, and Reynolds (2003) and Cross et al. (2007) provided evidence of the tendency of two αs_2-casein' peptides to form helical motifs, possible do the phosphoserine region and the presence of glutamic acid (Glu) within the structure of the examined peptides. Moreover, the importance of lysine (Lys) and Glu as the most prominent amino acids in αs_2-casein, should be taken into consideration. Both amino acids manifested a preference for building α-helixes, especially Glu that has been further classified as a strong helix accommodator (Malkov et al., 2008). Very recently, although the complete individual protein analysis was not performed, De Vitte et al. (2022) revealed that Lys was more abundant in both proteins containing

β-casein A1, that corroborates with the present results.

5. Can these structural changes realistically reflect behaviour of casein micelles and casein particles during milk digestion and its processing?

The scientific data described in the present study presents a combination of two related approaches. Developed conclusions: where the reported results are shown to be repeatable in both spectroscopy settings; and suggestive: where the results related to dairy processing or *in vitro* digestion models were based on the derivations, assumptions, and theories obtained from the structural casein analysis. Recently, both FTIR and NMR spectroscopies have been used to study cheese matrices (Tarapoulouzi & Theocharis, 2019) and have been associated to the caseins' interactions and network formation (Grewal, Vasiljevic, & Huppertz, 2021), also during *in vitro* digestion (Verma et al., 2022) that would open up new approaches for understanding these mechanisms.

Nevertheless, the present data lacks detailed information that can explain the conditions, which may influence the behaviour of casein micelle and its particles in the gastrointestinal tract or during cheese making, since only one temperature and three different pH values were used as part of the study. Within the cheese making and digestion patterns, not only changing of pH or temperature is involved, but rather a vast array of other factors, including milk composition, enzymes, motion of the samples during digestion, mechanical shear, ionic strength, starter cultures, rate and extent of acid development, moisture content and curd manipulation, to name a few (Brodkorb et al., 2019; Lucey, Johnson, & Horne, 2003).

The possible explanation for the journey of the caseins' confirmation should start from their initial structure, as in the present study, since caseins as well-studied model system could serve as the cornerstone of this knowledge base (Huppertz et al., 2018). To date, casein and casein-based systems have been examined using FTIR and NMR spectroscopies (Boiani et al., 2018; Daniloski et al., 2022a, 2022e), however, structural analysis must consider two crucial factors. The first is the extension of the length scale utilising spectroscopies to fully understand the breakdown of caseins, but also their interactions. Secondly, only by obtaining structural information and the factors that navigate those changes, it might be possible to determine the digestion behaviour and, consequently, the optimum method for milk processability. Therefore, while our understanding of structure, dynamics, and interactions of biological macromolecules, such as caseins, is improving by using spectroscopy methods (Markoska, Vasiljevic, & Huppertz, 2020), the behaviour of an environmentally sensitive casein system should be investigated *in situ* within the digestive environment. This also applies to casein gels important for cheese making impacted by factors mentioned above.

6. Conclusion

One- and two-dimensional NMR and FTIR in combination with chemometrics allowed for the structure of casein molecules to be determined at pH ranging between 6.7 and 2.3. The structural differences observed were due to casein protein profile and genetic variant and were significantly affected by pH. The intramolecular β-sheets and α-helixes were predominant in A1/A1 sample, simply due to the higher content of both β- and αs_2-caseins, however β-turns, aggregated β-sheets and PPII helixes dominated in A1/A2 and A2/A2 caseins, mainly as a result of β- and κ-casein phenotypes and the concentration of αs_1-casein. However, at pH 5.7 all samples behaved similarly, which opens a debate on the behaviour of these phenotypes during cheese processing or during the final phase of the intestinal *in vitro* human digestion (Fallingborg, 1999; McSweeney & Fox, 2013). The A1/A2 casein micelle showed similarity with A2/A2 casein micelle indicating that β-casein A2 dominated in the protein's structural organisation. In this regard, the structural differentiations were based on the presence of the additional Pro^{67}, which was most likely associated with the adoption of more PPII helixes in A2/A2 casein micelle.

Future structural and computational, including *in vitro* digestion studies will investigate the relationships among caseins, and their genetic variants themselves and within the casein micelle. Similar studies with more samples carrying β-caseins A1/A1, A1/A2, and A2/A2 with other casein phenotypes could be undertaken to determine their tendency to build the casein micelle and to what extent those proteins can influence the casein micelle structure and subsequently, food-functional properties.

Author statement

Davor Daniloski conceived the study and research question; designed and wrote the original draft, conceptualised, reviewed, edited the manuscript, designed the tables and the figures. **Davor Daniloski** prepared the methodology, formal analysis and investigation. **Todor Vasiljevic** and **Noel A. McCarthy** provided critical feedback and analysis, secured funding, reviewed and edited the manuscript and supervised the study. **Tatijana Markoska** gave critical feedback and analysis, reviewed and edited the manuscript. All authors have contributed to the manuscript and reviewed the final version.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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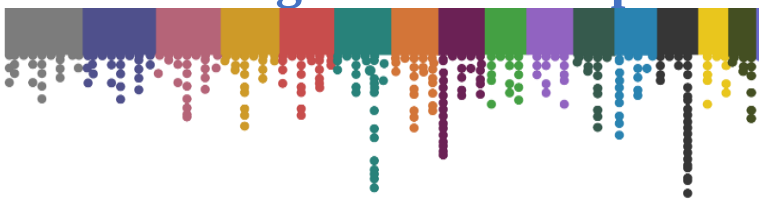

Conformational and physicochemical characteristics of bovine skim milk obtained from cows with different genetic variants of β-casein

- Fingerprinting conformational difference of milk and caseins using FTIR and ^{1}H NMR
- Conformational differences depended on β-casein proteoform and temperature
- β-casein A2 dominated in the protein conformation of samples with β-casein A1/A2 and A2
- Samples with β-caseins A1 and A2 possessed mainly α- and PPII helices, respectively
- Different proteoforms affected the micelle size but not the minerals among samples

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Conformational and physicochemical characteristics of bovine skim milk obtained from cows with different genetic variants of β-casein

Davor Daniloski [a,b], Noel A. McCarthy [b], Tatijana Markoska [a], Martin J. Auldist [c,d], Todor Vasiljevic [a,*]

[a] Victoria University, Advanced Food Systems Research Unit, Institute for Sustainable Industries and Liveable Cities and College of Health and Biomedicine, Melbourne, Victoria, 8001, Australia
[b] Teagasc Food Research Centre, Food Chemistry and Technology Department, Moorepark, Fermoy, P61 C996, Cork, Ireland
[c] Agriculture Victoria, Department of Jobs, Precincts and Regions, Ellinbank, Victoria, 3821, Australia
[d] University of Melbourne, Centre for Agricultural Innovation, School of Agriculture and Food, Faculty of Veterinary and Agricultural Sciences, Melbourne, Victoria, 3010, Australia

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ABSTRACT

This study highlights differences in conformational and physicochemical characteristics of bovine skim milk and micellar casein from cows of different β-casein phenotypes. These genetic variants have been one of the predominant topics among dairy researchers due to their differences in β-casein structure, and thus their potential effects on dairy processing and human health. For characterising differences in milk protein structure, Fourier Transform Infrared (FTIR) and Nuclear Magnetic Resonance (^{1}H NMR) spectroscopies combined with chemometric analysis were used. Additionally, physiochemical properties such as mineral content, particle size, and electrostatic charge in skim milk and micellar casein samples were analysed at 4 and 20 °C. Results showed variation in the secondary structure of all three genetic variants independent of temperature. Moreover, the main differences involved a higher level of β-turn and α-helical structures in A1/A1 β-casein milk, while intermolecular β-sheets were more numerous in A1/A2 β-casein milk, whereas random or polyproline II (PPII) structures were more common in A2/A2 β-casein milk. Temperature slightly affected these differences, which was due to the dissociation of β-casein from the micelle at low temperature. In addition, A2/A2 β-casein milk and its micellar casein had a larger average particle size, which resulted in a lower negative ζ-potential. The A2/A2 β-casein samples contained greater amounts of phosphorus and less calcium compared to the other genetic variants of milk and their micellar caseins. The results also indicated that a combination of FTIR and ^{1}H NMR spectroscopies could be used to establish conformational differences in milk and micellar caseins of different genetic variants.

1. Introduction

Bovine milk and derivatives thereof are an excellent source of energy and provide vital nutrients in animal and human nutrition, including proteins, carbohydrates (lactose), lipids, minerals, and vitamins (McSweeney & Fox, 2013). Several components of bovine milk, particularly the genetic variants of its proteins, are subject of an ongoing debate about their composition, nature, technological role in milk and dairy products, and biological significance to human health (Daniloski, Cunha, et al., 2021). Namely, 80% of these proteins are composed primarily of caseins (CNs), which originate from the family of phosphoproteins and are synthesised and assembled in the form of micelles. The remaining 20% of total milk proteins are known as whey proteins (WPs), composed mostly of α-lactalbumin (α-La) and β-lactoglobulin (β-Lg) (Huppertz, Fox, & Kelly, 2018).

Caseins may be further categorised into four different types, α_{s1}-, α_{s2}-, β- and κ-CN, all of which contribute to a spherical structure known as the CN micelle, with β-CN comprising about 30–40% of the total protein content. β-CN is encoded by the CSN2 gene mapped by chromosome BTA 6 in a tightly linked 250-kb cluster and is distinguished by its significant number of genetic polymorphisms (McSweeney & Fox, 2013). Currently, 12–15 different genetic variants of β-CN have been identified based on their amino acid sequence, of which there are two most commonly recognised groups, namely the A1 β-CN family (A1, B, and F

* Corresponding author.
E-mail address: todor.vasiljevic@vu.edu.au (T. Vasiljevic).

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genetic variants were used for further analysis. Individually, the further steps of samples preparation needed for a specific examination is explained within the description context of every procedure.

2.3. Physicochemical characteristics of milk proteoforms

2.3.1. Protein analysis

The Kjeldahl method (ISO, 2014) was used to determine the total nitrogen (TN), non-casein nitrogen (NCN), and non-protein nitrogen (NPN) of milk and micellar CN samples, with a nitrogen conversion factor of 6.38 used to calculate total protein content. The protein profile of skim milk samples (i.e., A1/A1, A1/A2, and A2/A2 β-CN variants) was established using a High Performance Liquid Chromatography (HPLC) equipped with a Varian 9012 system controller (Agilent Technologies Inc., Santa Clara, CA, USA) coupled with a RI detector (Varian, 9050). The method was previously described by Aprianita et al. (2014) with slight modifications. Briefly, skimmed milk samples (0.8 mL) were reduced using a denaturing urea solution (3.2 mL: 8 M urea, 165 mM Tris, 44 mM sodium citrate, and 0.3 mL v/v β-mercaptoethanol). The sample mixture was directly analysed with a C4 column for very hydrophobic proteins (Phenomenex Aeris WIDEPORE, 150 mm × 4.6 mm, 3.6 μm particle size, 300 Å porosity, Torrance, USA). The column temperature was adjusted between 25 °C (minimum) and 45 °C (maximum). Elution was attained with ultra-pure water (mobile phase [Eluent] A), and acetonitrile (ACN: Sigma-Aldrich, MO, USA) (mobile phase [Eluent] B), both containing 0.1% trifluoroacetic acid (TFA; Sigma-Aldrich, MO, USA). A gradient flow rate of 0.8 mL/min was used, with detection determined by absorbance at a wavelength of 240 nm. The sample injection volume was set at 20 μL, with a total runtime of 51 min per sample (including column re-equilibration).

2.3.2. Mineral composition analysis

The mineral composition (Ca, Mg, K, Na, and P) of milk and micellar CN samples was analysed using an inductively coupled plasma (ICP) atomic emission spectrometer (ICP Multitype, Shimadzu Corporation, Kyoto, Japan) (Grewal, Chandrapala, Donkor, Apostolopoulos, and Vasiljevic (2017). Before being dried and then ashed in a muffle furnace, milk and micellar CN aliquots were kept at 4 and 20 °C, to observe if any difference in the minerals content might be initiated by treating the samples at two different temperatures. Additionally, the ionic calcium (Ca^{2+}) was determined in the samples (Markoska et al., 2019a) using a calcium ion selective electrode along with a pH metre (InoLab, WTW GmbH, Ingolstadt, Germany) at 4 and 20 °C. Calcium chloride was used for the preparation of a standard curve of condensed solution varying between 0 mM and 2 mM with $R^2 = 0.996$. The strength was adjusted by the addition of 80 mM KCL to the standards.

2.3.3. Particle size and zeta potential (ζ)

The particle size and ζ-potential analyses of milk and micellar CN samples were conducted by dynamic light scattering Zetasizer-Nano ZS (Malvern Instruments, Malvern, UK) and the data was processed using a Dispersion Technology software (version 5, Malvern Instruments). All samples were dispersed in SMUF in a ratio of 1:100 (Markoska et al., 2019b). The solutions of every sample were placed in the temperature-controlled chamber at 4 or 20 °C. A refractive index of 1.349 and 1.570 was used for skim milk and micellar CN samples, respectively; and the refractive index for SMUF was set up at 1.342. In both cases, the samples had an absorbance of 0.001 AU (Markoska et al., 2019a). Each measurement was conducted at both temperatures in triplicate; every measurement was equivalent to auto-correlation functions recorded over a period of 40 s. Size distribution by the number was used for the analysis of the results.

2.4. Conformational characterisation of proteins

2.4.1. Fourier Transform Infrared (FTIR) spectroscopy

Structural characteristics of milk and micellar CN samples as a function of different genetic variants and temperatures were analysed using a PerkinElmer FTIR spectrometer (Frontier, PerkinElmer, Boston, MA, USA) in the range of 4000 cm^{-1} - 600 cm^{-1} with a resolution of 4 cm^{-1} and by averaging 64 scans of each spectrum (Markoska et al., 2019a). At the beginning of the measurements, the background spectrum was scanned with a blank diamond attenuated total reflectance (ATR) cell using the same instrumental conditions as for the sample spectra acquisition. The solvent spectra for both, milk samples (WP serum after centrifugation) and micellar CN samples (SMUF) were recorded and used for subtraction from the sample spectra. Particular consideration was paid for the second derivative of region 1700 cm^{-1} - 1600 cm^{-1} (Amide I) for evaluating structural differences of the proteins and greater resolution of the peaks. Based on the peak/protein secondary structure relationships, 9–13 peaks were obtained and established. In particular, intermolecular (aggregated) β-sheet at 1700 cm^{-1} - 1682 cm^{-1}; β-turn at 1681 cm^{-1} - 1665 cm^{-1}; α-helix at 1664 cm^{-1} - 1646 cm^{-1}; random coil at 1645 cm^{-1} - 1638 cm^{-1}; intramolecular β-sheet at 1637 cm^{-1} - 1615 cm^{-1}; and side chain at 1614 cm^{-1} - 1601 cm^{-1} (Grewal et al., 2017). The spectra of ten sub samples of each sample was taken by refilling the ATR cell.

2.4.2. Proton Nuclear Magnetic Resonance (^1H NMR) analysis

A Bruker Avance spectrometer (Bruker BioSpin GmbH, Rheinstetten, Karlsruhe, Germany) operating at 600 MHz transmitter frequency using 5 mm TXI probe with Z - gradient and XYZ - gradient, was used to conduct the NMR experiments. The ^1H NMR spectra (accumulated proton NMR) were recorded using a 9615 Hz spectral width and 64 k data point with 90° pulse sequence, 2 s relaxation delay, and 128 scan numbers, requiring about 6 min per sample. The ^1H NMR spectra was obtained at both temperatures, namely, 277.15 K (4 °C) and 293.15 K (20 °C). The reconstituted skim milk and micellar CN samples (2.5 mg/mL protein) were dispersed in 1 mL of a solution of ultra-pure water and deuterium oxide (D_2O [99.8 atom percent excess and pH 6.0: Sigma-Aldrich, MO, USA]) in a ratio of 9:1, and mixed in a 1.5 mL Eppendorf tube. The samples were vortexed for 30 s and sonicated at room temperature for 20 min before centrifugation at 10,000×*g* for 10 min to obtain a clear supernatant. The supernatants (0.6 mL) were transferred to NMR tubes (5 mm, Wilmad, economy, 500 MHz frequency and L 7 in, Sigma-Aldrich, MO, USA). Chemical shift assignment was performed utilising corresponding amino acids atoms values acquired from the biological magnetic resonance bank (BMRB) files and data from previous studies (Markoska et al., 2020; Sanchez et al., 2021; Zhao, Chen, Feng, Chen, & Cai, 2017). In all spectra, water suppression was achieved using excitation sculpting with gradients.

2.5. Spectral data and statistical analyses

The data in the FTIR spectra was initially corrected using a Spectragryph software (version 1.2.7, Oberstdorf, Germany), followed by a calculation and derivatisation (second derivative) in the region of 1700 cm^{-1} - 1600 cm^{-1} (Amide I) originally described by Militello et al. (2004) with some modifications. Utilising the Peak Fitting method with the Gaussian function for all deconvoluted spectra, enabled fit, identification, and quantification of related peak areas. In contrast, the NMR spectra was phase-corrected using 0 and 1st order correction for pk; the FIDs were corrected with a 0.3 Hz line-broadening parameter. NMR data processing was performed using a TopSpin (version 4.1.1) software (Bruker BioSpin, Billerica, Massachusetts, USA). To reduce the dimensionality of the original data and to observe the composition of skim milk and micellar CN, the matrices were exported for unsupervised chemometric analysis by Principal Component Analysis (PCA). The included PCA was with 95% confidence and the aim was to identify the

changes in both, FTIR and NMR regions, which classified different samples based on the β-CN genetic variant (milk or micellar CN) and temperature change (4 or 20 °C), and thus validating the observations performed on both spectra separately. The PCA data was evaluated and reported with the Origin software (Origin Pro 2021, v. 95 E, OriginLab Corporation, Northampton, MA, USA). Namely, PCA plots presented groupings; loading plots demonstrated the spectral markers (identification of wavenumbers), which have contributed the most in the samples' classification into different groups.

In addition, the statistical analysis was performed using a Minitab statistical software (Version 19; Minitab, Pennsylvania, USA) using Analysis of variance (1-way ANOVA) followed by Tukey - HSD post-hoc test in order to evaluate the structural and physicochemical parameters of the samples. The effects of the fixed factors (genetic variant and temperature) were taken into consideration for all data; the level of significance was considered at $p \leq 0.05$ (Supplementary Tables 3 [A, B, C] and 4 [A, B, C]). The entire experiment was conducted in triplicate and results were reported as the mean ± standard error.

3. Results and discussion

3.1. Protein profile and mineral composition of milk and micellar CN samples

Reversed-phase HPLC protein profiles of skim milk samples are shown in Fig. 1, with significant variations observed between protein chromatograms from skim milks of A1/A1, A1/A2, and A2/A2 β-CN variants. The peaks were confirmed by comparison with commercial standards of β-CN (Sigma-Aldrich, MO, USA) or by comparison with milk protein chromatograms from DNA-genotyped animals obtained from the literature (Day et al., 2015; Thomä, Krause, & Kulozik, 2006). The amount of β-CNs relative to the total protein concentrations in milk samples was 11.51 mg/mL for A1/A1 β-CN milk; 6.38 mg/mL and 5.77 mg/mL for A1/A2 β-CN milk; and 11.30 mg/mL for A2/A2 β-CN milk. The exact proteoforms of other major milk proteins were not established, including κ-CN and β-Lg. It is however well known that these two proteins and their proteoforms influence conformational and physicochemical characteristics of the milk samples (Bonfatti, Di Martino, Cecchinato, Vicario, & Carnier, 2010; Day et al., 2015). Notably from Fig. 1, κ-CN in A1/A2 and A2/A2 β-CN milk differed from that in A1/A1 β-CN milk. Interestingly, the temperature of the column and the hydrophobicity of both, the column and β-CNs, had a substantial effect on the protein resolution between A1/A1 β-CN, A1/A2 β-CN, and A2/A2 β-CN. Specifically, increasing the column temperature (minimum 25 °C and maximum 45 °C), known to possess a denaturing effect, improved the resolution between all three β-CN genetic variants. Similar results were found elsewhere in the literature (Bonfatti, Grigoletto, Cecchinato, Gallo, & Carnier, 2008). Furthermore, protein separation by reversed-phase HPLC is assumed to be based on the interaction of the protein's hydrophobic groups with insoluble hydrophobic groups immobilised on the matrix (Bertin et al., 2015; Cardamone & Puri, 1992). Based on the theoretical predictions, proteins' elution highly depends on their hydrophobicity (Boysen & Hearn, 2010). Namely, the order of elution of both A1 β-CN and A2 β-CN differed based on their hydrophobicity, since the hydrophobicity of the reversed-phase column utilised was same for both proteins (see section 2.3.1.). According to Raynes, Day, Augustin, and Carver (2015), A1 β-CN and A2 β-CN possess different hydrophobicity, thus the former eluted in 26.5 min in comparison with the latter, which eluted in less than 28 min (Fig. 1). Specifically, hydrophobic proteins adsorb to the hydrophobic surface after entering the column, and remain in that state until the organic modifier diminishes these interactions (Carr, 2002). Theoretically, the additional Pro^{67} (non-polar partially hydrophobic amino acid) (Morgan & Rubenstein, 2013; Schobert & Tschesche, 1978) within the protein structure which replaces the charged polar His^{67} is expected to increase hydrophobicity of A2 β-CN, thus enhancing its affinity for a C4 column

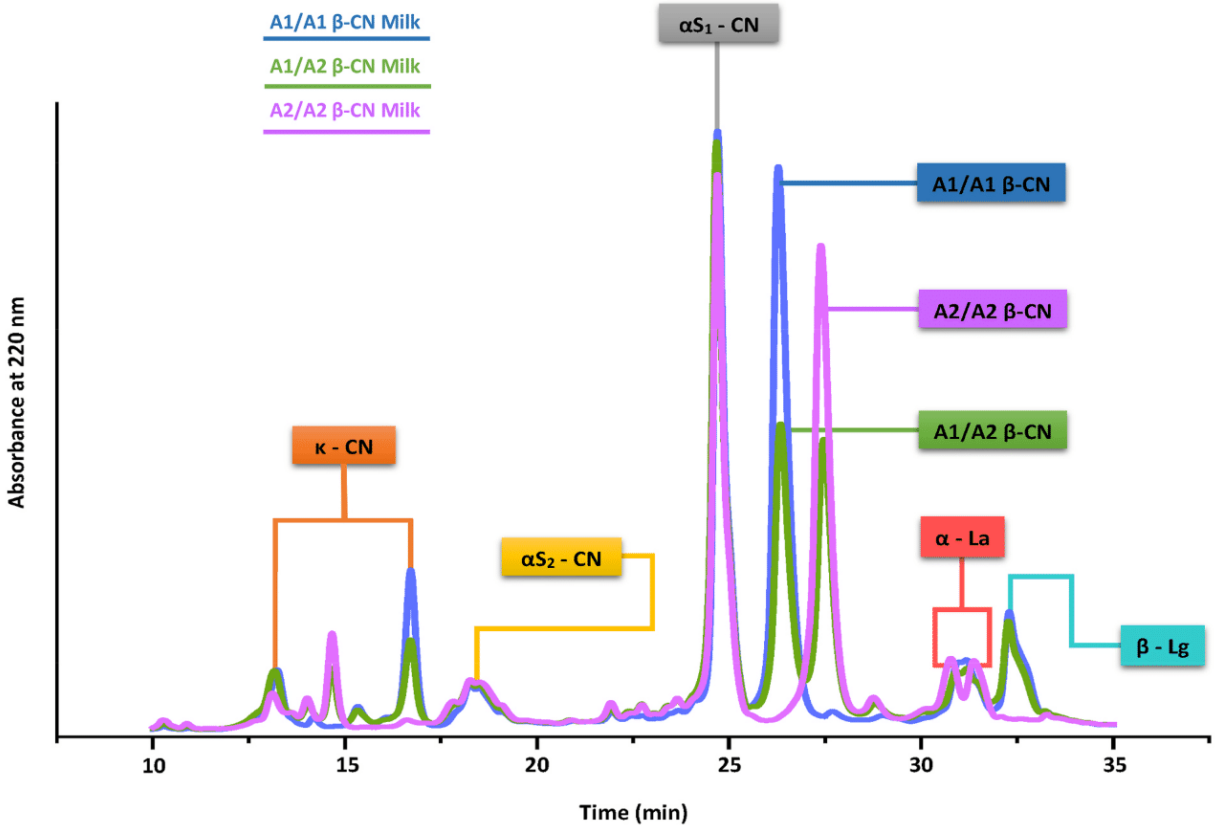

Fig. 1. Reverse phase-HPLC chromatograms of skim milk containing either the A1/A1, A1/A2, or A2/A2 β-CN genetic variant.

(Aguilar, 2004) and consequently extending the elution time from the column compared to that of A1 β-CN.

Concentrations of Ca, K, Mg, Na, P, and Ca^{2+} in milk and micellar CN samples are presented in Table 1. The values are comparable with expected values for bovine milk and micellar CN; some variations among the samples have been distinguished (Supplementary Tables 3A and 4A) (Huppertz, Heck, Bijl, Poulsen, & Larsen, 2021). Notably, for milk samples, Ca, Mg, Na, and P, and for micellar CN samples, Ca, K, Mg, Na, and P, had significant correlations ($p < 0.05$) with genetic variant and temperature. This is in contrast to Ketto et al. (2017) who reported that the mineral distribution in milk was not influenced by protein polymorphisms. Such varying observations could be due to differences in feeding regime, stage of lactation, genetic variants of CNs and WPs, temperature, and holding times (Devold, Brovold, Langsrud, & Vegarud, 2000). Previously published data agreed that these minerals were associated in the form of the CCP nanoclusters (Holt, 1982); these nanoclusters are the key elements in the CN micelle structure (Huppertz et al., 2021). As expected, the values of Ca and P in all three genetic variants of micellar CNs were higher compared to the other minerals, thus, being far more protein associated (Bijl, Van Valenberg, Huppertz, & Van Hooijdonk, 2013; Huppertz et al., 2021). The amounts of Ca and P in milk are highly related to the functionality of CN micelle and its size; such studies found that the level of P decreases proportionally with the decrease of the CN micelle size (Bijl, de Vries, van Valenberg, Huppertz, & Van Hooijdonk, 2014). Namely, A2/A2 β-CN milk and its micellar CN contained a greater amount of P and therefore their size was greater compared to the other genetic variants of milk and micellar CN samples. Additionally, the minerals' content was found to be higher in milk samples with better coagulation properties (Nguyen et al., 2018; Poulsen et al., 2013). However, little has been reported on the possible association of β-CN variants with the micelle size. Numerous studies, on the other hand, have related the size of CN micelle with the content and glycosylation of k-CN and its polymorphic variants (Bijl et al., 2014; Dalgleish, Horne, & Law, 1989; Ketto et al., 2017; Poulsen et al., 2013) and consequently with the coagulation properties of bovine milk. Furthermore, as determined in the current study A2/A2 β-CN milk and its micellar CN contained less total Ca than other two genetic variants, which may be indicative of a milk sample with poor coagulation properties (Nguyen et al., 2018). The total Ca and especially the concentration of Ca^{2+} are essential for the formation of rennet gels, since the para-CN micelles aggregate poorly if the concentration of Ca^{2+} is too low (Gustavsson et al., 2014). In most milk and micellar CN samples the amounts of total Ca and Ca^{2+} were slightly higher at 4 °C compared to that at 20 °C governed by temperature dependant solubility (Lewis, 2011). Contrarily, those samples examined at 20 °C that contained greater Ca and Ca^{2+} amounts may be due to re-equilibration and the mineral movement into the micellar phase from the serum mainly by association of Ca and P with the CN micelle (Bogahawaththa, Trajkovska, Markoska, & Vasiljevic, 2021).

3.2. Particle size and ζ-potential of milk and micellar CN samples

Particle size and ζ-potential of β-CN genetic variants of milk and micellar CN are presented in Table 2. The average particle size of A2/A2 β-CN milk and its micellar CN was greater than those of A1/A1 and A1/A2 β-CN milk, and their micellar CNs, at both temperatures ($p < 0.05$). The results shown by Day et al. (2015) are in line with the present study's results. The authors revealed that the milk containing A2/A2 β-CN was predominantly classified into a large CN micelle group, compared to milk containing either A1/A2 β-CN or A1/A1 β-CN (distributed between small and large CN micelle groups) (Day et al., 2015). Certainly, since it is known that A2/A2 β-CN contains an additional Pro in its structure (within its P,Q-rich region) in comparison with the other β-CN genetic variants, this phenomena might affect the size of the CN micelle. Namely, Pro is particularly prone to adopt polyproline II (PPII) conformation (it possesses the greatest tendency to create secondary PPII helical structural features), due to the restricted range of its backbone conformational angles (Raynes et al., 2015). Owing to the fact that the P,Q-rich sequences cannot be readily desolated, the PPII conformation favours to be conserved, leading to open and extended supramolecular structures (Thorn, Ecroyd, Carver, & Holt, 2015) and hence a larger CN micelle size. To confirm this hypothesis, a significantly larger set of milk samples from individual cows of known genotypes would need to be analysed. Once again, the effect of κ-CN and its proteoforms on the CN micelle size should also be considered. Namely, milk containing small CN micelles usually contain κ-CN B which is more glycosylated than the A variant of the same protein. Consequently, the amount of glycosylated κ-CN might be the major factor influencing CN micelle size, and may override the genotypic effects of β-CNs (Bijl et al., 2014; Day et al., 2015). Furthermore, these differences may also be influenced by numerous factors, other than a β-CN variant, including

Table 2
The average particle size and zeta potential concentrations of all three genetic variants of β-CN milk and micellar CN samples subjected at 4 °C and 20 °C.

Sample	Temperature (°C)	Particle size (nm)	Zeta potential (mV)
A1/A1 β-CN milk	4	196.93 ± 13.44^{c}	-16.63 ± 0.29^{bc}
	20	170.37 ± 2.15^{d}	-19.40 ± 0.44^{d}
A1/A2 β-CN milk	4	249.27 ± 6.07^{b}	-15.40 ± 1.51^{ab}
	20	158.33 ± 5.24^{d}	-17.87 ± 0.23^{cd}
A2/A2 β-CN milk	4	272.10 ± 0.55^{a}	-13.57 ± 1.16^{a}
	20	171.53 ± 1.84^{d}	-14.67 ± 0.15^{ab}
A1/A1 micellar CN	4	231.03 ± 44.55^{b}	-19.03 ± 1.00^{bc}
	20	186.63 ± 1.53^{b}	-22.23 ± 0.25^{d}
A1/A2 micellar CN	4	298.83 ± 4.80^{a}	-17.23 ± 0.70^{ab}
	20	187.43 ± 3.54^{b}	-19.50 ± 0.50^{c}
A2/A2 micellar CN	4	303.50 ± 7.64^{a}	-16.80 ± 1.37^{a}
	20	187.83 ± 1.85^{b}	-18.67 ± 0.25^{abc}

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$).

Table 1
The average total and insoluble mineral concentration of all three genetic variants of β-CN milk and micellar CN samples subjected at 4 °C and 20 °C.

Sample	Temperature (°C)	Ca (mM)	K (mM)	Mg (mM)	Na (mM)	P (mM)	Ca^{2+} (mM)
A1/A1 β-CN milk	4	37.93 ± 0.50^{a}	20.38 ± 0.25^{e}	5.07 ± 0.02^{b}	15.96 ± 0.19^{e}	28.36 ± 0.26^{c}	1.81 ± 0.02^{b}
	20	34.27 ± 0.63^{bc}	20.22 ± 0.193^{e}	5.02 ± 0.00^{b}	15.53 ± 0.08^{f}	26.26 ± 0.26^{d}	1.67 ± 0.07^{b}
A1/A2 β-CN milk	4	33.35 ± 0.14^{c}	62.07 ± 0.39^{d}	4.95 ± 0.02^{b}	22.20 ± 0.14^{d}	28.80 ± 0.17^{bc}	2.68 ± 0.42^{ab}
	20	34.93 ± 0.25^{b}	67.52 ± 1.11^{b}	5.57 ± 0.10^{a}	22.55 ± 0.11^{c}	30.76 ± 0.23^{a}	3.06 ± 0.76^{a}
A2/A2 β-CN milk	4	29.69 ± 0.01^{d}	69.48 ± 0.59^{a}	4.46 ± 0.05^{c}	23.10 ± 0.01^{b}	28.93 ± 0.09^{b}	2.10 ± 0.39^{ab}
	20	30.19 ± 0.00^{d}	64.11 ± 0.59^{c}	4.96 ± 0.02^{b}	26.94 ± 0.05^{a}	30.75 ± 0.16^{a}	2.11 ± 0.38^{ab}
A1/A1 micellar CN	4	16.31 ± 0.17^{c}	1.85 ± 0.02^{e}	1.10 ± 0.00^{d}	2.54 ± 0.02^{f}	10.19 ± 0.10^{d}	1.48 ± 0.04^{b}
	20	16.28 ± 0.14^{c}	1.71 ± 0.02^{f}	1.19 ± 0.01^{c}	2.77 ± 0.01^{e}	10.27 ± 0.09^{d}	1.87 ± 0.05^{ab}
A1/A2 micellar CN	4	18.19 ± 0.07^{a}	3.64 ± 0.01^{d}	1.22 ± 0.01^{c}	3.67 ± 0.02^{c}	13.34 ± 0.02^{a}	2.25 ± 0.04^{a}
	20	17.17 ± 0.03^{b}	3.77 ± 0.04^{c}	1.50 ± 0.01^{a}	4.19 ± 0.00^{a}	13.51 ± 0.07^{a}	2.32 ± 0.47^{a}
A2/A2 micellar CN	4	15.85 ± 0.09^{d}	4.14 ± 0.03^{b}	1.02 ± 0.03^{e}	3.26 ± 0.02^{d}	11.87 ± 0.04^{c}	2.22 ± 0.02^{a}
	20	15.37 ± 0.02^{e}	4.61 ± 0.01^{a}	1.28 ± 0.00^{b}	4.08 ± 0.01^{b}	12.85 ± 0.09^{b}	2.14 ± 0.20^{a}

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$).

cow genetics, protein post-translational modification (phosphorylation [κ-casein], and glycosylation of CN molecules), levels of minerals, farming practices, and environmental factors (feed and season) (Bijl, Holland, & Boland, 2020). Notably, average particle size of milk and micellar CN from all three genetic variants of β-CN were significantly greater at 4 °C as opposed to 20 °C ($p < 0.05$, Supplementary Tables 3B and 4B). Such an observation is likely to be in part due to dissociation of β-CN out of the CN micelle as a consequence of the absence of hydrophobic attractions leading to an increased particle size at 4 °C (Bijl et al., 2020). Furthermore, temperature dependant CCP solubilisation may be accompanied by dissociation of individual CNs or primary particles from the micelle at lower temperature (McMahon, Du, McManus, & Larsen, 2009) leading to greater interparticle distances and thus an increased particle size.

The ζ-potential values measured in milk and micellar CN samples were dependent on genetic variant of milk and temperature ($p < 0.05$, Supplementary Tables, 3 B and 4 B); however, the impact of different κ-CN proteoforms should not be discounted. The lower net negative charge observed in A2/A2 β-CN milk compared to other β-CN milks reflects a lower level of κ-CN on its micelle surface (2.73 mg/mL in A2/A2 β-CN milk vs 2.92 mg/mL or 3.94 mg/mL in A1/A2 or A1/A1 β-CN milks, respectively); κ-CN maintains the net negative charge of the CN micelles (McMahon & Oommen, 2013). As previously stated, κ-CN may possess a path-terminating role and an assumed importance for the CN micelle size, thus it was found an inverse linear relationship between them. In this regard, a decreased fraction of total κ-CN content appear to correlate well with larger CN micelle size (Day et al., 2015).

In both sets of samples (milk and micellar CN), the surface potential at 20 °C increased in absolute terms (became more negative) compared to those at 4 °C ($p < 0.05$, Table 2). This has been confirmed by numerous studies with the values numerically similar to the current study (between −13.57 mV and −22.23 mV) (Anema & Klostermeyer, 1996; Famelart, Tomazewski, Piot, & Pezennec, 2003; Markoska et al., 2019a).

3.3. Structural characteristics of milk and micellar CN samples

3.3.1. FTIR analysis of structural features of different genetic variants

Structural differences of milk and micellar CN, prediction of the protein fractions, and changes in the secondary structure of proteins were elaborated using FTIR. Since the Amide I region, particularly the region between 1700 cm^{-1} and 1600 cm^{-1} (C═O stretching vibrations of the peptide bonds), has the greatest contribution to assessment of the secondary structure of proteins and is the one that undergoes the largest changes, it was chosen for further analysis (Jackson & Mantsch, 1995). For the purpose of the hidden peaks' visualisation, and therefore separation of the overlapping bands into distinct peaks, the second derivatives of the FTIR spectra in the Amide I region were calculated for both, milk and micellar CN samples (Fig 2A and 3A).

The analysis of milk and micellar CN samples derived from all three β-CN genetic variants revealed considerable variations in the peaks' intensity, at 1695 cm^{-1}, 1684 cm^{-1} (aggregated β-sheet); 1677 cm^{-1}, 1665 cm^{-1} (β-turn); 1659 cm^{-1}, 1654 cm^{-1}, 1648 cm^{-1}, (α-helix); 1642 cm^{-1} (random coil); 1635 cm^{-1}, 1625 cm^{-1}, (intramolecular β-sheet);

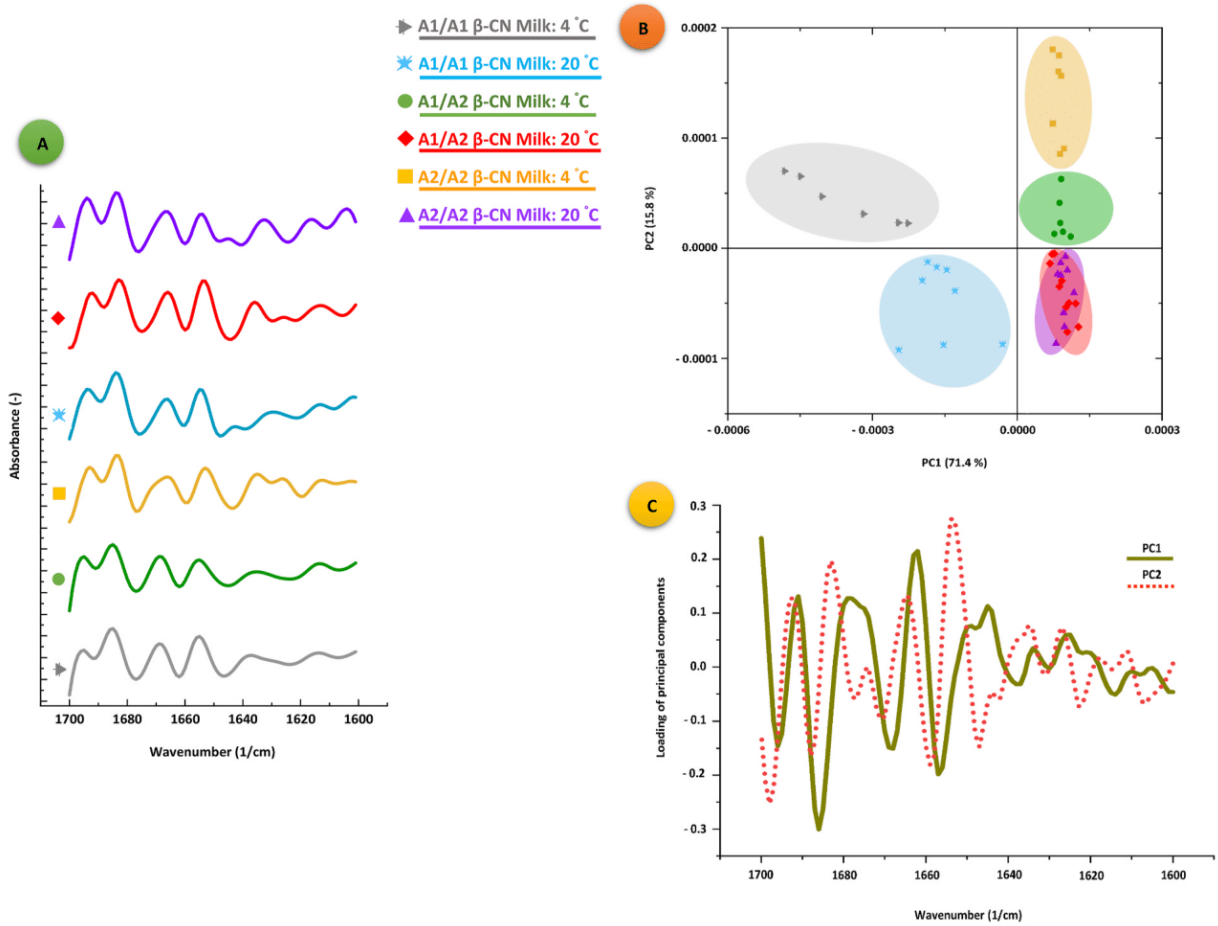

Fig. 2. A) Second derivative spectra of Amide I region of milk samples. Averaged spectra of ten repeated measurements on the subsamples from batches of milk. **B)** Principal Component Analysis score plot of milk samples in the 1700 cm^{-1} - 1600 cm^{-1} region. **C)** Principal Component Analysis loading plot of milk samples in the 1700 cm^{-1} - 1600 cm^{-1} region.

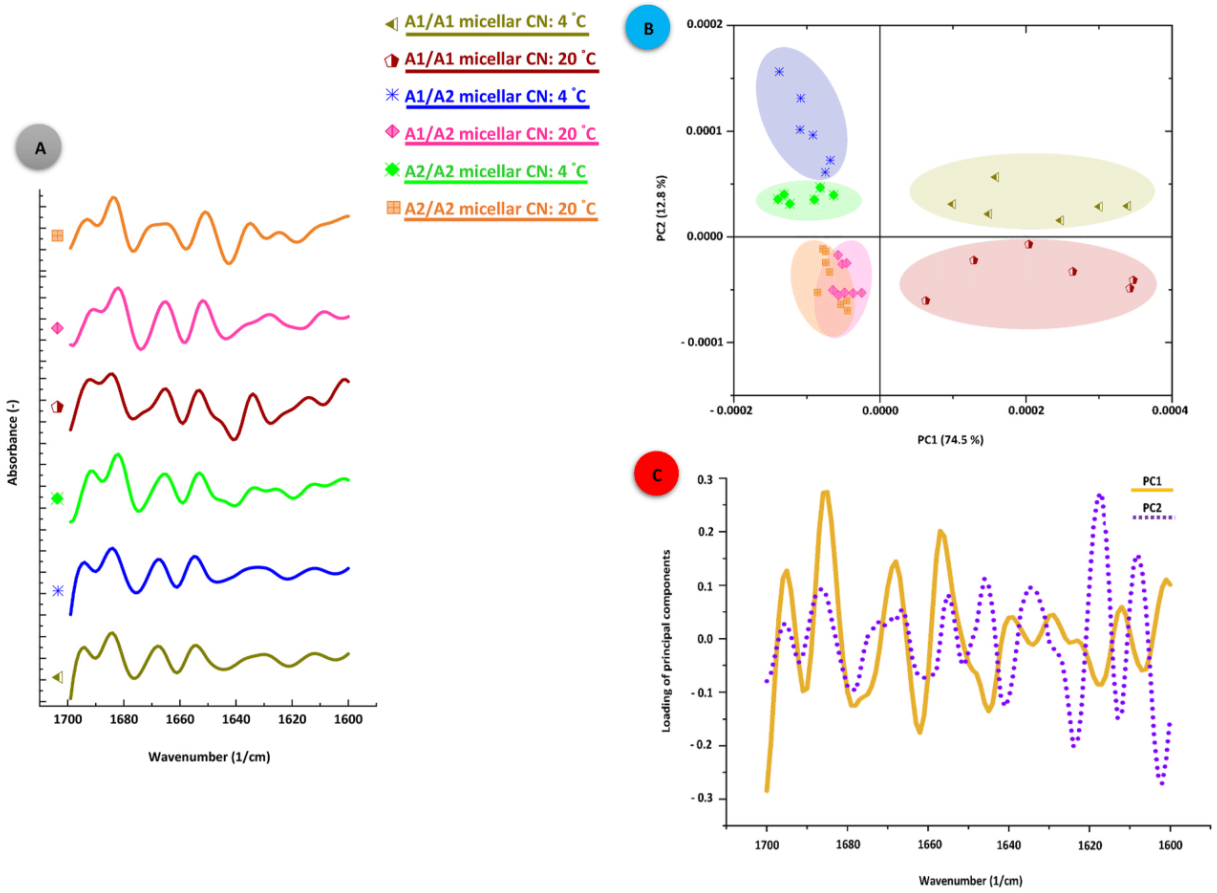

Fig. 3. A) Second derivative spectra of Amide I region of micellar CN samples. Averaged spectra of ten repeated measurements on the subsamples from batches of micellar CN. **B)** Principal Component Analysis score plot of micellar CN samples in the 1700 cm^{-1} - 1600 cm^{-1} region. **C)** Principal Component Analysis loading plot of micellar CN samples in the 1700 cm^{-1} - 1600 cm^{-1} region.

and 1611 cm^{-1} (side chain) (Grewal et al., 2017). The complete assignment of secondary structure components based on proportion of the peak area is presented in Tables 3 and 4. It is worth noting that these structural elements have previously been established on various globular proteins, while the CN micelles, which are assessed in the current work, have a rather very complex structure and these peak assignments could denote similar structural features.

The difference between genetic variants at room temperature was observed in greater proportion of β-turns and α-helical structures in A1/A1 β-CN milk compared to the other two genetic variants, which in the past was attributed to bonding between κ-CN and serum proteins (Dalgleish & Corredig, 2012). In addition, a high peak for κ-CN in A1/A1 β-CN milk was observed in the HPLC chromatogram (Fig. 1), which likely confirms its involvement in the secondary structure of this genetic variant. Nevertheless, as β-CN remains the main variant between observed samples, a high proportion of turns in A1/A1 β-CN milk and A1/A2 β-CN milk can also relate to involvement of β-CN in their formation. In fact, β-CN can form 20–30% of turns in milk (Huppertz, 2013) that can be Pro and non-Pro based turns (Kumosinski, Brown, & Farrell Jr, 1993). Presence of Pro residues favours formation of β-turns, presumably due to their cyclic structure (McSweeney & Fox, 2013), thus it would be expected to see a greater proportion of β-turns in A2/A2 β-CN milk as it contains more Pro residues. However, β-turns containing Pro residues may also result in van der Waals attractions with surrounding residues, leading to assignment of a single Pro initially to a β-turn conformation, depending on the neighbouring residues (Kumosinski, Brown, & Farrell, 1993). Graham, Malcolm, and McKenzie (1984) assessed differences in genetic variants of β-CN at residue 67 (His^{67} or

Table 3
Total percentage areas of different secondary structures in Amide I in genetic β-CN variants in skim milk.

Band Assessment	Band frequency (cm^{-2})	Peak area (%)					
		A1/A1 β-CN milk	A1/A2 β-CN milk	A2/A2 β-CN milk	A1/A1 β-CN milk	A1/A2 β-CN milk	A2/A2 β-CN milk
		4 °C			20 °C		
Side chain	1604–1608	4.10 ± 1.53^{b}	2.50 ± 1.30^{b}	5.20 ± 0.50 ^{ab}	5.80 ± 3.60 ^{ab}	5.80 ± 3.00 ^{ab}	9.90 ± 4.00^{a}
Intramolecular β-sheet	1622–1631	12.90 ± 2.71^{a}	17.80 ± 11.30^{a}	18.80 ± 15.50^{a}	13.60 ± 5.72^{a}	24.90 ± 10.10^{a}	14.30 ± 1.90^{a}
Random coil	1643	n/a	n/a	n/a	n/a	n/a	15.20 ± 6.80$^{n/a}$
α-helix	1647–1660	43.80 ± 1.30^{a}	26.60 ± 5.00 ^{cd}	41.90 ± 3.00^{a}	38.80 ± 1.31 ^{ab}	31.70 ± 8.00 ^{bc}	17.90 ± 7.00^{d}
β-turn	1974–1978	29.30 ± 2.94 ^{abc}	39.30 ± 5.60^{a}	18.40 ± 9.90^{d}	30.10 ± 1.30 ^{ab}	26.70 ± 2.50 ^{bcd}	24.90 ± 2.50 ^{cd}
Aggregated β-sheet	1697–1700	10.00 ± 0.98^{a}	13.90 ± 7.30^{a}	15.70 ± 5.40^{a}	12.50 ± 0.99^{a}	10.80 ± 4.50^{a}	17.90 ± 3.20^{a}

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$).

Table 4
Total percentage areas of different secondary structures in Amide I in genetic β-CN variants in micellar CN.

Band Assessment	Band frequency (cm^{-2})	Peak area (%)					
		A1/A1 micellar CN	A1/A2 micellar CN	A2/A2 micellar CN	A1/A1 micellar CN	A1/A2 micellar CN	A2/A2 micellar CN
		4 °C			20 °C		
Side chain	1602–1609	7.70 ± 0.75[a]	6.60 ± 4.00[a]	4.60 ± 2.50[a]	6.10 ± 2.25[a]	8.30 ± 4.30[a]	7.20 ± 4.40[a]
Intramolecular β-sheet	1622–1631	13.00 ± 0.95[a]	17.50 ± 7.20[a]	19.40 ± 11.20[a]	11.40 ± 0.94[a]	34.90 ± 12.80[b]	20.90 ± 5.40 [ab]
Random coil	1643–1645	n/a	16.20 ± 7.30[a]	n/a	n/a	n/a	22.70 ± 0.70[a]
α-helix	1652–1659	36.40 ± 1.36 [bc]	11.20 ± 4.30[a]	39.80 ± 7.30[c]	37.30 ± 8.58 [bc]	24.90 ± 12.70 [ab]	14.50 ± 4.80[a]
β-turn	1974–1978	30.60 ± 0.94[a]	30.70 ± 8.00[a]	24.70 ± 13.00[a]	26.80 ± 5.02[a]	21.90 ± 7.90[a]	22.60 ± 3.90[a]
Aggregated β-sheet	1693–1700	12.30 ± 1.33[a]	17.90 ± 4.60[a]	11.50 ± 5.00[a]	18.30 ± 15.64[a]	10.00 ± 3.20[a]	12.10 ± 5.40[a]

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$).

Pro^{67}) that caused the turn at f67-70 (fragment: His^{67} - Asn^{68} - Ser^{69} - Leu^{70}) observed only in the A1 β-CN genetic variant. This has led to an increased number of β-turns in milk containing A1 β-CN. Our FTIR results indicate similar observations as these showed that almost 29%–39% of the total turns originated from A1/A1 β-CN milk, A1/A2 β-CN milk and their micellar CNs, which is greater than that in A2/A2 β-CN samples (Tables 3 and 4).

Another difference between the genetic variants at room temperature was in the intramolecular β-sheets. The intramolecular β-sheets were approximately 10% more present in A1/A2 β-CN milk compared to other genetic variants of milk. The β-sheets in milk predominately are found in globular proteins, including WPs, mainly β-Lg, which was previously found to have eight β-sheets in its monomeric form (Uhrínová et al., 1998). However, since the samples were baseline corrected with the corresponding whey, it would be expected that these observations were clearly governed by the properties of the CN micelles in milk and the greater amount of β-sheets in A1/A2 β-CN milk could be related to the structural orientation of β-CN variant (Table 3). Similarly, 15–33% of β-sheets formations were also reported within the β-CN structure (Huppertz, 2013).

The A2/A2 β-CN milk is depicted by a great amount of random structures and fewer α-helical structures compared to the other two milk genetic variants. The peak referred to as random coil (1643 cm^{-1}) was previously assigned to short polyproline II helix/chain (PPII) (Dukor & Keiderling, 1991). Moreover, in A2/A2 β-CN milk the presence of Pro in position 67 favours the existence of PPII helix, disturbing formation of traditional α-helix, and thus possesses a crucial factor in hydrogen bonding interactions with the CN micelle (Raynes et al., 2015). As observed in Table 4, A2/A2 micellar CN contains ≈23% of random or PPII structures. Thus, in the micellar environment A2 β-CN appears to play an important part in the CN micelle formation through PPII mediated interactions leading to formation of CN micelles (Adzhubei, Sternberg, & Makarov, 2013). Furthermore, as the amount of α-helices decreased, it led to an exposed hydrophobic cavity of A2 β-CN leading to alternations in its interfacial properties and increase in hydrophobicity (Zhou, Zhu, Zhang, Hu, & Pan, 2021), also depicted by the RP-HPLC in the current study (section 3.1.).

The differences in structural components based on genetic variance were also identified by a multivariate analysis (PCA), which classified the samples into different groups. Namely, the Principal Component 1 (PC1) separated A1/A1 β-CN from A1/A2 β-CN and A2/A2 β-CN milk (71.4%) and micellar CN (74.5%) samples based on structural differences in proteins (Fig 2B and 3B). According to the PC1 loading plot, multiple peak loadings were present for all three genetic variants. Negative loading for A1/A1 β-CN milk was observed at 1618 cm^{-1} (side chain), 1658 cm^{-1} (α-helix), 1668 cm^{-1} (β-turn), 1683 cm^{-1} and 1695 cm^{-1} (native aggregated β-sheets). Contrarily, a positive loading for A1/A2 β-CN and A2/A2 β-CN milk samples was present at 1625 cm^{-1} and 1632 cm^{-1} (intramolecular β-sheets), 1645 cm^{-1} (random strain), 1665 cm^{-1} and 1679 cm^{-1} (β-turns) and 1690 cm^{-1} (aggregated β-sheet) (Fig 2C and 3C). The PC1 confirmed the differences among the genetic variants based on the structural features. In the loading score (Fig. 2B) the main difference was observed along PC1 by separating A1/A1 β-CN milk from the other two milk genetic variants. The PCA further confirmed these differences between variables to be induced by CNs (predominately β-CN) by separating the micellar CN samples along PC1 with 74.5% variance in the same trend as observed in the PCA of the milk samples. Nevertheless, A1/A1 micellar CN compared to other micellar CNs behaved similarly at both temperatures, owing to the fact that A1/A1 micellar CN does not contain A2 β-CN in its structure, leading to a possible different functioning. This can be directly associated to increased exposed hydrophobicity of A1 β-CN compared to A2 β-CN (Raynes et al., 2015). Since hydrogen bonding and hydrophobic interactions are important for the CN micelle formation (Holt, Carver, Ecroyd, & Thorn, 2013), the different polarity of both β-CNs would contribute to this altered equilibrium. As mentioned previously, the crucial factor that may affect β-CN interactions is the greater PPII helix conformation in A2 β-CN as a result of the amino acid mutation as part of its protein chain (Pro ^{67} [A2 β-CN] and His ^{67} [A1 β-CN]) (Raynes et al., 2015). This statement can further be bolstered by the importance of the A2 β-CN that contains additional Pro residue in its structure. Thus, A2 β-CN could increase the PPII helix formation and may show an influence on the protein-protein interactions (binding to other proteins) leading to an alternation of the proteins' function (Farrell, Wickham, Unruh, Qi, & Hoagland, 2001).

As expected, the intensity of the peaks at 4 °C significantly shifted, rearranged, decreased, or increased in comparison to the peaks and bands at 20 °C in all milk and micellar CN samples. At 4 °C, the native structural organisation between the genetic variants was slightly changed. Thus, β-turns were observed to be more dominant in A1/A2 β-CN milk ($p < 0.05$) and its micellar CN, α-helixes in both A1/A1 β-CN and A2/A2 β-CN milk and their CN micelles ($p < 0.05$), and intermolecular β-sheets for A1/A2 β-CN and A2/A2 β-CN milk and their micellar CNs ($p < 0.05$). Moreover, the PCA again supported the same grouping trend as in samples at room temperature by separating the A1/A1 β-CN milk from A1/A2 β-CN and A2/A2 β-CN milk along PC1. The temperature effect depicted the expected structural reorganisation. The β-CN milk samples established on temperature, namely all genetic variants of milk at 4 °C were divided from all genetic variants of milk at 20 °C along the direction of Principal Component 2 (PC2) (15.8%). This trend was identical for micellar CN samples by separating the micellar CN variants based on temperature change along PC2 with 12.8% variance. Therefore, the PCA clearly separated A1/A1, A1/A2, and A2/A2 β-CN milks and micellar CNs at 4 °C implying significant changes in milk and protein secondary structure in each group (Fig 2B and 3B). The PC2 included negative peaks at 1698 cm^{-1} and 1687 cm^{-1}, 1659 cm^{-1}, 1646 cm^{-1}, 1622 cm^{-1}, and 1607 cm^{-1} (aggregated β-sheet, α-helix, intramolecular β-sheet, and side chain); and positive peaks at 1692 cm^{-1}, 1683 cm^{-1} (aggregated β-sheets), 1665 cm^{-1} (β-turn), 1653 cm^{-1} (α-helix) and 1639 cm^{-1}, 1630 cm^{-1} (intramolecular β-sheets). Based on

the lower percentage of significance herein only the main changes are discussed. Consequently, in the loading plot for the β-CN milk and micellar CN genetic variants, the main difference was observed in the region between 1660 cm^{-1} and 1600 cm^{-1} (Fig 2C and 3C). Notably, it appears that transition from 4 °C to 20 °C led to changes in the α-helixes, random structures, intermolecular β-sheets, and side chains in the proteins. Most probably, this appeared as a result of the CN micelle dissolution, which occurred when milk samples were kept at 4 °C, leading to an absence of the hydrophobicity (rearrangement of revealed hydrophobic binding sites) and, therefore, partial liberation of β-CN into the serum phase (Bijl et al., 2020). Changes in the loading intensity for side chain at 1609 cm^{-1} at 20 °C compared to at 4 °C is likely due to amino acid side chain absorptions, primarily tyrosine (Tyr) residues (Matsuura, Hasegawa, & Miyazawa, 1986). For the other secondary structures, it has been found that neither the genetic variants nor the temperatures showed any effect ($p > 0.05$) (Supplementary Tables 3C and 4C).

3.3.2. 1D ^1H NMR analysis of milk of different genetic variants

In the current study to elucidate the structural differences between the milk and the micellar CN samples, two major regions within the NMR spectra were analysed. The included regions for both sets of samples were the aliphatic region, between 5 ppm and 0 ppm (including the carbohydrate region found from 4.7 ppm to 3.1 ppm); and the aromatic/H^N region from 9 ppm to 5.5 ppm (Garwolińska, Hewelt-Belka, Kot-Wasik, & Sundekilde, 2020). Thus, most non-related milk molecules and metabolites (if not significant for the structure) were excluded from the discussion.

In this study, we used ^1H NMR to observe the proton changes in milk and micellar CN samples with different genetic variants and temperature at 4 and 20 °C. NMR highlighted the difference among genetic variants as presented in Fig 4A and 5A. Hence, the observed milk samples have shown similar chemical shift distribution regardless of temperature. Slight difference between these three milk samples was noted in the aliphatic region of the NMR spectra (2 ppm–0 ppm). The peak at 1.9 ppm appeared as a doublet in A2/A2 β-CN milk and singlet in other milk samples. In the A1/A1 β-CN milk, more pronounced changes were observed in chemical shifts in the aliphatic region compared to other variants. Thus, the side chains of some amino acids (Ile, Val, and Leu) (Devold et al., 2000) have shown lower peak intensity in comparison to the other observed milk variables. Similar to FTIR spectra, NMR analysis confirmed presence of more turns and helical structures in the A1/A1 β-CN milk. This can relate to the fact that protons of these amino acids may be involved into hydrophobic bonds with other hydrogens indicating to different structural organisation compared to the other two genetic variants. Noteworthy, in A1/A1 β-CN milk the sidechains of the amino acids played important role in hydrophobic interactions and thus structural reorganisation in molecules, which differentiated this milk from both, A1/A2 β-CN milk and A2/A2 β-CN milk. These observations followed similar trend among the genetic variants in micellar CN samples (Fig. 5).

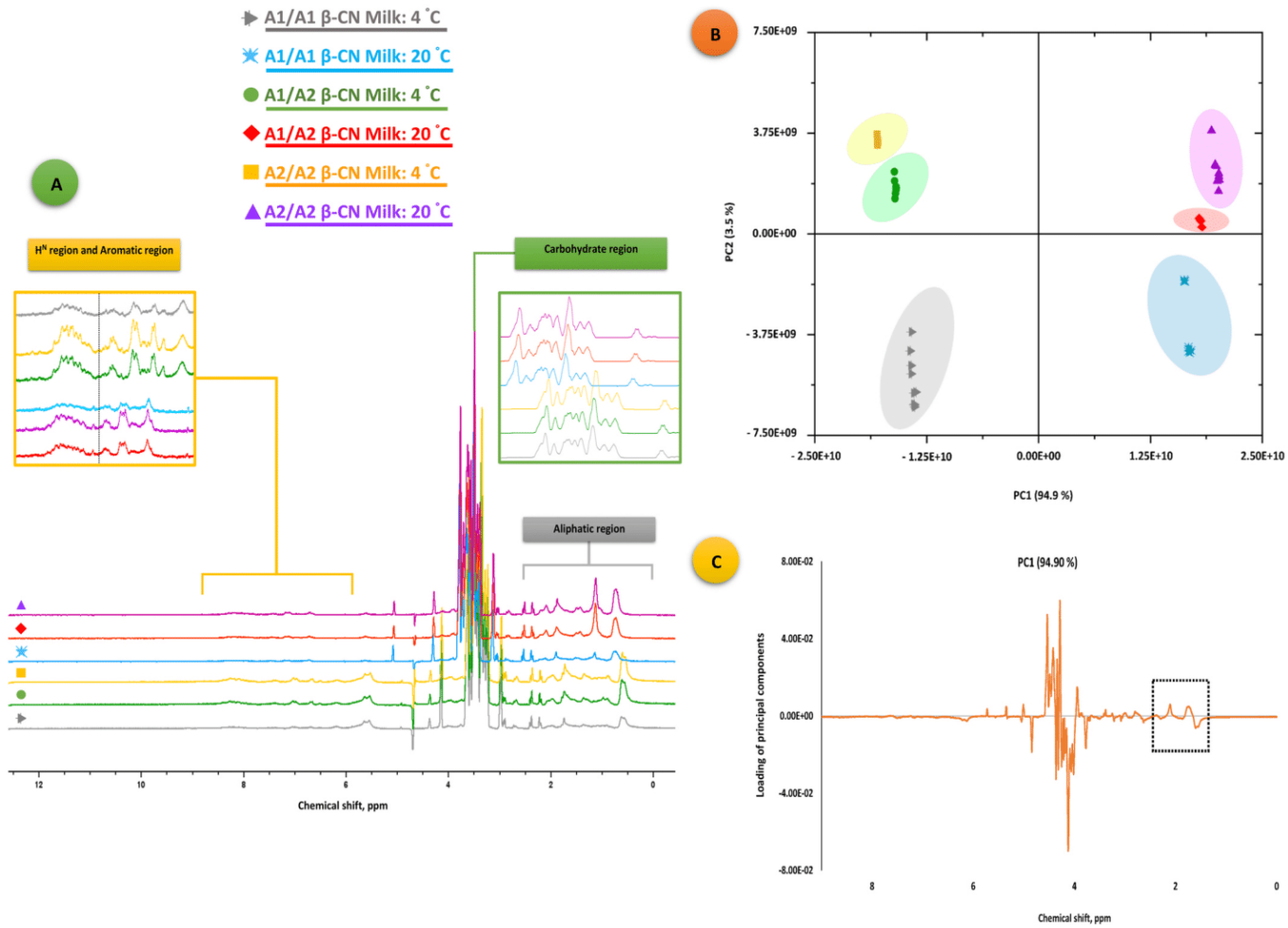

Fig. 4. A) ^1H NMR spectrum of milk samples. Averaged spectra of ten repeated measurements on the subsamples from batches of milk. **B)** Principal Component Analysis score plot of milk samples obtained by the ^1H NMR spectrum. **C)** Principal Component Analysis loading plot of milk samples obtained by the ^1H NMR spectrum.

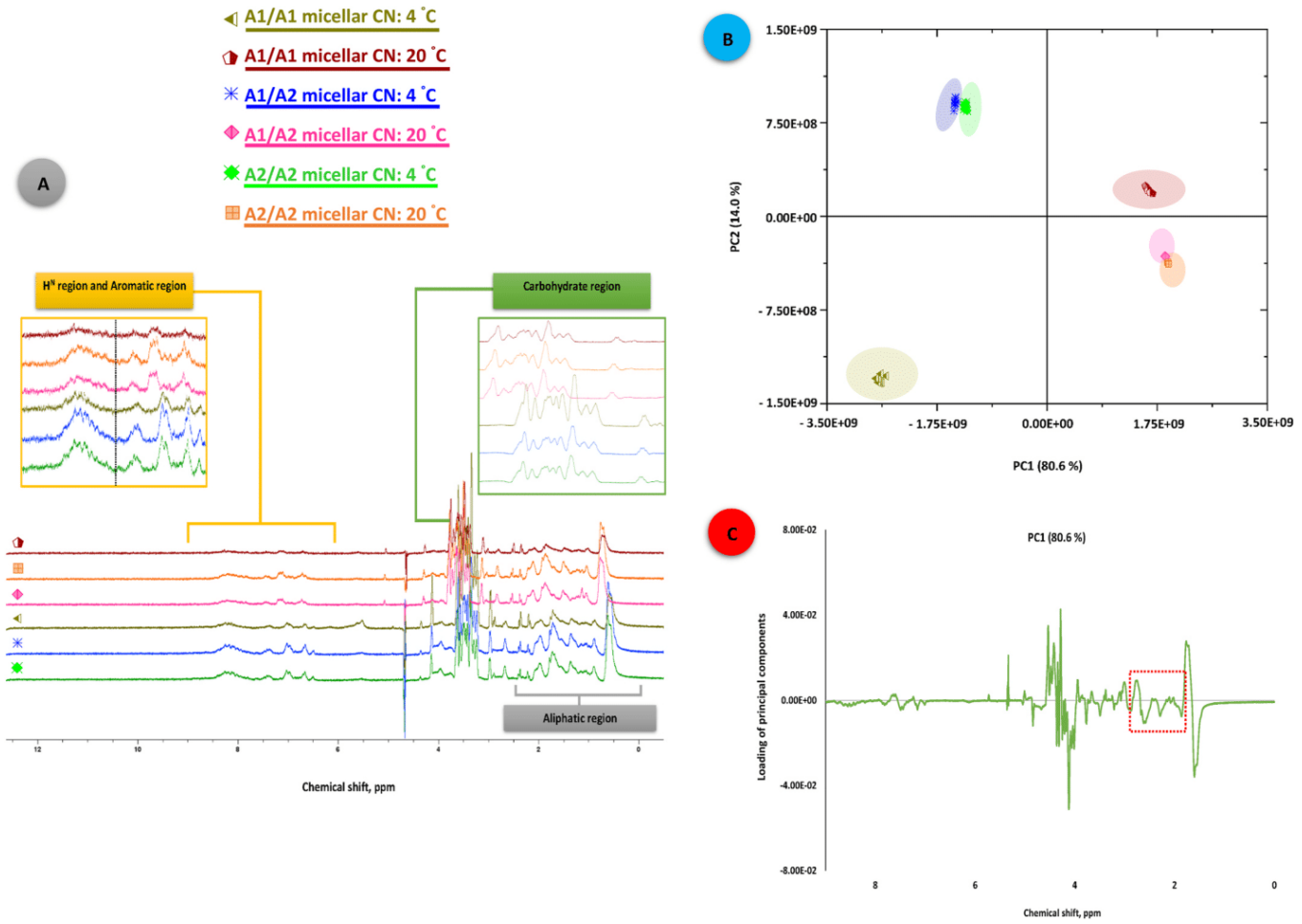

Fig. 5. A) ^{1}H NMR spectrum of micellar CN samples. Averaged spectra of ten repeated measurements on the subsamples from batches of micellar CN. **B)** Principal Component Analysis score plot of micellar CN samples obtained by the ^{1}H NMR spectrum. **C)** Principal Component Analysis loading plot of micellar CN samples obtained by the ^{1}H NMR spectrum.

The results in Fig 4A and 5A indicate a substantial difference between milk and micellar CN samples due to temperature, respectively. For all samples, a constant up-field shifting of peaks was noted when the samples were cooled at 4 °C. Temperature dependant proton shifting was likely due to changes in hydrogen bonding, temperature dependant changes in the structure, and exchange between district protein conformations (Trainor, Palumbo, MacKenzie, & Meiering, 2020). Moreover, it was previously observed that shielding and change in the peak intensity in milk due to the temperature change is a result of conformational changes of CN micelle and dissociation of CNs components from the micelle (Rollema & Brinkhuis, 1989). In the aliphatic region up-field shielding of the peaks is observed ≈0.3 ppm related to the alkyl groups of proteins at 4 °C (Farahani et al., 2014). Another substantial difference in the aliphatic region is related to the peak at 1.17 ppm, which appears for A2/A2 β-CN milk and A1/A2 β-CN milk and micellar CN samples at 20 °C with high intensity, and A1/A1 β-CN milk and micellar CN samples at 20 °C with lesser intensity. However, this peak is not present in any of the samples at 4 °C. The presence of these peaks relates to changes in the β-turns in the polypeptide chains of proteins (Farahani et al., 2014) resulted from cis/trans isomerism of X-Pro bond and changes in the proximity between alkyl fragments (CH_2–CH_3) of amino acids and H^{α} protons of Pro. This change in peaks distribution may be due to shifting of intermolecular distances between chromophoric groups (Perminova et al., 2018).

The most abundant molecule detected in milk and micellar CN samples treated at 4 and 20 °C was lactose; the carbohydrate (middle) region dominated by the broad lactose peak between 4 ppm and 3 ppm (Dangat et al., 2016). In Fig 4A and 5A it was observed up-field shift of lactose peak at 4 °C, for all samples with small change in the peak intensity. In this region α-protons (H^{α}) of amino acids were also observed, however, the broad signal of lactose overlapped with the H^{α} signal.

The aromatic region follows similar shielding trend as previously observed in the aliphatic and carbohydrate regions. The observed shielding for the protons in the above observed regions occurs due to extending of the intermolecular bonds at low temperature. This led to weakening of ^{1}H-X electron polarisation and promoting up-field shifting in chemical shifts (Hong, Jing, & Yao, 2013). However, in the Amide region (H^{N}) of the spectra, the H^{N} peaks were observed at both temperatures. Moreover, this region in all samples (milks and micellar CNs) remained stable due to the temperature change. The Amide region of the polypeptide chains in proteins have strongly bonded hydrogen that was not affected by the temperature change. Nevertheless, the main difference in this region was in the peak's intensity. Thus, samples at 4 °C had higher peak loading compared to the samples at 20 °C. This is further confirmed in the PCA loading plots indicating a greater presence of Amide protons for samples at 4 °C (Fig 4C and 5C). The temperature induced changes by increase in peak's intensity can indicate that the rigid core of the CN micelle became mobile (Rollema & Brinkhuis, 1989).

PCA was performed separately for milk and micellar CN samples. In milk, two principal components (PCs) were able to explain 98.4% of the total variability. The first PC (PC1), representing 94.9%, while PC2

accounted for 3.5% of the total variance. In micellar CN samples, both PCs explained 80.6% and 14.0% of the variance, respectively. In milk and micellar CN samples, PC1 was positively linked to all three genetic variants at 20 °C, while negatively associated with all three genetic variants at 4 °C. Thus, PC1 has separated the milk and micellar CN samples based on the temperature difference. Additionally, PC2 positively correlated with A1/A2 β-CN milk and A2/A2 β-CN milk, at 4 °C and 20 °C, whereas it was inversely correlated to A1/A1 β-CN milk at both temperatures. On the contrary, PC2 divided A1/A1 micellar CN at 20 °C, and A1/A2 micellar CN, A2/A2 micellar CN at 4 °C (positive), out of A1/A1 micellar CN at 4 °C, and A1/A2 micellar CN, A2/A2 micellar CN at 20 °C (negative) (Fig 4B and 5B). In addition, the difference in peak intensity between milk samples with different genetic variations and temperature of 20 °C is related to different structural orientation of the polypeptide chain in proteins mainly between A1 β-CN and A2 β-CN milk and micellar CN samples. The difference in the aliphatic region was also confirmed by PCA loading plot and score (Fig 4C and 5C), where two positive loadings are presented for all samples at room temperature and only one negative loading for samples at 4 °C. Additionally, in the same region, two positive loadings are present for all micellar CN samples at 20 °C and three negative loadings for samples at 4 °C.

The present study, using the set of three genetic variants of milks and micellar CNs, found that it would likely be possible to authenticate these samples by means of FTIR and ^{1}H NMR fingerprinting. Nevertheless, larger scale and extensive data studies with larger sample sizes that would contain A1/A1, A1/A2, or A2/A2 β-CNs are needed to further elaborate on the structural impact of these genetic variants on both milk and micellar CN. This would also include the impact of κ-CN or β-Lg levels, their polymorphic variants, degree of κ-CN glycosylation, composite αs_1-β-κ-CN variants, which were not assessed in the current study, all of which influence various interactions, mineral levels in milk or micellar CNs, protein conformation, and most importantly the size of CN micelle. Particularly, the genetic variants of both, κ-CN and β-Lg have been related to milk production traits, coagulation time, curd firmness, and cheese yield (Bonfatti et al., 2010). Regardless of the genetic variant of β-CN, numerous studies have associated the genetic nature of κ-CN with the CN micelle size of individual bovine milk samples (Bijl et al., 2014; Bonfatti, Chiarot, & Carnier, 2014; Day et al., 2015; Hallén, Wedholm, Andrén, & Lundén, 2008; Ng-Kwai-Hang, Otter, Lowe, Boland, & Auldist, 2002; Vallas et al., 2012). While κ-CN appears crucial for the behaviour of the CN micelle, a role of β-CN genetic variants should not be disregarded. It is known that phosphorylation sites of β-CN, critical for the internal micellar formation via calcium phosphate nanoparticles (Huppertz, 2013), may be influenced by the single amino acid mutation, which might directly or indirectly control the asymmetric growth of the CN micelles (Day et al., 2015). Respectively, further studies might correlate the genetic variants of both, β-CN and κ-CN to illuminate this conundrum and thus reveal the robustness of FTIR and ^{1}H NMR methods for conformational classifications of β-CN milks and their CNs. Thus, A1/A1, A1/A2, or A2/A2 β-CN milks coupled with only a homozygous κ-CN might additionally establish the nature of these types of milk, particularly when assessing their physicochemical properties. While the current study has shed some light to conformational properties of CN micelles of these three genetic variants, it still remains to be seen whether β-CN variants influence the internal structure of the CN micelle, as well as functional characteristics of milk.

4. Conclusions

This study brings new insights on the conformational differences among A1/A1, A1/A2, and A2/A2 β-CN milk genetic variants. In both milk and micellar CN samples, structural differences were observed and the similar pattern trend between these two samples indicated that differences were due to composition of the CN micelle and properties of β-CN genetic variants. The β-turn and α-helix structures were predominant in the A1/A1 β-CN genetic variant, intermolecular β-sheets dominated in the A1/A2 β-CN variant, while the A2/A2 β-CN genetic variants of milk and micellar CNs possessed more random structures. The latter are classified as PPII helix based on the band absorption. These structural characters are based on the genetic variant of β-CN in the samples and its involvement in different structural formations in the CN micelle. Thus, for both milk and micellar CN samples, A1/A1 β-CN variant clearly separates from A2/A2 β-CN variant. However, A1/A2 β-CN variant showed similarity with A2/A2 β-CN variant indicating that A2 β-CN dominates in the protein's structural organisation. Their differentiation was consistent regardless of the temperature, which expectedly affected the structural organisation of the samples.

Milk and micellar CN samples originating from A2/A2 β-CN genetic variant had greater average micelle size than those from A1/A2 β-CN and A1/A1 β-CN genetic variants. In addition, A2/A2 CN micelles had a lower net negative ζ-potential compared to the other β-CN genetic variants, possibly due to fewer κ-CNs on the micelle surface. All samples were similar in terms of mineral composition and their balance and equilibration was only affected by temperature.

This is the first structural description and differentiation of A1/A1 β-CN milk, A1/A2 β-CN milk, and A2/A2 β-CN milk and their micellar CNs using FTIR in tandem with ^{1}H NMR. These techniques were capable of distinguishing between the structures of these milk samples and their corresponding micellar CNs originating from different β-CN genetic variants. Thus, these tools, especially application of FTIR, could be used as an effective and fast approach in determining different genetic variants of milk and micellar CN samples. It will be interesting to monitor future trends to gain better insights into how the structure of CN micelles can be influenced by changing nature of some conditions, particularly temperature treatment, pH modification, and addition of salts. This study has added useful knowledge regarding the main conformational similarities and differentiations between these three genetic variants of milk and their micellar CNs, which can further lead to further shedding more light on the impact of structural differences of these genotypes onto technological properties of corresponding milk and human health.

Author contributions

Davor Daniloski conceived the study and research question; designed and wrote the original draft, conceptualised, reviewed, edited the manuscript, designed the tables and the figures. Davor Daniloski prepared the methodology, formal analysis and investigation. Todor Vasiljevic and Noel A. McCarthy provided critical feedback and analysis, secured funding, reviewed and edited the manuscript and supervised the study. Tatijana Markoska and Martin J. Auldist gave critical feedback and analysis, reviewed and edited the manuscript. Martin J. Auldist supplied the milk samples. All authors have contributed to the manuscript and reviewed the final version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2021.107186.

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Main findings: Part I. Chapter 5.

Impact of heating on the properties of A1/A1, A1/A2, and A2/A2 β-casein milk phenotypes

- FTIR and 1H NMR identified structural variations of heat-treated milks
- Heat treatment decreased random coils and α-helices amount in all milks
- Aggregated β-sheets in A1/A2 milk related with the tyrosine residues
- A1/A1 and A1/A2 milks were similar; A2/A2 milk possessed unique traits
- β-casein proteoforms influenced the micelle size and the amount of minerals

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Impact of heating on the properties of A1/A1, A1/A2, and A2/A2 β-casein milk phenotypes

Davor Daniloski [a,b], Noel A. McCarthy [b], Todor Vasiljevic [a,*]

[a] Victoria University, Advanced Food Systems Research Unit, Institute for Sustainable Industries and Liveable Cities and College of Health and Biomedicine, Melbourne, Victoria, 8001, Australia

[b] Teagasc Food Research Centre, Food Chemistry and Technology Department, Moorepark, Fermoy, P61 C996, Cork, Ireland

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ABSTRACT

This study assessed the effect of heat treatment on the physical properties of bovine milk samples possesing different β-casein (β-CN) phenotypes. After heat treatment at 72 °C/15 s, 121 °C/2.6 min, or 140 °C/3 s, β-CN milks were analysed using Fourier Transform Infrared (FTIR) spectroscopy, Nuclear Magnetic (^{1}H NMR) resonance, and a number of physicochemical measurements. Principal Component Analysis (PCA) was used to provide a discrimination of samples. In contrast to significant amounts of intramolecular β-sheet, β-turn, and random coil, in A1/A1, A1/A2, and A2/A2 β-CN milks, respectively, increasing the heat treatment temperature decreased the level of intramolecular β-sheets in all three types of bovine milk. The main difference involved a higher presence of aggregated β-sheet structures in A1/A2 β-CN milk likely due to the presence of tyrosine. A1/A1 and A1/A2 β-CN milks were characterised with greater amounts of calcium and phosphorus, and a higher net negative zeta potential than A2/A2 β-CN milk. Furthermore, A2/A2 β-CN milk was composed of larger casein micelle particles with lower levels of κ-CN compared to the other β-CN milk phenotypes. These findings may assist in predicting the behaviour of β-CN milks during relevant industrial processing.

1. Introduction

Bovine milk is a perishable and complex food system, and if not properly processed, can rapidly lose its nutritional quality. Heat treatment is the standard means of meeting safety criteria by reducing bacterial and enzymatic activity, thus prolonging the shelf-life and functionality of milk and dairy products (Anema, 2019; Bogahawaththa, Trajkovska, Markoska, & Vasiljevic, 2021; Deeth & Lewis, 2016; Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015a). Depending on the application and industrial needs, a wide range of temperatures are employed during thermal heat treatment, from moderate levels of heating aimed at the inactivation of pathogens (pasteurisation at ~ 72 °C) to levels surpassing 121 °C and 140 °C as in the case of in-container sterilisation and ultra-high temperature processing, respectively (Dumpler, Huppertz, & Kulozik, 2020). Particularly, the heat treatment above 70 °C may alter the nutritional and physicochemical properties of milk, including the mineral equilibria (calcium phosphate precipitation), lactose isomerisation (Maillard reactions), and pH variations that may affect the functionality of some dairy products (Bogahawaththa et al., 2021). Over the last two decades, tremendous research effort has been applied into understanding how heat treatment of milk influences the nature and behaviour of milk proteins (Anema, 2021; Deeth & Lewis, 2017; Huppertz, 2016; Singh & Latham, 1993). Therefore, it was postulated that the diversified functionalities of milk proteins arise from their conformational state upon heat treatment (McSweeney & Fox, 2013). In this regard, heat treatment of bovine milk affected the secondary structure of proteins, which led to formation of more random protein structures (Markoska, Huppertz, Grewal, & Vasiljevic, 2019) and intermolecular β-sheet aggregates (Mediwaththe, Bogahawaththa, Grewal, Chandrapala, & Vasiljevic, 2018).

Milk proteins can be distinguished into two major groups, caseins (CNs) and whey proteins (WPs), by their solubility at pH 4.6. Caseins, denoted as αs_1-, αs_2-, β- and κ-CNs, make up 80% of bovine milk proteins and are mostly present in milk in the form of CN micelles; the surface of the micelles is composed of the amphiphilic κ-CNs, which stabilise them against aggregation (De Kruif, Huppertz, Urban, & Petukhov, 2012; Holt, Carver, Ecroyd, & Thorn, 2013; Huppertz, 2013). Caseins are heat stable, both in the form of CN micelles and caseinates, due to their unstructured nature. In contrast, α-lactalbumin (α-La) and β-lactoglobulin (β-Lg), known as the major WPs are heat labile and are readily denatured

* Corresponding author.
E-mail address: todor.vasiljevic@vu.edu.au (T. Vasiljevic).

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under heating at around 70 °C (McSweeney & Fox, 2013). Specifically, β-Lg contains a free thiol group that is readily exposed by thermal unfolding; it is responsible for the heat-induced aggregation between different WPs and between WPs and CN micelles in milk because of the formation of disulphide bonds (Anema, 2021). Hence, WPs especially β-Lg can denature/aggregate and potentially associate with CN micelles via κ-CN linkages or form κ-CN/β-Lg complexes in the serum phase depending on pH (Bogahawaththa et al., 2021). Thus, upon the heat treatment of bovine milk, the proteins undergo different reversible and irreversible changes, such as significant denaturation of WPs, and destabilisation of CN micelles, that can alter the milk proteins' structure and functionality at the molecular level, all of which may influence the stability and quality of the manufactured dairy products (Anema, 2021; Huppertz, 2016).

Additionally, the raw milk quality and its safety depends on its gross composition, microbial and somatic cell count, and absence of various biological contaminates (Akkerman et al., 2021); moreover, based on the genetics, its quality has recently been associated to a single nucleotide polymorphism in the *CSN2* gene, essential for β-CN coding (Daniloski, Cunha, et al., 2021). Different phenotypes of β-CN in bovine milk have been postulated to be related with breeding goals, affecting the functionality of dairy products, and possibly having an impact on human health (Daniloski, McCarthy, & Vasiljevic, 2021). Accordingly, due to the mutation of a single amino acid in the β-CN polypeptide chain at position 67, (proline [Pro] to histidine [His]), 12 to 15 phenotypes of β-CN exist, with A1 and A2 β-CNs being the most abundant in modern cattle (Daniloski, Cunha, et al., 2021). Previous studies (Lambers, Broeren, Heck, Bragt, & Huppertz, 2021; Nguyen, Busetti, Smolenski, Johnson, & Solah, 2021) have shown that heat treatment appears to affect the release of opioid peptides from bovine milk carrying either A1/A1, A1/A2, or A2/A2 β-CN phenotype during its simulated gastrointestinal digestion. Nevertheless, it is not known how heat treatment impacts the structure of milk proteins in bovine milk of various β-CN phenotypes. Thus, the objective of this study was to establish how the structural properties of milk proteins obtained from three of the most common β-CN phenotypes were affected by thermal heat treatment.

2. Materials and methods

2.1. Milk sampling and heat treatment

Raw bovine milk was provided by The Agriculture Victoria's Ellinbank Centre in Victoria, Australia in three occasions obtained from 114 Holstein Friesian dairy cows in mid lactation (calved in late winter or early spring). All cows were healthy, showed no clinical signs of mastitis, and they were previously genotyped using the capillary electrophoresis method established by Raynes, Day, Augustin, and Carver (2015). Only cows with A1/A1, A1/A2, or A2/A2 β-CN phenotypes were further analysed (n = 64). Particularly, 5 milk samples were found to carry A1/A1 β-CN, while 9 and 50 milk samples possessed A1/A2 and A2/A2 β-CN, respectively. Upon assessment of the milk samples, the high frequency of milk from A2 β-CN family, especially A2/A2 β-CN milk, was apparent confirming previously published data (Daniloski, McCarthy, Markoska, Auldist, & Vasiljevic, 2022; Nguyen, Solah, Busetti, Smolenski, & Cooney, 2020; Schettini, Lambert, da Silva Souza, Costa, & de Camargo, 2020). To minimise the biological bias for further analysis, 5 milk samples per each β-CN phenotype were selected from individual cows. The samples were collected as per a phenotype, kept at 4 °C and delivered after a day to the Victoria University pilot plant for further processing. The raw whole milk samples were skimmed by centrifugation (Avanti J-26XP, Beckman instrument Australia Pty. Ltd, Gladesville, NSW, Australia) at 3225 g x for 20 min at 20 °C. Each skimmed milk sample was separated into three 10 mL aliquots for heat treatment and the forth was considered as a control (skim milk kept at 20 °C) (Markoska et al., 2019). The heat treatment conditions applied reflected those at a commercial scale, specifically 72 °C, 121 °C, and 140 °C with the processing time of 15 s, 2.6 min, and 3 s, respectively (Fox et al., 2015a). Ten mL of each milk sample was transferred into borosilicate Pyrex tubes with black screw cap lids (diameter 10 mm and height 85 mm, Lab Direct, Sydney, NSW, Australia). To heat the milk samples an oil bath was set at 72 °C, 121 °C, or 140 °C and the calculated average heating rates for 72 °C, 121 °C, and 140 °C were 0.43, 0.50, and 0.59 °C/s, respectively. Subsequently, the samples were removed from the oil bath when the required temperature/time combination was achieved and immediately cooled in an ice bath down to 20 °C. After the thermal heat treatment, sub-aliquots from the control and treated skim milk samples were ultra-centrifuged (Ultra L - 70 Centrifuge, Beckman Coulter, Indianapolis, IN, USA) at 100,000×*g* for 1 h at 20 °C. The serum phase was carefully separated from the pellet, keeping the other aliquot as the intact milk, and further analysed directly for physicochemical and structural differences. The milk samples were denominated as A1/A1 β-CN milk, A1/A2 β-CN milk, and A2/A2 β-CN milk.

2.2. Analytical methods

2.2.1. Gross milk composition

The general composition of the bovine milk from A1/A1, A1/A2, and A2/A2 phenotypes of β-CN before and after skimming and heat treatments were analysed using an automatic Lactoscan Milk Analyser (Lactoscan LS-60, Milkotronic Ltd., Nova Zagora, Bulgaria) (Supplementary Table 1 [A, B, and C]). The moisture content and total solids were estimated by placing the samples in an oven at 105 °C until a constant weight was obtained (AOAC, 2016). The protein content was calculated by following the Kjeldahl method where a nitrogen to protein conversion factor of 6.38 was used (ISO, 2014).

2.2.2. Reverse Phase-High Performance Liquid Chromatography for identification and quantification of proteins in milk samples

The identification, separation, and quantification of proteins, particularly β-CN in untreated and heat-treated skim milk and serum samples was determined by Reverse Phase-High Performance Liquid Chromatography equipped with a UV detector (RP-HPLC: LC-2030C, Shimadzu Corporation, Kyoto, Japan) following a method of Daniloski, McCarthy, Markoska, et al. (2022). Briefly, an Aeris WIDEPORE C4 column supplied by Phenomenex (150 mm × 4.6 mm, 3.6 μm particle size, 300 Å porosity, Torrance, USA) was used for the chromatographic separation of target proteins (stationary phase). A constant flow rate of 0.8 mL/min and an absorbance at a wavelength of 240 nm were used. The sample injection volume was set at 20 μL and the temperature of the column maintained at 45 °C for 51 min per sample. For calibration, the bovine αs-CN, β-CN, κ-CN, α-La, and β-Lg standard proteins were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Particularly, the protein content of either A1/A1, A1/A2, and A2/A2 β-CN milks obtained by RP-HPLC was calculated by previously prepared standard solutions as described elsewhere in the literature (Bonizzi, Buffoni, & Feligini, 2009; Mediwaththe, Chandrapala, & Vasiljevic, 2018). In addition, β-CN chromatograms obtained from the literature were used for further confirmation of either A1 or A2 β-CN (Akkerman et al., 2021; Day, Williams, Otter, & Augustin, 2015; Raynes et al., 2015).

2.2.3. Gel electrophoresis

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out under non-reducing and reducing conditions on all milk (25 μL/mL) and serum (0.7 mg/mL) samples as described by Bogahawaththa et al. (2021) using the gels containing 30% acrylamide/bis solution (Sigma-Aldrich, St. Louis, MO, USA) and 10% SDS (Merck, Darmstadt, Germany). Reducing SDS-PAGE was performed with the addition of a reducing agent, β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA). After electrophoresis (210 V, 70 mA, 6.5 W for 60 min) the gels were stained with Coomassive Brilliant Blue (Sigma-Aldrich, St. Louis, MO, USA) and the intensities of the major protein bands were scanned on an Image Lab. 2.1 software connected with a densitometer

(BioRad ChemiDoc MP Imaging System, Gladesville, NSW, Australia).

2.3. Physicochemical and structural characteristics

2.3.1. Mineral content

The total mineral content of the skim milk and soluble mineral content in the ultracentrifuged supernatant milk serum (Ca, Mg, and P, before and after heating) were determined using Inductively Coupled Plasma mass spectrometry (ICP Multitype, Shimadzu Corporation, Kyoto, Japan). All samples were ashed and dissolved in 1 M nitric acid (Sigma-Aldrich, St. Louis, MO, USA) before analysing the mineral content (Bogahawaththa et al., 2021). Ionic calcium (Ca^{2+}) was measured with an ion-selective electrode (InoLab, WTW GmbH, Ingolstadt, Germany). Ionic calcium concentration of both samples was calculated relative to a standard curve of condensed solution varying between 0 and 2 mM with $R^2 = 0.996$, prepared by use of 80 mM KCL to the standards (Markoska et al., 2019).

2.3.2. Particle size, zeta (ζ) potential, and pH analyses

The particle size and ζ potential of untreated and treated skim milk samples were analysed and processed by a light scattering technique (Zetasizer-Nano ZS, Malvern Instruments, Malvern, Worcestershire UK) coupled with a dispersion technology software (Malvern Instruments, Version 5). Samples were prepared by 100-fold dilution with simulated milk ultrafiltrate (SMUF) (Daniloski, McCarthy, Markoska, et al., 2022). A refractive index of 1.57 for the CN micelle and 1.33 for dispersant (SMUF) were used. All measurements were done within 15 min after dilution (Mediwaththe, Bogahawaththa, et al., 2018). The pH of milk samples was determined in 20 mL of control (unheated) or heat-treated milk samples using a calibrated pH meter (Metrohm AG, Oberdorfatrasse, Herisau, Switzerland) at room temperature (Markoska, Huppertz, & Vasiljevic, 2021a).

2.3.3. Fourier Transform Infrared spectroscopy

The changes of protein conformation were analysed with a PerkinElmer Frontier FTIR spectrometer (PerkinElmer, Boston, MA, USA). The IR spectra were collected between 4000 and 600 cm^{-1}, with 16 scans per position (for both untreated and treated milk) and spectral resolution of 4 cm^{-1}. Onto an attenuated total reflectance (ATR) cell, approximately 0.5 mL of sample was added. The corresponding serum was used as a background spectrum scanned at the beginning of the measurements. The spectra of ten sub-aliquots of each sample were taken by refiling the ATR cell (Daniloski, McCarthy, O'Callaghan, & Vasiljevic, 2022).

2.3.4. Nuclear Magnetic resonance spectroscopy

The ^{1}H NMR spectra were collected using a Bruker Avance spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) at 20 °C, operating at a 600 MHz transmitter frequency using 5 mm TXI probe with Z- and XYZ-gradients. A volume of 0.9 mL of a milk sample was added to 0.1 mL of deuterium oxide (D_2O: 99.8 atom percent excess and pH 6.0: Sigma-Aldrich, St. Louis, MO, USA) and vortexed for 20 s. An aliquot of 0.6 mL of the mix was transferred into a 5 mm NMR tube (Wilmad, economy, 500 MHz frequency and L 7 in, Sigma-Aldrich, St. Louis, MO, USA). The accumulated proton NMR was acquired using 16 scans, spectra width of 9615 Hz (12 ppm), acquisition time 4 s, relaxation delay 2 s (Daniloski, McCarthy, Markoska, et al., 2022). Chemical shift assignment was accomplished by using the biological magnetic resonance data bank (BMRB) files and data from previous studies (Erich et al., 2015; Markoska, Huppertz, & Vasiljevic, 2021b; Sanchez, Zhu, Frew, & Kebede, 2021).

2.4. Spectral data and statistical analyses

The FTIR spectra were initially baseline corrected and normalised using a Spectragryph software (version 1.2.7, Oberstdorf, Germany) followed by a derivatisation (second derivative form of all the spectra) within broad Amide I region (C═O stretching of proteins: 1700 - 1600 cm^{-1}). Amide I region possesses the purest contribution from changes in the second structure of proteins and is the one that undergoes the largest changes, thus it was chosen for further analysis (Mediwaththe, Bogahawaththa, et al., 2018). These data of Amide I region were further quantified by Origin software (Origin Pro 2021, v. 95E, OriginLab Corporation, Northampton, MA, USA) based on the method previously described by Grewal, Huppertz, and Vasiljevic (2018). The ^{1}H NMR spectra were processed using a TopSpin (version 4.1.1) software (Bruker BioSpin, Billerica, Massachusetts, USA) followed by the field intensity decays correction with a 0.3 Hz line-broadening parameter. Before further statistical analysis, all spectra were phase-corrected using 0th and 1st order correction for pk (Markoska et al., 2021b).

As this study aimed at demonstrating the conformational changes between milk samples influenced by the difference in β-CN phenotype and heat treatment, the Principal Component Analysis (PCA) was also employed to separate the observations obtained using FTIR and ^{1}H NMR. The grouping of the samples was demonstrated with the PCA score plots whereas the identification of the wavenumbers, which contributed the most in sample categorisation into different groups, was obtained by PCA loading plots. The evaluation of the PCA data was conducted with the Origin software (Origin Pro 2021, v. 95E, OriginLab Corporation, Northampton, MA, USA). All physicochemical and structural analyses were carried in triplicate for each milk sample. A two-way ANOVA was conducted using β-CN phenotype and temperature as fixed factors, to determine statistical significance among samples using Minitab statistical software version 19 (Minitab Inc., State College, Pennsylvania, USA). The level of significance was stablished at $p < 0.05$ with Tukey - HSD as a post-hoc test.

3. Results

3.1. Milk protein characterisation

Protein characterisation of the three milk pools representing different β-CN phenotypes (A1/A1, A1/A2, and A2/A2) were assessed by RP-HPLC (Figs. 1 and 2, Supplementary Tables 2 and 3). The four CN and two WP fractions were effectively resolved in approximately 35 min, and in particular, the selected method allowed unambiguous separation of both β-CNs (Fig. 1B and C). The separation was likely due to the hydrophobic nature and a great resolving power of the C4 column aimed in separation of highly hydrophobic proteins, such as β-CNs (Vincent, Elkins, Condina, Ezernieks, & Rochfort, 2016). Specifically, A1 β-CN eluted in 25 min in comparison with A2 β-CN, which eluted in 27.5 min (Fig. 1 A, B, C). The analysis first confirmed the presence of κ-CN, which amount at all temperatures was 20–50% higher in A1/A1 and A1/A2 β-CN milks compared to its amount in A2/A2 β-CN milk ($p < 0.05$) (Supplementary Table 2). Furthermore, κ-CN in A1/A2 and A2/A2 β-CN milks appeared to be different from that in A1/A1 β-CN milk (Fig. 1). Despite the subtle differences, such as decrease of protein concentrations and peak areas, in all β-CN milks the amount of β-CNs remained similar at all temperatures ($p > 0.05$) (Fig. 1B).

The behaviour of milk proteins in β-CN milks and their corresponding serums upon the heat treatment have been visualised in lanes 1 to 8 of the SDS PAGE gels (Fig. 2). The extent of aggregation was concomitant with the magnitude of the heat treatment with A1/A2 milk showing greater aggregation in comparison to those of A1/A1 and A2/A2 β-CN milks (Fig. 2C and D). Interestingly, despite the utilisation of a reducing agent in SDS-PAGE, compared to the other phenotypes of β-CN milk, the presence of aggregates in A1/A1 β-CN at 121 °C was highly visible. Furthermore, the shift of the bands representing the CNs was obvious. Nonetheless, the CN bands became less featured after the heat treatment (especially the bands representing κ-CN), which indicates the creation of conjugates between CNs and WPs. Furthermore, it was observed that shift of the bands indicating αs-CNs and β-CN was limited in the samples

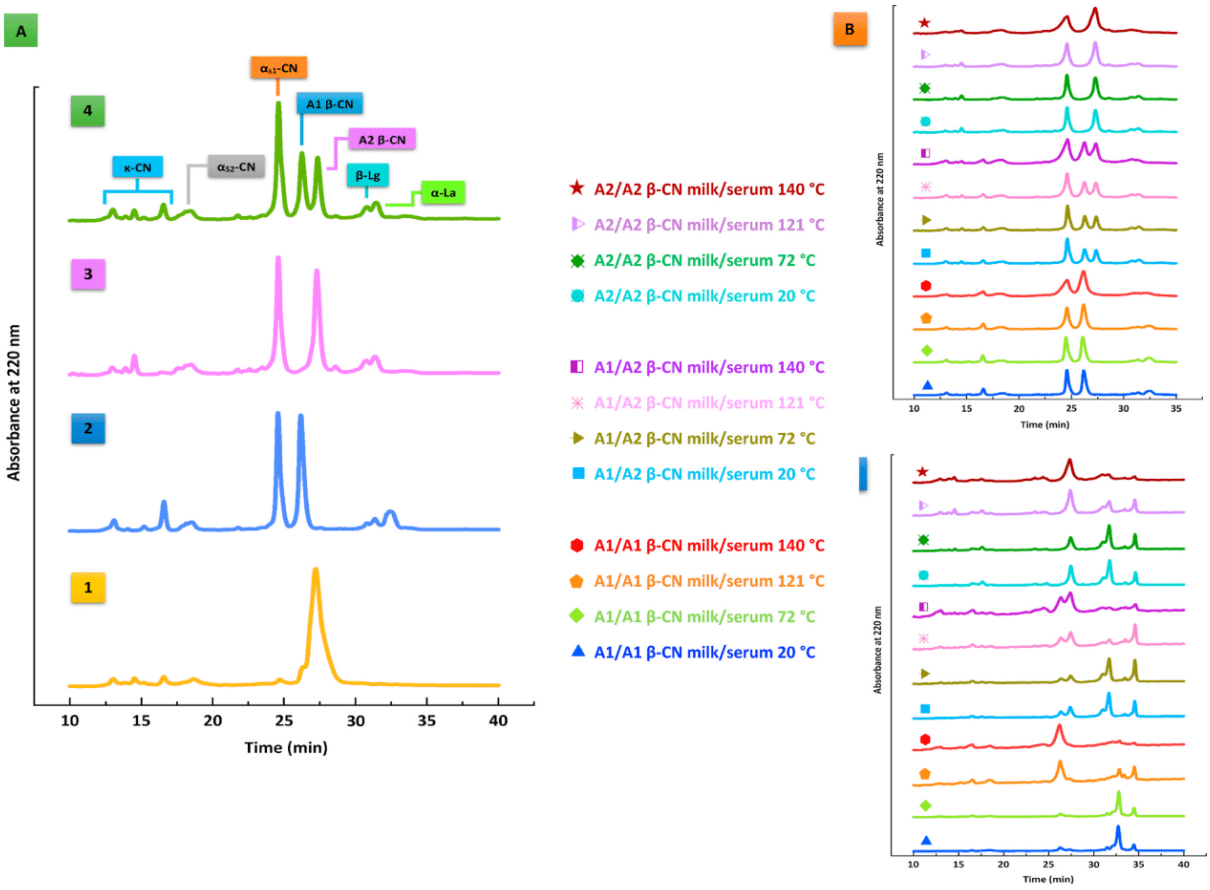

Fig. 1. A) Reverse phase-HPLC chromatographic profiles for identification of different β-CN milk samples. 1. Standard solutions containing β-CN; 2. A1/A1 β-CN milk; 3. A2/A2 β-CN milk; 4. A1/A2 β-CN milk. **B)** Reverse phase-HPLC chromatogram of A1/A1, A1/A2, and A2/A2 β-CN untreated and heat-treated milk samples. **C)** Reverse phase-HPLC chromatogram of A1/A1, A1/A2, and A2/A2 β-CN untreated and heat-treated serum samples.

at 121 and 140 °C (non-reducing and reducing gels, lanes 7 and 8) in all β-CN milks. In contrast, κ-CN (reducing gels) almost disappeared, especially in A2/A2 β-CN milk, which is in line with the results obtained by RP-HPLC (Fig. 1 and Supplementary Tables 2 and 3). As compared to the CNs, the bands' intensity representing WPs decreased in all three β-CN milks with the increase in the temperature of heat treatment. Overall, both CN and WP concentrations in the serum phase decreased with thermal treatment and therefore were likely engaged in a conjugation, thus forming insoluble CN-WP complexes.

3.2. Physicochemical properties of different β-CN milk samples

Any changes of the physicochemical characteristics, including pH, mineral content, particle size, and ζ potential might be related to the different β-CN phenotypes and impact of heat treatment (Tables 1–3). The pH of untreated and heat-treated samples did not differ substantially among β-CN milk phenotypes, with all milks having a pH within the normal range (6.58–6.83) (Grewal, Chandrapala, Donkor, Apostolopoulos, & Vasiljevic, 2017). However there was a slight decrease in pH after heat treatment (A1/A1 β-CN milk: 6.76–6.58; A1/A2 β-CN milk: 6.79–6.63; and A2/A2 β-CN milk: 6.83–6.69). The decrease in pH was more prominent in the samples treated at 72 °C, i.e., by 0.07–0.11 pH units. In general, the reduction of pH in milk with heat treatment is due to creation of organic acids from lactose degradation and movement of Ca and P from the serum into the micelle, forming insoluble calcium phosphate (Markoska et al., 2021a).

The mineral partitioning of milks between sedimentable and soluble phases before and after heat treatment are shown in Tables 2 and 3. The amounts of Ca and P in A1/A1 β-CN milk and serum (untreated and heat-treated) were significantly greater ($p < 0.05$) compared to the other two phenotype milks and their corresponding supernatants. There were only relatively small changes in Mg between the β-CN milk phenotypes and their serums. In comparison with A1/A1 and A2/A2 β-CN milk samples, A1/A2 β-CN milk and its serum showed slightly higher amounts of Ca^{2+}.

Table 1 shows the average particle size and ζ potential of the milk samples. The average particle size of A1/A1 β-CN milk (164.07–218.47 nm) at all temperatures (excluding at 121 °C) were smaller compared to those of A1/A2 β-CN (172.27–226.57 nm) and A2/A2 β-CN milks (179.90–238.60 nm) before and after heating ($p < 0.05$). Additionally, increasing the temperature significantly influenced the particle size of all three milk samples ($p < 0.05$) following the proportional rise in the average particle size. The major increase in particle size was noticed at and above 121 °C. The ζ potential (Table 1) for all three β-CN milk phenotypes showed that the net negative charge at most temperatures was greater for A1/A1 and A1/A2 β-CN milks than that in A2/A2 β-CN milk. Comparatively the ζ potential values for heat-treated samples showed the same trend among all three β-CN milk samples. Hence, the apparent surface potential of the particles increased in absolute terms ($p < 0.05$) by approximately 1–3 mV from the initial values. The only different trend was observed in A2/A2 β-CN milk upon heat treatment where the ζ potential initially increased from - 12.07 at 20 °C to - 13.07 and - 13.63 mV at 72 and 121 °C, but subsequently heating at 140 °C resulted in a significant decline ($p < 0.05$) down to - 12.67 mV. These observations and trends among the milk samples carrying various β-CN phenotypes may be correlated to the protein interactions and changes in the mineral balance (Markoska et al., 2019).

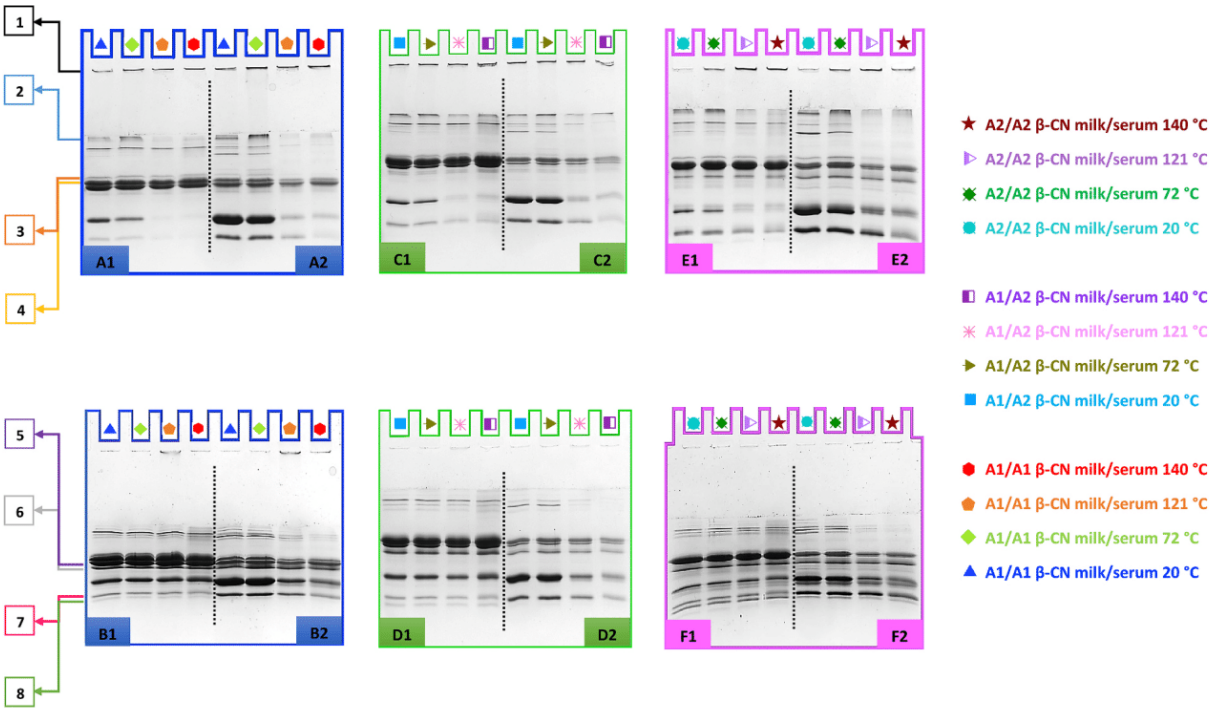

Fig. 2. SDS-PAGE electropherograms of skim milk and supernatants. The proteins were resolved under non-reducing (A1/A1 β-CN: **A** [A1: milk and A2: serum]; A1/A2 β-CN: **C** [C1: milk and C2: serum]; A2/A2 β-CN: **E** [E1: milk and E2: serum]) or reducing (A1/A1 β-CN: **B** [B1: milk and B2: serum]; A1/A2 β-CN: **D** [D1: milk and D2: serum]; A2/A2 β-CN: **F** [F1: milk and F2: serum]) conditions. 1. Aggregates; 2. Immunoglobulins and bovine serum albumin; 3. αs_2-casein; 4. αs_1-casein; 5. β-casein; 6. κ-casein; 7. β-Lactoglobulin; 8. α-Lactalbumin.

Table 1
The average particle size and zeta potential in all three milk types (n = 5 per phenotype) determined upon heat treatment.

Sample	Temperature (°C)	Particle size (nm)	Zeta potential (mV)	pH
A1/A1	20	164.07 ± 3.38 ^{h}	−12.67 ± 0.32 ^{bc}	6.76 ± 0.01 ^{c}
A1/A2		172.27 ± 1.12 ^{g}	−12.90 ± 1.37 ^{bc}	6.79 ± 0.01 ^{b}
A2/A2		179.90 ± 0.89 ^{ef}	−12.07 ± 0.51 ^{c}	6.83 ± 0.00 ^{a}
A1/A1	72	160.20 ± 0.89 ^{de}	−13.07 ± 0.61 ^{bc}	6.66 ± 0.01 ^{f}
A1/A2		166.57 ± 1.53 ^{gh}	−12.90 ± 1.37 ^{bc}	6.68 ± 0.00 ^{e}
A2/A2		168.77 ± 1.22 ^{gh}	−12.85 ± 0.27 ^{bc}	6.76 ± 0.01 ^{c}
A1/A1	121	188.18 ± 2.33 ^{c}	−13.63 ± 0.36 ^{abc}	6.63 ± 0.01 ^{g}
A1/A2		184.03 ± 2.16 ^{f}	−13.47 ± 0.25 ^{bc}	6.65 ± 0.01 ^{f}
A2/A2		186.57 ± 1.26 ^{cd}	−13.37 ± 0.21 ^{bc}	6.73 ± 0.01 ^{d}
A1/A1	140	218.47 ± 0.68 ^{b}	−13.98 ± 0.79 ^{bc}	6.58 ± 0.00 ^{h}
A1/A2		226.57 ± 3.43 ^{b}	−15.87 ± 0.32 ^{a}	6.63 ± 0.01 ^{g}
A2/A2		238.60 ± 5.51 ^{a}	−12.67 ± 0.67 ^{bc}	6.69 ± 0.00 ^{e}

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$); n/a - not applicable.

Table 2
The total mineral concentration of all three types of milk samples (n = 5 per phenotype) determined upon heat treatment.

Sample	Temperature (°C)	Ca (mM)	Mg (mM)	P (mM)	Ca^{2+} (mM)
A1/A1	20	42.92 ± 0.60 ^{a}	5.20 ± 0.08 ^{a}	40.24 ± 0.16 ^{a}	3.69 ± 0.01 ^{b}
A1/A2		30.23 ± 0.55 ^{g}	3.77 ± 0.04 ^{d}	28.17 ± 0.15 ^{g}	3.84 ± 0.04 ^{a}
A2/A2		33.52 ± 0.44 ^{d}	3.82 ± 0.06 ^{d}	30.82 ± 0.12 ^{d}	3.49 ± 0.06 ^{c}
A1/A1	72	41.03 ± 0.63 ^{b}	5.01 ± 0.09 ^{b}	38.24 ± 0.17 ^{b}	3.15 ± 0.02 ^{d}
A1/A2		29.14 ± 0.40 ^{h}	3.62 ± 0.05 ^{e}	27.22 ± 0.13 ^{h}	3.47 ± 0.04 ^{c}
A2/A2		32.10 ± 0.36 ^{ef}	3.38 ± 0.05 ^{f}	28.88 ± 0.11 ^{f}	2.97 ± 0.05 ^{e}
A1/A1	121	40.73 ± 0.46 ^{b}	4.90 ± 0.07 ^{b}	37.99 ± 0.20 ^{b}	2.70 ± 0.06 ^{f}
A1/A2		29.99 ± 0.41 ^{gh}	3.73 ± 0.04 ^{de}	27.97 ± 0.14 ^{g}	3.14 ± 0.04 ^{d}
A2/A2		32.89 ± 0.26 ^{de}	3.47 ± 0.03 ^{f}	29.66 ± 0.07 ^{e}	2.94 ± 0.05 ^{e}
A1/A1	140	38.07 ± 0.45 ^{c}	4.53 ± 0.06 ^{c}	35.42 ± 0.12 ^{c}	2.54 ± 0.04 ^{g}
A1/A2		29.31 ± 0.36 ^{h}	3.64 ± 0.05 ^{e}	27.28 ± 0.09 ^{h}	3.11 ± 0.02 ^{d}
A2/A2		31.78 ± 0.20 ^{f}	3.36 ± 0.02 ^{f}	28.67 ± 0.08 ^{f}	2.93 ± 0.02 ^{e}

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$); n/a - not applicable.

3.3. Conformational fingerprinting of β-casein milk phenotypes during heating

3.3.1. Fourier Transform Infrared resonance structural configurations

To elucidate the conformational differences between milks, FTIR second-derivative spectra were used to find the number of secondary structure components and the approximate positions of these peaks. Thus, the comparison between different β-CN milk phenotypes as a function of the heat treatment was presented. The absorbance difference between all three β-CN milk phenotypes can be seen by focusing on the

Table 3
The soluble mineral concentration of all three types serum samples (n = 5 per phenotype) determined upon heat treatment.

Sample	Temperature (°C)	Ca (mM)	Mg (mM)	P (mM)	Ca^{2+} (mM)
A1/A1	20	13.32 ± 0.07 ^{a}	2.95 ± 0.03 ^{a}	17.11 ± 0.09 ^{a}	2.73 ± 0.03 ^{b}
A1/A2		10.58 ± 0.08 ^{d}	2.35 ± 0.02 ^{d}	13.37 ± 0.08 ^{d}	2.86 ± 0.02 ^{a}
A2/A2		10.16 ± 0.07 ^{g}	1.97 ± 0.01 ^{g}	12.45 ± 0.05 ^{f}	2.57 ± 0.06 ^{c}
A1/A1	72	12.53 ± 0.03 ^{b}	2.91 ± 0.02 ^{b}	17.08 ± 0.08 ^{a}	2.43 ± 0.07 ^{ef}
A1/A2		10.31 ± 0.05 ^{f}	2.32 ± 0.01 ^{e}	13.31 ± 0.12 ^{d}	2.79 ± 0.04 ^{ab}
A2/A2		10.80 ± 0.05 ^{c}	2.38 ± 0.02 ^{d}	13.23 ± 0.05 ^{d}	2.55 ± 0.04 ^{cd}
A1/A1	121	10.83 ± 0.06 ^{c}	2.53 ± 0.01 ^{c}	15.41 ± 0.07 ^{b}	2.30 ± 0.06 ^{gh}
A1/A2		9.34 ± 0.04 ^{h}	2.09 ± 0.02 ^{f}	12.42 ± 0.09 ^{f}	2.48 ± 0.04 ^{de}
A2/A2		10.45 ± 0.08 ^{e}	1.92 ± 0.01 ^{h}	13.01 ± 0.11 ^{e}	2.54 ± 0.03 ^{cd}
A1/A1	140	10.08 ± 0.04 ^{g}	2.30 ± 0.02 ^{d}	14.88 ± 0.05 ^{c}	2.33 ± 0.04 ^{gh}
A1/A2		8.59 ± 0.06 ^{i}	1.93 ± 0.01 ^{h}	11.87 ± 0.06 ^{h}	2.93 ± 0.05 ^{fg}
A2/A2		9.34 ± 0.10 ^{h}	1.86 ± 0.01 ^{i}	12.14 ± 0.10 ^{g}	2.26 ± 0.01 ^{h}

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$); n/a - not applicable.

spectral window from 1700 to 1600 cm^{-1} (Fig. 3A). Both phenotype and temperature affected the variations of peak intensities observed at 1700 cm^{-1} - 1682 cm^{-1} (intermolecular/aggregated β-sheet); 1681 cm^{-1} - 1665 cm^{-1} (β-turn); 1664 cm^{-1} - 1646 cm^{-1} (α-helix); 1645 cm^{-1} - 1638 cm^{-1} (random coil); 1637 cm^{-1} - 1615 cm^{-1} (intramolecular β-sheet); and 1614 cm^{-1} - 1601 cm^{-1} (side chain) (Daniloski, McCarthy, Markoska, et al., 2022). Particularly, upon heating at 140 °C, more intense variations in milk proteins appeared at 1650 cm^{-1}, 1640 cm^{-1}, and 1634 cm^{-1} with increase in the intensity of α-helix, random, and intramolecular β-sheet structures in A1/A1 β-CN milk compared to the other phenotypes of milk (Fig. 3A). In contrast, the intensity of aggregated β-sheets (1691 cm^{-1}) and β-turns (1678 - 1675 cm^{-1}) were greater in A1/A2 β-CN milk and after prolonged heating, they further increased in the intensity. The major observed change in the second derivative of the A2/A2 β-CN milks' spectra was a marked increase in a band around 1653 cm^{-1}, indicating increase of α-helical structure compared to the other milk phenotypes regardless of the temperature. In the same milk, β-turns (1678 cm^{-1}) followed similar pattern presented in A1/A2 β-CN milk.

PCA clearly separated A2/A2 β-CN milk from A1/A1 and A1/A2 β-CN milk samples in both unheated controls and heat-treated samples. As observed from the PCA scatter plot (Fig. 3B), PC1 was positively correlated for A2/A2 β-CN milks at all heat treatment temperatures and for A1/A2 β-CN milk at 121 °C, and was negatively associated with A1/A1 and A1/A2 β-CN milks (for all remaining temperatures). The loading on PC1 included positive peaks at 1679 cm^{-1} (β-turn), 1662 cm^{-1}, and 1648 cm^{-1} (α-helix), and 1624 cm^{-1} (intramolecular β-sheet); and negative peaks at 1687 cm^{-1} (aggregated β-sheet), 1656 cm^{-1} (α-helix), and 1640 (random coil). The PC2 separated the scores of A2/A2 β-CN milks (all temperatures) and heat-treated A1/A1 β-CN milk at 140 °C (negative), from the scores of A1/A1 and A1/A2 β-CN milks at all remaining temperatures (positive). According to the loading plot, PC2 component included positive peaks at 1690 cm^{-1} (aggregated β-sheet), 1678 cm^{-1} (β-turn), 1657 cm^{-1} (α-helix), and 1624 cm^{-1} (intramolecular β-sheet); and negative peaks at 1684 cm^{-1} (aggregated β-sheet), 1672 cm^{-1} (β-turn), 1655 cm^{-1} (α-helix), 1640 cm^{-1} (random

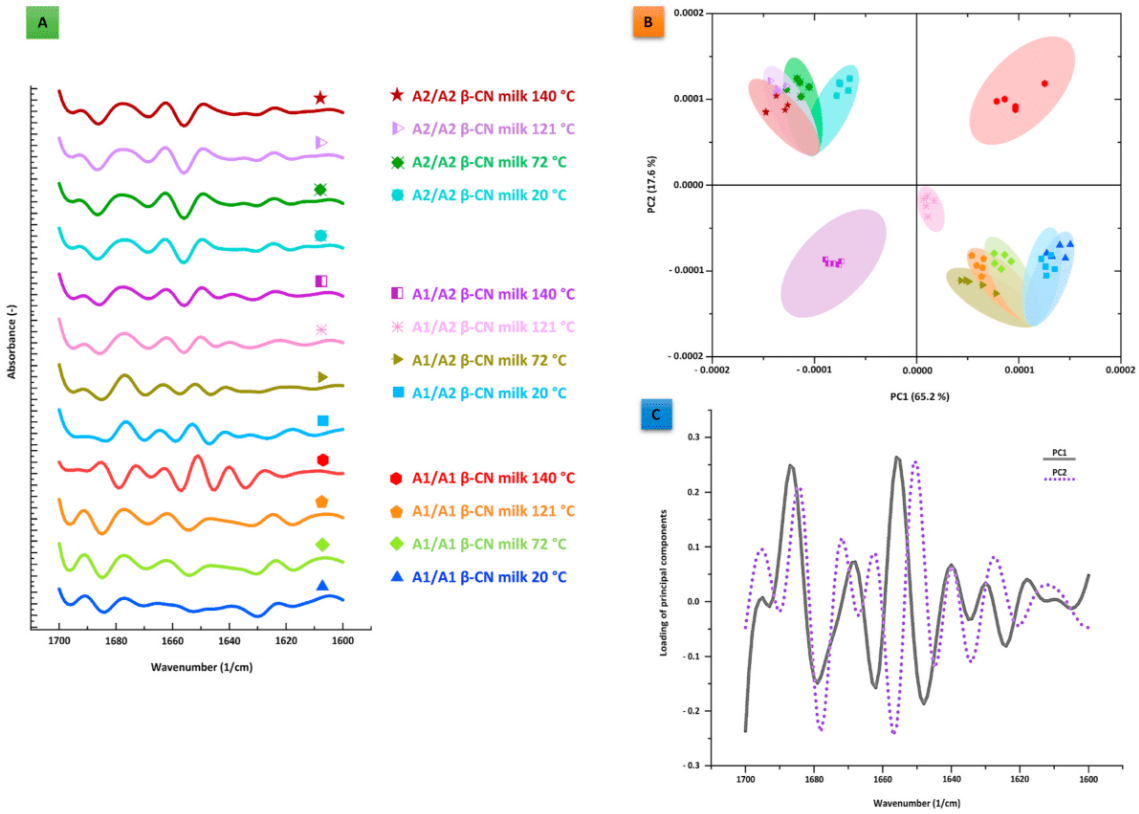

Fig. 3. A) Second derivative spectra of Amide I region of milk samples. **B)** Scatter plot of the PCA scores of FTIR spectra of milk samples. **C)** The plot of the PCA loadings of FTIR spectra of milk samples.

coil), and 1624 cm^{-1} (intramolecular β-sheet). Notably, both negative peaks at 1640 cm^{-1} in PC1 and PC2, might be associated with the random coil structures present in A1/A1 β-CN milk at 140 °C (approximately 34%) (Fig. 3C and Table 4). Interestingly, A2/A2 β-CN milks at all temperatures only appeared at the left axis of both, PC1 and PC2, presumably due to its structural difference compared to the other samples.

Collectively, this work demonstrates that A1/A1, A1/A2, and A2/A2 β-CN milks signified some similarities and differences of the protein secondary structures in each group. The quantification of changes in the secondary structure was obtained through the Gauss curve fitting in the Amide I region. Table 4 tabulates a proportion of individual components of aggregated β-sheet, β-turn, α-helix, random coil, intramolecular β-sheet, and side chain. Relatively, in all three milks the contents of β-sheet, β-turn, and α-helix were greater compared with the other features of the secondary protein structure. The difference between the milk phenotypes was reflected in a significantly greater proportions of intramolecular β-sheets in A1/A1 β-CN milk ($p < 0.05$) and aggregated β-sheets in A1/A2 β-CN milks ($p < 0.05$) compared to those in A2/A2 β-CN milks at all temperatures. Noteworthy, with the rise in temperature the intramolecular β-sheets decreased simultaneously in all three milks; these structures completely disappeared in A1/A2 β-CN milk at 140 °C. In contrast, random structures increased proportionally with the temperature increase; A2/A2 β-CN milk was characterised by a great amount of random structures. Interestingly, before and upon heating, in A2/A2 β-CN milk the α-helical structures were more present (10–15%) compared to the other two milk phenotypes, excluding A1/A1 β-CN milk at 140 °C, where the amount of α-helical structures increased substantially by approximately 14% from that determined at the initial temperature ($p < 0.05$, Table 4). Although the overall trend of β-turns in all milk samples (untreated and heated) was more or less similar, A1/A1 β-CN milk contained less β-turns then the other milk samples. Similarly to the α-helical structures, here again, in A1/A1 β-CN milk at 140 °C the amount of β-turns increased significantly from 10 to 21% at 1679 cm^{-1}.

3.3.2. *One-dimensional patterns in Nuclear Magnetic resonance spectra*

In this study ^{1}H NMR spectroscopy was used as a complementary method to evaluate the conformational differences and proton changes between A1/A1, A1/A2, and A2/A2 β-CN milk phenotypes, before and after heat treatment. The average spectra of all three β-CN milk phenotypes are presented in Fig. 4A. The spectra mainly consist of the signals of numerous resonances assigned among three different regions. Hence, the aliphatic region is represented at between 5 and 0 ppm, with the carbohydrate region from 4.7 to 3.1 ppm, and the aromatic/H^N region from 9 to 5.5 ppm (Garwolińska, Hewelt-Belka, Kot-Wasik, & Sundekilde, 2020). Hence, only proteins in milk related to its structure were further explained. The ^{1}H NMR spectra of all samples revealed several similarities, as well as a number of differences in structural features between all three samples. Thus, in the aliphatic region, particularly between 2 and 0 ppm, slight variations were observed. In A1/A1 and A1/A2 β-CN milks at all temperatures the peaks around 1.9 ppm seemed to be singlets in comparison with those in A2/A2 β-CN milk which appeared as doublets. Namely, the different appearance of these peaks might initiate the presence of β-turn structures (de Angelis Curtis et al., 2000). Additionally, an important variation has been observed within the aliphatic region associated to the prominent sharpening of the signal and a high intense peak at 1.17 ppm which again demonstrates the presence of β-turn structures (Daniloski, McCarthy, Markoska, et al., 2022; de Angelis Curtis et al., 2000) in A1/A1 β-CN milk at 72 and 140 °C compared to the other milk phenotypes. Thus, NMR confirmed the conformational variation in β-turns and their high presence mainly in A2/A2 β-CN milk, and their high presence in A1/A1 β-CN milk only at elevated temperatures, as confirmed by FTIR spectra (section 3.3.1., Table 4).

In principle, ^{1}H NMR detects α-protons (H^α) of amino acids in the region between 4 and 3 ppm, which is in the same region as lactose and because of the high concentration of lactose in milk, their signals are expected to overlap with the amino acids profile (Dangat et al., 2016). Therefore, an overlapping of these peaks has been detected. Nevertheless, around 3 ppm we were able to detect single peaks only for A1/A2 β-CN milk at all temperatures compared to the remaining milk phenotypes where the peaks at the same positions were presented as bimodal (Fig. 4A); these peaks might be related to the presence of tyrosine (Tyr) residues in the polypeptide chain (Leslie, Irons, & Chapman, 1969).

Despite this finding, the overlap of proton lines, together with the presence of the underlying broad signals of lactose, makes a precise measurement of the α-protons' linewidths extremely difficult. As presented in Fig. 4A, regarding the milk phenotype and the temperature used, the lactose peaks experienced up-field shift. This shielding might be due to changes in proton distribution as a function of the changed environment (temperature) (Markoska et al., 2021b). The shielding trend presented in the aromatic/Amide (H^N) region was similar as noticed in both remaining regions, mostly as a result of the intramolecular bonds enlargement (Hong, Jing, & Yao, 2013). Therefore, the polypeptide chains in proteins remained stable regardless of phenotype or temperature of heat treatment.

Any specific resonances in ^{1}H NMR spectra of all samples, which could provide clear structural differentiations between A1/A1, A1/A2, and A2/A2 β-CN milks were not detected (Fig. 4A). Therefore, the chemometric methods were used to interpret the ^{1}H NMR signals and to reveal hidden information in ^{1}H NMR spectra of samples, such as milk from cows of different genetic heritage. The PCA score plots of PC1 (59.8%) and PC2 (19.7%) visualise the separation of all three β-CN milk phenotypes based on ^{1}H NMR full-spectral data (Fig. 4B). According to PC1, milk samples were divided based on the temperature. Specifically, PC1 was positively linked to all β-CN milk phenotypes at 72 °C and

Table 4
Total percentage areas of different secondary structures in Amide I in all three phenotypes of milk samples (n = 5 per phenotype) determined upon heat treatment.

Sample	Temperature (°C)	Side chain	Intramolecular β-sheet	Random coil	α-helix	β-turn	Aggregated β-sheet
A1/A1	20	4.26 ± 1.83^{ab}	29.61 ± 6.68^{c}	7.78 ± 2.86^{bcd}	19.93 ± 4.88^{ab}	10.43 ± 3.03^{a}	28.00 ± 9.82^{bcd}
A1/A2		8.09 ± 3.07^{b}	11.63 ± 4.58^{b}	n/a	25.62 ± 3.80^{bc}	15.81 ± 3.82^{ab}	38.85 ± 11.47^{bcd}
A2/A2		2.81 ± 1.18^{a}	15.00 ± 0.81^{b}	13.37 ± 5.01^{d}	28.44 ± 3.39^{cd}	15.82 ± 3.41^{ab}	24.56 ± 3.38^{b}
A1/A1	72	4.83 ± 4.34^{ab}	23.44 ± 1.89^{c}	7.90 ± 3.06^{bcd}	15.94 ± 1.88^{a}	16.19 ± 1.24^{ab}	31.70 ± 7.89^{bcd}
A1/A2		4.80 ± 2.00^{ab}	11.19 ± 3.22^{b}	7.60 ± 2.53^{bcd}	15.92 ± 3.69^{a}	16.87 ± 1.59^{b}	40.41 ± 7.56^{cd}
A2/A2		2.97 ± 0.68^{a}	13.48 ± 1.37^{b}	13.93 ± 2.20^{d}	28.98 ± 1.76^{cd}	20.08 ± 5.02^{b}	23.77 ± 1.83^{ab}
A1/A1	121	3.48 ± 1.85^{a}	27.41 ± 2.04^{c}	n/a	16.22 ± 1.92^{a}	16.42 ± 2.51^{ab}	36.47 ± 3.13^{bcd}
A1/A2		6.08 ± 1.37^{a}	13.45 ± 3.67^{b}	5.00 ± 2.33^{ab}	17.32 ± 4.16^{a}	16.60 ± 1.41^{ab}	41.55 ± 11.01^{d}
A2/A2		3.45 ± 0.72^{a}	13.03 ± 4.16^{b}	12.55 ± 4.49^{cd}	27.71 ± 2.19^{cd}	17.39 ± 3.22^{b}	25.87 ± 3.07^{bc}
A1/A1	140	3.28 ± 1.34^{a}	13.76 ± 2.15^{b}	11.26 ± 3.08^{bcd}	27.63 ± 2.18^{cd}	19.08 ± 2.22^{b}	26.23 ± 3.77^{bcd}
A1/A2		4.95 ± 0.87^{ab}	n/a	6.69 ± 1.03^{bc}	20.48 ± 4.32^{ab}	15.40 ± 2.95^{ab}	38.71 ± 9.88^{bcd}
A2/A2		3.77 ± 1.90^{ab}	12.02 ± 4.43^{b}	33.93 ± 3.70^{e}	33.73 ± 3.05^{d}	20.55 ± 2.72^{b}	8.50 ± 1.76^{a}
Band frequency (cm^{-1})		1600–1614	1626–1633	1638–1644	1647–1656	1668–1679	1689–1692

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$); n/a - not applicable.

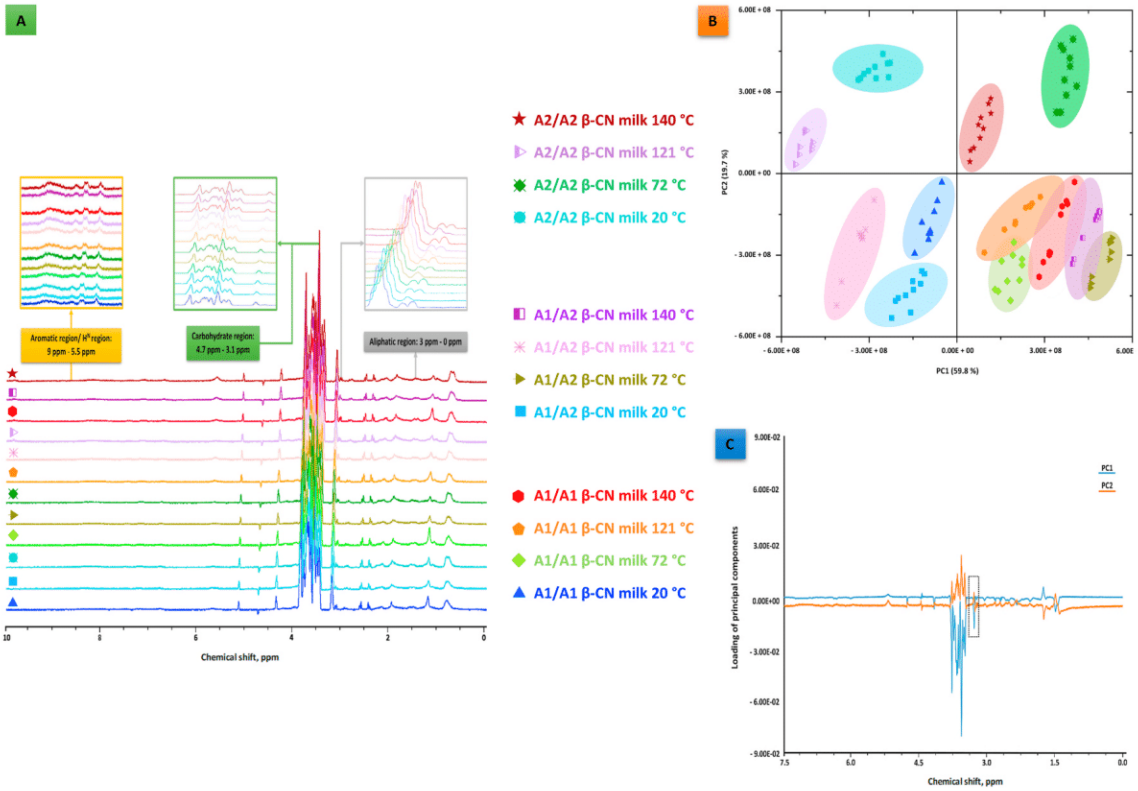

Fig. 4. A) ^{1}H NMR spectra of milk samples. **B)** Scatter plot of the PCA scores of ^{1}H NMR spectra of milk samples. **C)** The plot of the PCA loadings of ^{1}H NMR spectra of milk samples.

140 °C (including A1/A1 β-CN milk at 121 °C), and negatively associated with β-CN milks at 20 °C and 121 °C. In contrast, A2/A2 β-CN milk samples at all temperatures were predominantly positioned in the region of positive PC2 values, whereas A1/A1 and A1/A2 β-CN milk samples (at all temperatures) were assigned in the region of negative PC2 values. Therefore, PC2 has separated the milk samples based on the β-CN milk phenotypes, indicating that A1 β-CN might have the prior influence in both milks containing this protein in their structures. Similar clusters were observed based on the analysis of derivatised Amide I region of the FTIR spectral data (Section 3.3. and Fig. 3B). The difference in the aliphatic region was also confirmed by the PCA loading plot (Fig. 4C), where an intensive negative loading around 3 ppm has been shown for A1/A1 and A1/A2 β-CN milks and small, not intense peak at the same area for A2/A2 β-CN milk.

4. Discussion

The behaviour between β-CN phenotypes upon heat treatment appeared substantially different in the current study. The β-CN milk phenotype appears to drive, at least in part, the observed differences between milk samples in terms of structural and physiochemical responses to applied thermal processing. Namely, at 72 °C, the SDS-PAGE results depicted some protein aggregates containing mainly β-Lg, α-La, and κ-CN, induced by heat treatment, as was also observed in the study of Markoska et al. (2021a). These observations were established by a greater band intensity of aggregates with increasing heating temperature (i.e., sterilisation temperature and UHT) and reduction of band related to β-Lg in SDS-PAGE patterns and further supported by the chromatographic data (Figs. 1 and 2, and Supplementary Tables 2 and 3), particularly in A1/A1 and A1/A2 β-CN milks. In contrast, the most visible observation in A2/A2 β-CN milk was the presence of non-denatured WPs, even though is known that most WPs would undergo reversible (heated at 72 °C) or irreversible (at and above 121 °C) denaturation initiated by unfolding (Mediwaththe, Chandrapala, & Vasiljevic, 2018). These results were additionally supported by the WP content data obtained by RP-HPLC. Herein, despite the fact that WPs decreases with the temperature rise in all β-CN milks, A2/A2 β-CN milk contained the greatest amount of soluble WPs (Supplementary Table 3). Further, the SDS-PAGE bands presenting soluble β-CN in A2/A2 β-CN milk serum were more intense. This was confirmed with RP-HPLC data showing that upon heating more A2 β-CN is released from the micelle, compared to A1 β-CN and A1/A2 β-CN. Previously, it was found that β-CN at elevated temperatures can interact with partially unfolded WPs via hydrophobic domains, preventing their normal thiol-disulphide interchange with other WPs and eventual aggregation; a mechanism also referred to as a chaperone-like activity (Liyanaarachchi & Vasiljevic, 2018). Hence, the greater presence of non-denatured WPs and soluble β-CN in A2/A2 β-CN serum compared to the other β-CN serums might be explained by the finding of Raynes et al. (2015) in which A2 β-CN showed an enhanced chaperon like activity compared to A1 β-CN, which may be the case in the current study. Supposedly, the presence of A1 β-CN can initiate the formation of aggregates (Anema, 2021), also depicted as more intense bands in both milks containing A1 β-CN (Fig. 2 A and D).

Whereas the protein aggregates formed by disulphide bonds should be cleaved under the reducing conditions by β-mercaptoethanol (Bogahawaththa et al., 2021), aggregates due to lactosylation might remain present, particularly after the heating process (e.g. in A1/A1 β-CN milk at 121 °C, Fig. 2B). These aggregates are normally used as a marker of thermally-induced damage due to milk processing. Furthermore, they might be a consequence of the cross-linking of the CN micelles at the particle surfaces due to non-covalent interactions (Anema, 2021), which again shows the differences between A1/A1, A1/A2, and A2/A2 CN micelles. In particular, protein-bound amino acids may undergo modifications in their side chains, that may require the participation of sugars, as in the Maillard reaction, or not, as in the formation of

cross-linked amino acids (Boschin, D'Agostina, Rinaldi, & Arnoldi, 2003; Henle, Walter, Krause, & Klostermeyer, 1991). These protein-bound amino acids may undergo reduction due to the reaction of dehydroalanine (formed during milk heating) with His residues, leading to a histidinoalanine formation (Anema, 2021). In this regard, since A1/A1 β-CN milk contains His^{67} it is expected that those compounds found only at 121 °C in the reducing SDS-PAGE in this milk may be due to formation of histidinoalanines, thus explaining the possibility that different amino acids might influence the whole behaviour of the milk system during thermal processing.

Milk phenotypes containing His^{67} in their β-CN structure had a high proportion of intramolecular β-sheets (A1/A1 β-CN milks) and aggregated β-sheets (A1/A2 β-CN milks) after heating (Fig. 3 and Table 4). Previously, it has been shown that their increased content was found to affect the conformational rearrangements of β-Lg during denaturation (Qi, Ren, Xiao, & Tomasula, 2015). Hence, these structures were particularly found in β-Lg, which is known to have eight β-sheet structures in its monomeric form. Nevertheless, since the samples were baseline corrected with the corresponding serum, it would be expected that these observations were reflective of the properties of the CN micelles in milk, particularly, the structural orientation of β-CN and κ-CN variants. Approximately, 17–49% and 20–35% of β-sheets found in bovine milk are related to the different structural orientation of αs_1-CN and κ-CN, respectively (Huppertz, 2013; Uhrínová et al., 1998). Thus, there is a high possibility that the greater amount of aggregated β-sheet structures upon heating could be induced due to the conformational orientation of colloidal αs_1-CN in A1/A2 β-CN milk and colloidal κ-CN in A1/A1 β-CN milk and their association or dissociation with the CN micelle. In the past, it was found that β-sheet is a common conformational motif of Tyr residues in proteins (Huang & Nau, 2003; Lokstejn, Dzwolak, & Krysiński, 2008; Malkov, Živković, Beljanski, Hall, & Zarić, 2008). Huppertz (2013) revealed that 10 Tyr residues are positioned in the polypeptide chain of αs_1-CN. In this regard, since αs_1-CN was most present in A1/A2 β-CN milk upon heat treatment compared to other milk samples, the aggregated β-sheet structures might be initiated by greater presence of Tyr residues, also depicted by the FTIR and NMR spectra in this study.

Anema (2008) proposed that heat-induced dissociation of κ-CN from the micelle precedes interactions with denatured WPs in the serum, thus the structural differences of the CN micelle due to presence of different CN phenotypes may affect the distribution of κ-CN between the serum and micellar phases and its interaction with denatured WPs (Fig. 2). Although the content of CNs in all raw β-CN milks and their serums varied, most prominent differences observed were predominately in the concentration of soluble κ-CN in the sample after heating (Fig. 2 and Supplementary Tables 2 and 3). These differences in total and soluble κ-CN levels prior to heating should not be discounted, particularly when considering the CN micelle size and its electrostatic charge (Bijl, de Vries, van Valenberg, Huppertz, & van Hooijdonk, 2014). As observed from RP-HPLC and SDS-PAGE data (Figs. 1 and 2; Supplementary Tables 2 and 3) A2/A2 β-CN milk contained the lowest κ-CN content before and after heat treatment at a particular temperature, compared to the other phenotypes of β-CN milks. Day et al. (2015) and Bijl et al. (2014) generally agreed that higher κ-CN content was negatively correlated with the CN micelle size, explaining the path-terminating character of this protein. This is in line with the present study, since the particle size of A1/A1 and A1/A2 β-CN milks were shown to be smaller than that of A2/A2 β-CN milk ($p < 0.05$) (Supplementary Table 2). The decrease in particle size on heating samples in milks containing A1 β-CN (Fig. 2 A and D) could be explained by the heat-induced dissociation of κ-CN from the micelle (Anema, 2007). Namely, as the temperature increased from 72 to 140 °C in the milk phenotypes containing A1 β-CN in their structures, κ-CN progressively dissociated from the CN micelles ($p < 0.05$). As for the A2/A2 β-CN milks, little or no-induced dissociation of κ-CN occurred (Fig. 2 E and F), thus their particle sizes were greater compared to those of other β-CN milks (untreated and heat-treated).

Previously, dissociation of micellar κ-CN and its aggregation with WPs due to heating of milk was related with appearance of β-turn structures (Grewal et al., 2018). Namely, the high proportion of β-turns were found in A2/A2 β-CN milk (15.82–20.55%, Table 4), thus their content might be owing to the effect of A2 β-CN in β-turns' formation. Huppertz (2013) explained that 20–30% of turns in milk might be formed by β-CN and can be found as Pro or non-Pro based. In this regard, β-CN is known to contain 209 amino acid residues, out of which 16.7% is Pro. As a result of their cyclic structures, the presence of this amino acid residue favours the formation of β-turns (Daniloski, McCarthy, Markoska, et al., 2022; Kumosinski, Brown, & Farrell, 1993; McSweeney & Fox, 2013). The A2/A2 β-CN milk contains an additional Pro^{67} in its structure, and therefore it is expected that this milk will possess a greater amount of β-turns. According to the FTIR and ^{1}H NMR results, A2/A2 β-CN milk had approximately 5% greater β-turns compared to the other β-CN milks upon heating ($p < 0.05$) (Fig. 2 and Table 4).

Additionally, the lower κ-CN presented in A2/A2 β-CN milks and their serums may imply their lower net negative charge compared to the other phenotypes, since it has previously been reported that κ-CN is essential for maintaining the net negative charge of CN micelle (McSweeney & Fox, 2013). In particular, ζ potential significantly decreased (became more negative) as the temperature increased (Bogahawaththa et al., 2021), explaining the influence of both, phenotype and heat treatment on the net charge of the CN micelle ($p < 0.05$). Specifically, the existence of His^{67} (positively charged basic amino acid) leads to an increase of the net positive charge of A1 and A1/A2 β-CNs compared with A2 β-CN (Vigolo, Franzoi, Penasa, & De Marchi, 2021), which is known to contain Pro^{67} (neutral charged amino acid) (Damodaran & Parkin, 2017). The net charge of a protein at physiological conditions is dependent on a relative number of basic and acidic amino acid residues in the protein. In this regard, A1 β-CN contains more basic amino acids due to presence of His thus behaves as a positively charged (basic) protein at neutral pH (Damodaran & Parkin, 2017). It is well known that the net negative charge increases while reducing κ-CN dissociation during heat treatment (Crowley et al., 2014), as the case may be parallel to the present study which presented higher micellar κ-CN in A1/A1 and A1/A2 β-CN milks than A2/A2 β-CN milk.

The intense CN bands observed at 72, 121, and 140 °C in A2/A2 β-CN serum phenotypes (Fig. 2) shows destabilisation of CN micelles and dissociation of CNs (particularly A2 β-CN) as a results of the colloidal calcium phosphate (CCP) solubilisation from the micelle and interruption of hydrogen bonds, which were clearly influenced by heat treatment temperature (Anema, 2008). The amount of Ca, P, and Ca^{2+} in all β-CN milks decreased with the temperature increase ($p < 0.05$), which likely suggests that soluble Ca and P reached their solubility limits and created insoluble calcium phosphate that precipitated (Table 2) (Wang & Ma, 2020). Consequently, heat treatment caused a change in the mineral equilibrium (Tables 2 and 3) with mineral shifting into the micelle observed for A1/A2 β-CN milk which confirmed a previously reported observation (Bogahawaththa et al., 2021). Regarding both homozygous β-CN milks, there is a possibility that the minerals precipitated on the surface of the equipment (Tables 2 and 3), thus leading to a decrease of both micellar and soluble minerals with the rise in temperature. This has been also reported by Nieuwenhuijse and Huppertz (2021) indicating that not all Ca and P that precipitates at high temperature is stabilised by CN, at least not at the mineral:CN ratio as found in CN micelles, so that during heating of milk fouling of heating equipment can occur.

Additionally, as the CNs contain phosphoserine residues, they can readily bind Ca ions to their phosphate cluster residues in the order of $\alpha s_2 > \alpha s_1 > \beta >$ κ-CN, due to different levels of phosphorylation and number of ester groups present in each specific CN (O'Mahony & Fox, 2013). It is worth mentioning that both A1 and A2 β-CNs contain 5 phosphoseryl groups (Farrell et al., 2004), however αs_2, αs_1, and β-CN were found more in the colloidal phase of both milks carrying A1 β-CN upon heating. Therefore, this could explain why A1/A2 β-CN milks had

higher levels of minerals (Ca, P, and Ca^{2+}) relative to A1/A1 and A2/A2 β-CN milks (Table 2, $p < 0.05$) when measured after heating in the current study.

Upon heating at sterilisation temperatures (121 and 140 °C), the changes in β-sheet and α-helical fractions in all β-CN milks may indicate substantial unfolding of the secondary structure of proteins, thus proteins behave as random coils (Markoska, Daniloski, Vasiljevic, & Huppertz, 2021; Nishinari, Zhang, & Ikeda, 2000), particularly shown in some A1/A1 β-CN milks, but mainly in A2/A2 β-CN milks upon heating (Table 4). Fox, Uniacke-Lowe, McSweeney, and O'Mahony (2015b) and Huppertz (2013) assigned the random coil secondary structure motif largely to β-CN (23–70%) and αs_1-CN (23%). In the present study, upon heating, the A1/A1 and A2/A2 micellar CNs contained a higher amount of β-CN and αs_1-CN, respectively, compared to the A1/A2 micellar CN, which might indicate that these two proteins may govern the formation of the aforementioned secondary protein structures (Supplementary Tables 2 and 3). Previously, the random structure peaks were assigned to short polyproline II (PPII) helix/chains (Dukor & Keiderling, 1991), and were specifically found in unheated A2/A2 β-CN milk (Daniloski, McCarthy, Markoska et al., 2022; Daniloski, McCarthy, O'Callaghan et al., 2022). Moreover, with the temperature increase the content of β-sheets in A2/A2 β-CN milks significantly decreased ($p < 0.05$). In the past, Farrell, Qi, Wickham, and Unruh (2002) stated that the conversion from β-sheet to PPII conformations may happen when β-CN was heated at pasteurisation temperatures, with the additional Pro^{67} in A2/A2 β-CN milk favouring the formation of these structures (Daniloski, McCarthy, O'Callaghan et al., 2022; Raynes et al., 2015). Overall, the relative insensitivity to temperature, as shown by FTIR, suggests that this milk is stabilised by its predicted and persistent PPII structure. Indeed, the additional Pro^{67} in A2/A2 β-CN milk favours the presence of PPII helical structures, which theoretically precludes the formation of α-helices and other ordered secondary structures, which are important for the hydrogen bonding interactions with the CN micelle (Daniloski, McCarthy, O'Callaghan et al., 2022). Therefore, it appears that A2 β-CN may have an essential role in the CN micelle formation through the PPII mediated interactions (Adzhubei, Sternberg, & Makarov, 2013).

Interestingly, in the present study, A2/A2 β-CN milk possessed a greater amount of α-helical structures than the other β-CN milks, particularly upon heating at 72 and 121 °C (Table 4). This is in-line with a previous finding that the loss of β-sheets in the milk (explained above) upon heating may contribute to the formation of α-helixes (Ye, Zhou, Shi, Chen, & Du, 2017). In particular, their presence in both milks containing Pro^{67} might be due to the high content of both insoluble αs_2-CN and A2 β-CN, since it was found that both proteins contain the highest amount of α-helical structures (54 and 25%, respectively) (Huppertz, 2013). Despite the fact that Pro is known as a α-helical breaker (Damodaran & Parkin, 2017), two possible findings might explain this phenomenon in the present study. Firstly, Grewal et al. (2018) observed the structural changes of milk proteins induced by heating and explained that the Amide I vibration might not be able to completely differentiate between α-helical and random structures, and thus the increase in α-helixes (1656 - 1653 cm^{-2}, Table 4) might also correspond to the increase in random coils. Secondly, polyproline (PP) can form two types of helical structures, PPI: peptide bonds in *cis* configuration and PPII: peptide bonds in *trans* configuration (Damodaran & Parkin, 2017; Markoska et al., 2021b), which may directly or indirectly influence the formation of a higher amount of helical structures. Namely, upon heating the PPI conformations tend to transition to PPII helixes (Kuemin, Engel, & Wennemers, 2010), thus their increased content was expected in A2/A2 β-CN milk as recently shown (Daniloski, McCarthy, Markoska, et al., 2022). Collagen is another example that exists as PPII-type helix due to the high amount of Pro units (i.e., the same as A2 β-CN). At the molecular level, collagen forms a right-handed triple helix consisting of three entwined peptide strands held together by interchain hydrogen bonds, resulting in higher amount of helical structures (Maaβen et al., 2020), which might be the case with A2 β-CN in A2/A2 β-CN milk.

Even though not assessed in the present study, the secondary structure of proteins and various interactions among diverse milk components including proteins and minerals may be also impacted by αs_1-CN, κ-CN or β-Lg levels, their polymorphic variants, degree of κ-CN glycosylation, composite αs_1-β-κ-CN variants, to name a few (Bijl et al., 2014; Bijl, Holland, & Boland, 2020; Day et al., 2015). Furthermore, the phenotypes of both, κ-CN and β-Lg, have been related to functionality and stability of heat-treated bovine milk and manufactured dairy products. In this regard, Bienvenue, Jiménez-Flores, and Singh (2003) showed that β-Lg A variant associated to a greater extent with the CN micelles in comparison to β-Lg B upon heat treatment leading to greater crosslinking between the dispersed particles and much enhanced viscosity of the heat treated milk during storage. Additionally, upon heat treatment of bovine milk, the B variant of κ-CN possessed some ability to stabilise β-Lg against heat-induced denaturation (Choi & Ng-Kwai-Huang & Nau, 2003), however, the same genetic variant of κ-CN was found to be a less effective stabiliser of the CN micelle compared to κ-CN A (Imafidon, Ng-Kwai-Hang, Harwalkar, & Ma, 1991; Jensen, Holland, Poulsen, & Larsen, 2012). Daniloski, McCarthy, Markoska, et al. (2022) confirmed that κ-CN could have the path-terminating function and thus impact the CN micelle size; a lower proportion of total κ-CN content was found to correspond well with greater CN micelle size in A2/A2 β-CN milk compared to that of A1/A1 or A1/A2 β-CN milks. More recently (Daniloski, McCarthy, O'Callaghan et al., 2022), showed that up to 60% of structural variation could be attributed to the effect of present β-CN variants. Therefore, some of the aforementioned studies have shown that not only the β-CN phenotypes, but also the content and genetic variations of other CNs and WPs, may affect the structure and functionality of the A1/A1, A1/A2, and A2/A2 β-CN heat-treated milks (Day et al., 2015; Jensen et al., 2012). In order to fully address these assumptions and consequently elucidate the structural influence of these phenotypes on milk, bigger scale data and thorough investigations with greater sample sizes containing not only various phenotypes of β-CN, but also other CNs, are required.

5. Conclusions

This study reports on the structural characterisation of untreated and thermally treated β-CN milk phenotypes using FTIR and ^{1}H NMR. Through this work, the qualitative and quantitative spectral investigation identified dominant presence of intramolecular β-sheets in A1/A1 β-CN milk, aggregated β-sheets in A1/A2 β-CN milk, and β-turns, random coils, and α-helices in A2/A2 β-CN milk. Particularly, in the latter milk the structural differentiations were based on the presence of the additional Pro^{67}, which was most likely associated with the adoption of more PPII helices in A2 β-CN. Although β-CN and κ-CN appeared to be continually impacted by temperature effects, other CNs appeared to experience variations between two phases, which may potentially be attributed to a change in the mineral balance towards the micellar phase at higher heat treatment temperatures. Hence, the amino acid mutation and decreased κ-CN content in A2/A2 β-CN milk might lead to an increased micelle size, lower net negative charge, and decreased amount of minerals compared to the other β-CN milks. Although A1/A1 and A1/A2 β-CN milks had similar structural properties, in specific areas A2/A2 β-CN milk possessed unique traits, thus PCA clearly separated A2/A2 β-CN milk from the prior milks.

Therefore, FTIR associated with ^{1}H NMR and chemometric tools can potentially be used in the determination of conformational properties of milk samples initiated by the prevalence of β-CN phenotypes and heat treatment. Such information should be useful in explaining the functional behaviour of heat-treated β-CN milks in food systems. In order to understand this complex process of structural differentiations between β-CN milk samples, the influence of different parameters, e.g. nature of surrounding milk proteins, processing conditions of thermal heat treatment, and sample preparation, should be taken into account. Future

prospects of the work would allow for the effect of the various β-CN phenotypes on milk and dairy products to be unravelled. In addition, these protocols, particularly FTIR, can be easily adopted in the control of industrial processes, as common instrumentation is only needed.

Author contributions

Davor Daniloski conceived the study and research question; designed and wrote the original draft, conceptualised, reviewed, edited the manuscript, designed the tables and the figures. **Davor Daniloski** prepared the methodology, formal analysis and investigation. **Todor Vasiljevic** and **Noel A. McCarthy** provided critical feedback and analysis, secured funding, reviewed and edited the manuscript, and supervised the study. All authors have contributed to the manuscript and reviewed the final version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2022.107604.

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Main findings: Part I. Chapter 6.

Authentication of β-casein milk phenotypes using FTIR spectroscopy

- Use of FTIR was assessed for authentication of β-casein milks
- A2 β-casein milk family possessed higher proportions of random coils and β-sheets
- Milks containing A1 β-casein were govern by α-helix and β-turn conformations
- Additional proline in milks potentially elevated the formation of PPII helices
- Data visualisation was performed and confirmed by PCA and PLS-DA chemometrics
- Potential for β-CN milk families to be discriminated by FTIR

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Authentication of β-casein milk phenotypes using FTIR spectroscopy

Davor Daniloski [a, b], Noel A. McCarthy [b], Tom F. O'Callaghan [c], Todor Vasiljevic [a, *]

[a] Victoria University, Advanced Food Systems Research Unit, Institute for Sustainable Industries and Liveable Cities and College of Health and Biomedicine, Melbourne, Victoria 8001, Australia
[b] Teagasc Food Research Centre, Food Chemistry and Technology Department, Moorepark, Fermoy, Cork, P61 C996, Ireland
[c] University College Cork, School of Food and Nutritional Sciences, Cork, T12 YT20, Ireland

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ABSTRACT

The ability to accurately and cost-effectively distinguish between different genetic protein variants in milk is of pivotal significance to the dairy industry as the natural proteoforms of caseins affect the composition and functionality of dairy products. Recently, β-casein proteoforms have received increased consumer attention in regards to A1 and A2 milk families. Fourier transform infrared (FTIR) spectroscopy coupled with multivariate analysis was used to discriminate milk samples from 114 Holstein-Friesian cows possessing different β-casein genetic variants. Principal component and partial least squares-discriminant analyses were used to characterise and distinguish A1 and A2 β-casein genetic families based on spectral data. Milk containing A2 β-casein had higher proportions of random coil and β-sheet conformations than milk containing A1 β-casein and the lowest amount of α-helical and β-turn structures, due to additional proline. This may provide a feature to distinguish between these phenotypes using FTIR spectroscopy in association with chemometric analyses.

1. Introduction

Bovine milk is a complex biological fluid defined as the sole source of nutrients required for neonate development, but is also an important food in the human diet. It is well known that bovine milk is generally composed of many different protein fractions, including the four caseins (CNs: α_{S1}, α_{S2}, β, and κ) and two major whey proteins [WPs: α-La (α-lactalbumin) and β-Lg (β-lactoglobulin)], expressed by the genomic organisation of *CSN* and *WAP* genes' locii, respectively (McSweeney & Fox, 2013).

Caseins, which occur in bovine milk as micelles (i.e., spherical, loosely packed, colloidal complexes of proteins and salts) are highly polymorphic due to the presence of numerous proteoforms (Bijl, Holland, & Boland, 2020; de Kruif, Huppertz, Urban, & Petukhov, 2012; Goulding, Fox, & O'Mahony, 2020). Hence, the genetic polymorphism of CNs can either affect the structural, physicochemical and biological properties of milk, or may have an influence on the functionality of milk and dairy products (Bijl et al., 2020). Recently, milk quality has been associated to a single nucleotide polymorphism in the *CSN2* gene, essential for β-CN coding (Nguyen, Schwendel, Harland, & Day, 2018). Various proteoforms of β-CN in milk were associated with breeding goals and were hypothesised to impair dairy product functionality, including gelation properties, yoghurt manufacturing, foam and emulsion formation capabilities (Nguyen et al., 2018; Poulsen et al., 2013, 2016). Furthermore, consumers, the scientific community, and dairy industry stakeholders have focused their attention on the β-CN proteoforms as a result of their potential impact on human health (Daniloski et al., 2021a; EFSA, 2009; Swinburn, 2004).

In this regard, the current nomenclature of milk proteins in the *Bos* genus describes fifteen different β-CN proteoforms; A1 and A2 are the most common and investigated β-CNs (Daniloski et al., 2021a). The evolution of A1 β-CN proteoform appeared through mutation in the amino acid polypeptide chain at position 67, grouping the β-CN proteoforms into two families, namely A1 family with A1, B, and F variants; and A2 family with A2, A3, and I variants (Daniloski, McCarthy, Markoska, Auldist, & Vasiljevic, 2022). In this regard, all proteoforms of A1 β-CN family share the same mutation of proline (Pro) into histidine (His) at position 67. Furthermore, B and F β-CNs have undergone an additional mutation from arginine (Arg) to serine (Ser) at position 122 and leucine (Leu) to proline (Pro) at position 152, respectively. On the contrary, A2 β-CN family contains Pro at position 67, with only one mutation from methionine (Met) to Leu at position 93 detected in the polypeptide chain

* Corresponding author.
E-mail address: todor.vasiljevic@vu.edu.au (T. Vasiljevic).

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of I/I β-CN (Farrell et al., 2004; Huppertz, 2013; Huppertz, Fox, & Kelly, 2018).

Due to the greater commercial interest in certain parts of the world, including Australia, New Zealand, China, the USA and Canada over the last decade, the selection of cows for the production of A2/A2 β-CN milk has increased (Milan et al., 2019). As a result, the industry has a greater need for reliable, rapid, and non-expensive methods to discriminate between A1 and A2 β-CN milk samples, and to identify potential cross contamination or fraud (De Poi et al., 2020). Methods conventionally used to distinguish β-CN proteoforms have been primarily based on liquid chromatography and electrophoresis (Broadbent, Condina, & Colgrave, 2021; De Poi et al., 2020; Fuerer et al., 2020; Givens, Aikman, Gibson, & Brown, 2013; Nguyen, Solah, Busetti, Smolenski, & Cooney, 2020). These methods are, however, time consuming, laborious and thus expensive, hence, may not be feasible for an industrial scale analysis of β-CN proteoforms (De Poi et al., 2020; Duarte-Vázquez et al., 2018).

On the other hand, the authentication of milk samples could be achieved by utilising fast, non-destructive, and user-friendly techniques such as Fourier Transform Infrared (FTIR) spectroscopy (Daniloski et al., 2022). Hence, FTIR has been identified as a suitable candidate to assess milk characteristics, including its gross chemical composition, density, freezing point depression, prediction of the economic worth of raw milk delivery and, as a result, farmer payments (Andrade et al., 2019; Capuano, Rademaker, van den Bijgaart, & van Ruth, 2014a; Capuano et al., 2014b; Wang et al., 2021).

FTIR is a well-established technique for investigating conformational properties of milk proteins. Namely, the amide I region (1700–1600 cm^{-1}) depicts alterations in protein secondary structures (Daniloski et al., 2022; Grewal, Huppertz, & Vasiljevic, 2018; Markoska, Huppertz, Grewal, & Vasiljevic, 2019). In addition, chemometrics has been successfully used for identification of milk protein adulterations that cannot be accomplished through the study of a single milk property, but necessitates the collection of a multivariate dataset (Capuano, et al., 2014a). Hence, the major advantages of coupling FTIR with multivariate approaches, instead of using it as a standalone spectroscopic analysis, involve improved sensitivity due to the higher signal to noise ratio, improved throughput, superior accuracy, and capability to discriminate among samples (Capuano et al., 2014a; Grewal et al., 2018; Markoska et al., 2019).

Despite these advantages, to our knowledge there are few studies that have evaluated the potential of FTIR or another infrared spectroscopy for discriminating different β-CN genetic variants, and therefore their authentication (Cendron, Franzoi, Penasa, De Marchi, & Cassandro, 2021; Daniloski et al., 2022; Joshi, Mansuri, Kulkarni, & Jamkhedkar, 2021; Xiao et al., 2022). Previously we have reported structural differences between these proteoforms using FTIR and one dimensional proton nuclear magnetic resonance (^{1}H-NMR) on a limited number of samples (Daniloski et al., 2022), which indicated that these techniques were capable of differentiating milk based on the polymorphic forms of β-CN. The objective of the present work was to further assess the potential of FTIR to be used for a rapid and easy discrimination of bovine β-CN milk types.

2. Materials and methods

2.1. Collection of milk samples

Raw bovine milk samples from individual cows (n = 114) were collected from Australian Holstein Friesian cows carrying different β-CN proteoforms. The Agriculture Victoria's Ellinbank Centre in Victoria, Australia, generously provided the milk samples. Milk samples were collected from evening milking using sterile 50 mL plastic screw-top containers and cooled (4 °C) immediately. Each milk sample was analysed using a Lactoscan Milk Analyser (Lactoscan LS-60, Milkotronic Ltd., Nova Zagora, Bulgaria; milk composition data shown in Supplementary material Tables S1–S6), and skimmed by centrifugation at 3225 × *g* at 20 °C for 20 min (Avanti J-26XP, Beckman instrument Australia Pty. Ltd, Gladesville, NSW, Australia). In addition, 10 mL of each sample was ultracentrifuged (Ultra L-70 Centrifuge, Beckman Coulter, Indianapolis, IN, USA) at 100,000 × *g* for 1 h at 20 °C to obtain a CN-free serum required for a baseline correction during FTIR analysis, and finally frozen at −80 °C prior to further analytical tests. Fig. 1 outlines the experimental design of the study.

2.2. Apparatus, methodology, and chemical reagents

2.2.1. Identification of β-casein proteoforms by reversed phase-high performance liquid chromatography

The proteoforms of β-CN in the milk samples were clarified using reversed phase-high performance liquid chromatography (RP-HPLC), performed with a Shimadzu LC-2030C system (RP-HPLC: Shimadzu, Europa GmbH, Duisburg, Germany) equipped with an auto sampler maintained at 45 °C and a wavelength detector (240 nm). The elution conditions were optimised to ensure the best separation and identification of different β-CN proteoforms. At room temperature, 800 μL of each sample was dissolved in 3200 μL of a urea solution (Mediwaththe, Chandrapala, & Vasiljevic, 2018b) to dissociate CN micelles. The sample mixture (1000 μL) was filtered through a 0.45 μm filter and injected into an Aeris WIDEPORE C4 column supplied by Phenomenex (150 mm × 4.6 mm, 3.6 μm particle size, 300 Å porosity, Torrance, USA). The wash of the injection needle was obtained by two mobile phases, including "A" eluent: 0.1% trifluoroacetic acid (TFA: Sigma–Aldrich, St. Louis, MO, USA) in ultra-pure water; and "B" eluent: 0.1% acetonitrile (ACN: Sigma–Aldrich) in ultra-pure water. A single β-CN standard commercially available (Sigma–Aldrich) was used for calibration and identification of the examined samples. Since the β-CN standard was a mixture of various proteoforms, further comparisons with the available literature information were performed (Day, Williams, Otter, & Augustin, 2015; Poulsen et al., 2016). Proteoforms of other major milk proteins were not considered in the current study.

2.2.2. Spectral measurements

The spectral measurements were carried out using an ATR-FTIR spectrometer (Frontier 1, PerkinElmer, Boston, MA, USA). Specifically, FTIR spectroscopy is based on molecular vibrations; a molecule has a variety of chemical bonds, and each bond has different vibration modes, so the FTIR spectrum of a compound usually show multiple absorption bands (Haris & Severcan, 1999). Although the absorbance (vertical axis) does not possess true units (Fig. 3), the horizontal axis indicates the position of an absorption band. Instead of using frequency to show the absorbed radiation, wavenumbers (cm^{-1}) are used as a conventional way in FTIR spectra (Gallagher, 2009; Haris & Severcan, 1999). In the current study, the infrared spectra were recorded with 16 scans in the range 4000 to 650 cm^{-1} with a resolution of 4 cm^{-1}. The analyses were performed at ambient temperature (20 °C). During spectral measurement, each milk sample was scanned 5 times. The spectra were obtained in the absorbance mode. In addition, the background (ultracentrifuged serum, Section 2.1) was collected before every sample, and was measured with a blank Diamond ATR cell utilising the same instrumental conditions as for the sample spectra acquisition (Grewal et al., 2018). The FTIR spectra were derived

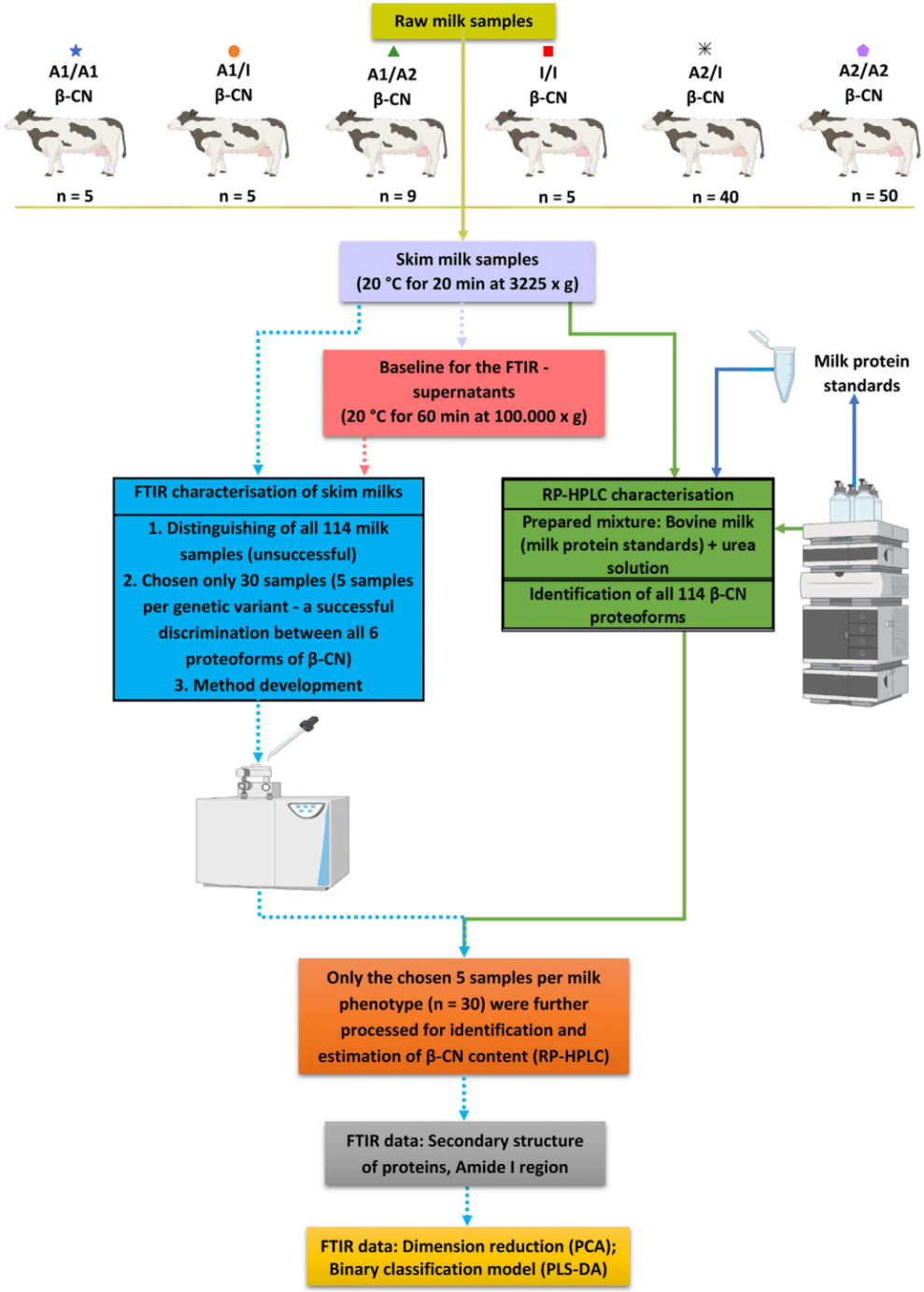

Fig. 1. Schematic illustration showing the general approach of sample selection and analysis.

upon baseline corrections allowing the results to be focused on the CNs and only on a specific region.

2.3. Pre-procession for spectra

Data processing of the chosen spectral measurements was performed in accordance with a previously published method of Grewal et al. (2018). The method included mean centring which was carried out by Spectragryph software (version 1.2.7, Oberstdorf, Germany) followed by a second-order Savitzky–Golay derivative form of all the spectra within broad Amide I envelope (C═O stretching of proteins: 1700–1600 cm^{-1}), before building the classification model. A curve-fitting procedure was used to estimate the area of each component (in unit of percentage, Table 1) representing secondary structures of the included proteins (Mediwaththe, Bogahawaththa, Grewal, Chandrapala, & Vasiljevic, 2018a).

2.4. Multivariate (chemometric) approaches

For multivariate analysis, the second derivate algorithms of the FTIR interferograms were subjected to principal component

analysis (PCA) from the collated data of all individual 114 samples, nevertheless, the chromatogram was over-crowded and a proper clustering between the samples was not observed (Supplementary material Fig. S1). Due to the high number of variables in spectra, it is essential to select the most useful variables for plotting to avoid overcrowding (Beattie & Esmonde-White, 2021). Six different genetic variants of β-CNs were observed among all 114 milk samples, which were firstly examined by RP-HPLC (Section 2.2.1.). In particular, 5 milk samples were found to carry A1/A1 β-CN while 50 milk samples possessed A2/A2 β-CN. Further, A1/A2, A1/I, A2/I, and I/I β-CNs were found in 9, 5, 40 and 5 milk samples, respectively. Upon assessment of these samples it can be noted the high frequency of milk from A2 β-CN family, especially A2/A2 β-CN milk, confirming previously published data (Daniloski et al., 2022; Nguyen et al., 2020; Schettini, Lambert, da Silva Souza, Costa, & de Camargo, 2020). To minimise the bias for further analysis, 5 milk samples per each β-CN proteoform were randomly selected from individual cows.

Subsequently, PCA was performed again on only the chosen 30 samples and these results were further discussed (see Section 3). Partial least squares-discriminant analysis (PLS-DA) was also performed to develop a classification model and distinguish between milk samples containing various genetic forms of β-CNs (only the chosen five samples per proteoform: n = 30). By summarising the information in a lower number of newly built features, these two multivariate techniques (PCA and PLS-DA) were employed for predictive purposes, dimension reduction, and model validation (Capuano et al., 2014a; Grewal et al., 2018). Origin (Origin Pro 2021, v. 95E, OriginLab Corporation, Northampton, MA, USA) and MetaboAnalyst 5.0 [www.metaboanalyst.ca/ (Pang et al., 2021);] software were used to develop PCA and PLS-DA multivariate classification models (Daniloski et al., 2022; Pang et al., 2021). A permutation test with 2000 repetitions was performed to check that the model differed from a random model. Also, the R^2 and Q^2 parameters were obtained and reported to assess the performance of the model using a 10 fold cross-validation approach as well as to estimate the number of components to analyse. The variable importance plot (VIP) shows which variables have a larger influence to the latent variables of the built PLS-DA model.

2.5. Statistical analyses

Minitab 19 software (version 19, Minitab Inc., USA) was used for the analysis of analytical and structural data and replicated on two occasions. Means for continuous variables (diverse β-CNs) were compared using Tukey's Studentised Range post-hoc (HDS) test and one-way analysis of variance (ANOVA), thus establishing effects of the main factor (β-CN proteoform). The significance level was fixed at $p = 0.05$ for all tests. Tables and graphs were also used to illustrate patterns in the variables.

3. Results and discussion

3.1. Determination of β-casein milk phenotypes using HPLC

Prior to the spectroscopic and multivariate analyses, RP-HPLC determination of all β-CN milk samples was carried out on the β-CN dataset. As expected, this analytical technique allowed a satisfactory discrimination between all milk samples. An important feature was the different time of elution between all milk samples. Namely, samples that were part of the A1 β-CN milk family (A1/A1 β-CN) eluted before the milk proteoforms that originated from A2 β-CN milk family (A2/A2 and I/I β-CNs) (Fig. 2). This outcome is related to the different affinity of β-CN with the hydrophobicity of the used column. The presence of a non-polar partially hydrophobic amino acid such as Pro^{67} (Morgan & Rubenstein, 2013) within the structure of proteins from A2 β-CN family is expected to increase the hydrophobicity of these proteins (Daniloski et al., 2022). Hence, it leads to an increased attraction of A2/A2, A2/I, and I/I β-CNs to C_4 column (Aguilar, 2004) and consequently elute later from the column compared with the proteins that carry His^{67} in their structure (A1 β-CN family). Overall, five samples per proteoform (explained in Section 2.4.), which were considered for further conformational analysis are presented in Fig. 2.

3.2. Conformational fingerprinting of β-casein milk phenotypes: infrared spectra collection

To investigate structural features of different β-CN proteoforms, a comparison was performed among A1/A1, A1/I, A1/A2, A2/I, I/I, and A2/A2 β-CN milk samples. In this regard, serum subtracted, smoothed, and derivatised spectra of the chosen milk samples included in the study were considered for further examination; overlapping bands were separated into distinct peaks. Furthermore, in the spectral range studied from 4000 to 650 cm^{-1}, numerous peaks corresponded to the different molecular bonds of milk components interacting with IR radiation. Therefore, to emphasise differences between the milk samples, the amide I region (1700–1600 cm^{-1}) was chosen for further analysis. It is commonly known that the most relevant peaks for the identification of protein secondary structure is the amide I region due to high absorption in this range (Grewal et al., 2018) (Fig. 3A). Accordingly, peaks between 1700 and 1682 cm^{-1} were assigned to intermolecular/aggregated β-sheet; 1681–1665 cm^{-1} to β-turn; 1664–1646 cm^{-1} to α-helix; and 1645–1636 cm^{-1} to random coil. The peaks between 1635 and 1615 cm^{-1} were ascribed to intramolecular β-sheet, and 1614–1601 cm^{-1} to side chains (Daniloski et al., 2022). Owing to the fact that second-derivative spectra are not detailed but qualitative in nature, quantitation of the changes through fitting with Gaussian bands was performed (Mediwaththe et al., 2018a). Thus, an estimation of the secondary structural elements that encompass this broad distribution is given in Table 1.

Table 1
Proportion of the total areas assigned to different secondary structures in the Amide I region for all six genetic variants of β-CN milk.[a]

Sample	Peak area (%)					
	Side chain	Intramolecular β-sheet	Random coil	α-helix	β-turn	Aggregated β-sheet
A1/A1 β-CN milk	5.12 ± 0.12^{abc}	14.99 ± 1.78^{b}	10.42 ± 1.78^{c}	20.24 ± 1.22^{bc}	40.36 ± 5.06^{a}	8.87 ± 0.15^{c}
A1/I β-CN milk	6.00 ± 0.07^{ab}	22.59 ± 1.05^{ab}	14.43 ± 1.62^{bc}	26.27 ± 2.99^{b}	13.80 ± 1.90^{c}	16.91 ± 1.85^{ab}
A1/A2 β-CN milk	3.29 ± 0.21^{c}	19.00 ± 1.39^{ab}	n/a	40.05 ± 7.84^{a}	19.01 ± 2.68^{bc}	18.65 ± 2.00^{a}
I/I β-CN milk	3.60 ± 0.18^{c}	29.79 ± 2.39^{a}	11.90 ± 1.15^{c}	24.24 ± 4.28^{b}	10.95 ± 0.92^{cd}	19.52 ± 3.85^{a}
A2/I β-CN milk	7.69 ± 0.44^{a}	19.23 ± 1.71^{ab}	18.16 ± 2.66^{b}	25.51 ± 3.60^{b}	10.90 ± 1.27^{cd}	18.51 ± 3.37^{a}
A2/A2 β-CN milk	7.00 ± 0.37^{a}	18.92 ± 1.58^{ab}	36.23 ± 5.84^{a}	n/a	26.84 ± 2.21^{b}	11.01 ± 1.08^{bc}
Band frequency (cm^{-1})	1610–1614	1626–1628	1640–1645	1646–1650	1674–1681	1687–1695

[a] Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$).

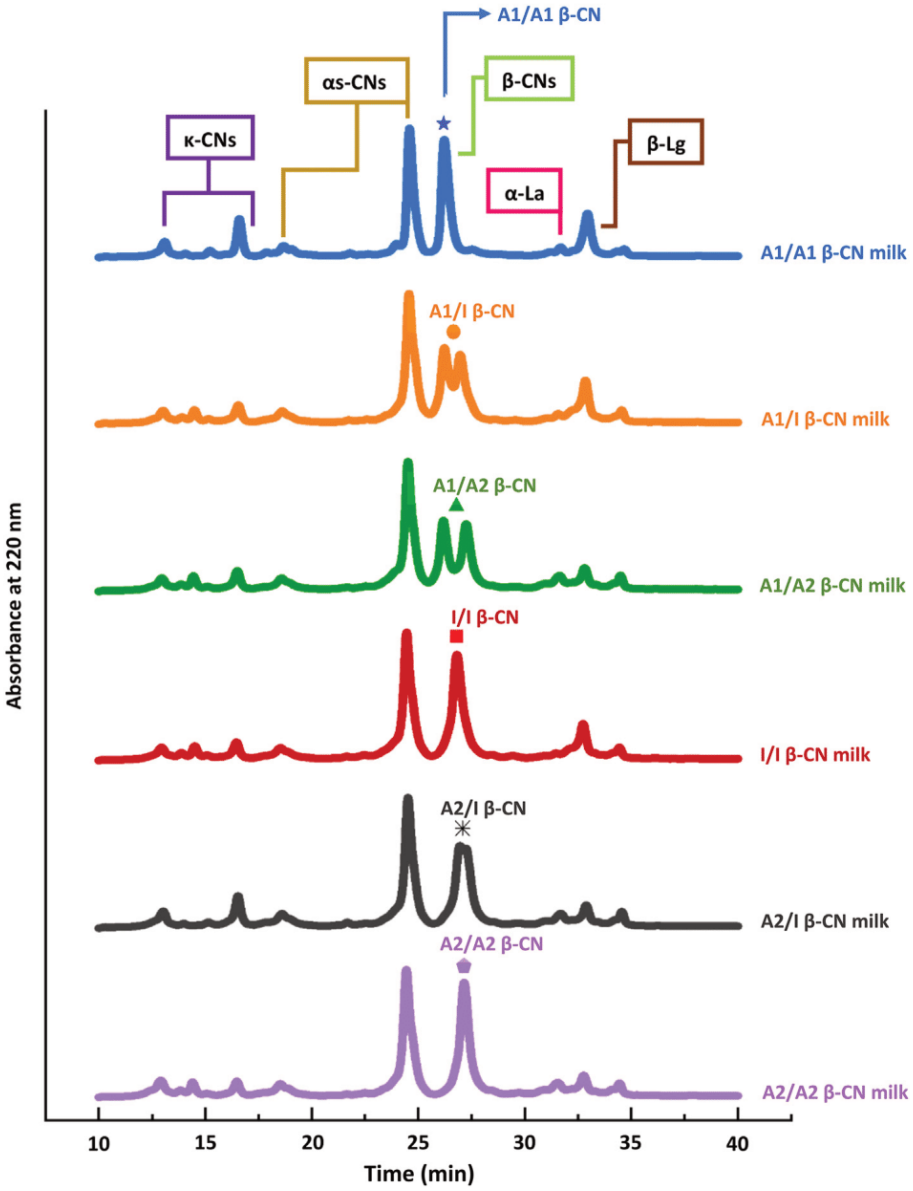

Fig. 2. RP-HPLC chromatographic profiles used for identification of different β-CN milk samples: ★, A1/A1 β-CN milk; ●, A1/I β-CN milk; ▲, A1/A2 β-CN milk; ■, I/I β-CN milk; ✳, A2 A2/I β-CN milk; ⬟, A2/A2 β-CN milk.

At ambient temperature, intramolecular β-sheets were more obvious in milk samples that contained the I/I β-CN proteoform. Namely, these structures were most present in I/I β-CN milk (30%), followed by A1/I (23%), and A2/I β-CN (19%) milks (Table 1; Fig. 3A). Although β-sheet structures are predominantly present in WPs, particularly β-Lg that possesses eight β-sheet structures in its monomeric form (Uhrínová et al., 1998); in the present study, since the baseline was corrected with the corresponding serum, this was not likely the case. Therefore, it should be expected that these findings were dictated by the CN micelle, conformational orientation of the particular β-CN proteoform, or the surrounding CNs. Huppertz (2013) and Fox, Uniacke-Lowe, McSweeney, and O'Mahony (2015) outlined that β-, κ-, α_{S1}- and α_{S2}-CNs contain 15–33%, 31%, 17–20%, and 6–37% β-sheet structures, respectively. Furthermore, a distinguishing feature of both β-CN families was the greater proportion of β-turn structures in A1/A1 β-CN milk compared with all other milk samples.

Consequently, it can be an indication that β-CN might govern the presence and formation of these structures in the casein micelle. In this regard, Huppertz (2013) explained that 20–30% of the turns in bovine milk can be formed by β-CN. Moreover, it was previously shown that β-turns can originate from proline (Pro) or non-Pro nature (Kumosinski, Brown, & Farrell, 1993). Due to its cyclic structure as an imino acid, either Pro residue control development of β-turn structures (Farrell, Brown, & Malin, 2013) or it is statistically preferred at several β-turn positions, presumably because its unique side chains contribute favourably to conformational stability in certain β-turn positions (Shapovalov, Vucetic, & Dunbrack, 2019). Therefore, since A2 β-CN possesses additional Pro at position 67, it is expected that A2 β-CN milk family would have a greater amount of these structures. Interestingly, in the present study, A1 β-CN milk family, specifically A1/A1 β-CN milk, were characterised by elevated amounts of β-turns (almost double) compared with the other milk samples (Table 1).

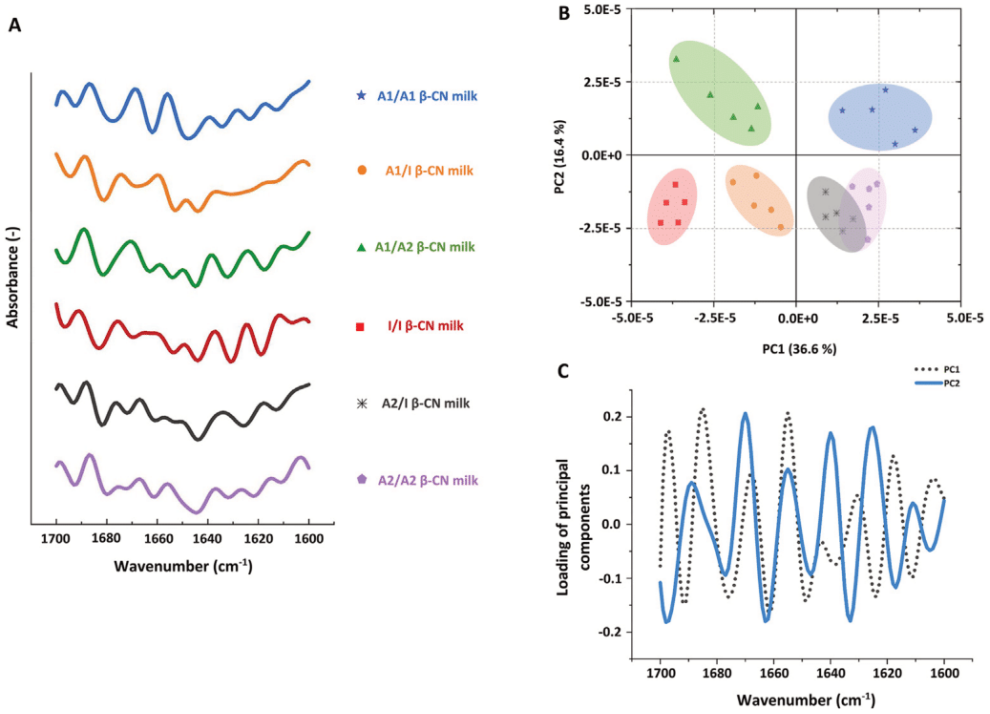

Fig. 3. Second derivative spectra of Amide I region of milk samples (A), with scatter plot (B) of the PCA scores of FTIR spectra of milk samples (★, A1/A1 β-CN milk; ●, A1/I β-CN milk; ▲, A1/A2 β-CN milk; ■, I/I β-CN milk; ✳, A2 A2/I β-CN milk; ⬟, A2/A2 β-CN milk) and (C) the plot of the PCA loadings (⋯⋯, PC1; ——, PC2) of FTIR spectra of milk samples.

One of the explanations for such an observation might be provided by Kumosinski et al. (1993) who assigned a single Pro initially to increased β-turn structures that resulted in the van der Waals attractions with the neighbouring residues. This was further supported by the findings of Graham, Malcolm, and McKenzie (1984) who examined the turns that appeared only in the polypeptide fragment of A1 β-CN (f67–70: His^{67}–Asn^{68}–Ser^{69}–Leu^{70}), resulting in increased levels of β-turns in this protein. Hence, the increase in intensity between 1680 and 1674 cm^{-1} could be tentatively associated with the formation of β-turns in milk samples containing A1 β-CN in their structures (Fig. 3A); whereas from the present study it could be proposed that decrease (or non-existence) in intensity from 1650 to 1646 cm^{-1} region arises from the loss of α-helices, mainly in milk comprised of A2 β-CN.

A significant peak at ≈1650 cm^{-1} (Fig. 3A) may be taken as an indication of the presence of a substantial amount of α-helical secondary structures in milk samples that carried A1 β-CN, in particular A1/A2 β-CN milk that had almost 40% of this structure (Table 1). Additionally, the spectra clearly show the exact position and line shape of the amide I vibrational transition of His (Ghosh, Tucker, & Gai, 2014). Previously, the protonation of the imidazole ring of this amino acid was found in the range 1650 to 1642 cm^{-1}, making the FTIR measurements useful for qualitatively monitoring of the protonation/deprotonation of the His side chains (Ghosh et al., 2014). Hence it is logical to interpret the spectral changes observed in this study to be possibly connected to the conformational changes of the His side chains. As expected, A2/A2 β-CN milk showed an absence of α-helices, likely due to the presence of the additional Pro^{67} in A2 β-CN polypeptide chain; in fact, Pro has been considered as a potent α-helical breaker (Li, Goto, Williams, & Deber, 1996). Hence, due to the high rigidity imposed by the Pro cyclic structure that prevents rotations about the N-C^{α} bond, led to attenuation of the helical structures (Huang & Nau, 2003). Interestingly, proteins that consist of a high amount of Pro residues tend to assume random structures (Damodaran & Parkin, 2017), which is in agreement to our results. Namely, A2/A2 β-CN milk contained 36% of random coils compared with A1/A1 or A1/A2 β-CN milk types, which presented 10% or a complete absence of these structures, respectively.

Considering all details and elements, including the lack of α-helical and β-turn structures, increased content of random coil and β-sheet conformations in all samples that contained an additional Pro^{67} within the structure of their β-CNs, it appears that this amino acid residue plays a pivotal role in the observed conformations. Notably, these results suggested that the helix degree decreased and the hydrophobic cavity of A2 β-CN was more exposed, which may lead to the changes in interfacial properties, increase in hydrophobicity and decrease in solubility of this protein (Zhou, Zhu, Zhang, Hu, & Pan, 2021).

The tendency of Pro to create polyproline (PP) secondary structures is driven by their presence in β-CN, containing approximately 17% of Pro residues (McSweeney & Fox, 2013). The PP, which is a tri-Pro sequence, may fold into stable right-handed or left-handed secondary structures, including type I (PPI) and type II helices (PPII) (Morgan & Rubenstein, 2013). These two PP structures can be found in *cis* (PPI) or *trans* (PPII) configurations that might differently influence the protein structure (Damodaran & Parkin, 2017). Recently, a peptide from β-CN (BCM7: Tyr^{60}–Pro^{61}–Phe^{62}–Pro^{63}–Gly^{64}–Pro^{65}–Ile^{66}) was also found to form *cis* and *trans* isomers, thus specifying the potential polymorphism that may arise from this amino acid (Markoska, Huppertz, & Vasiljevic, 2021). However, the high conformational stability, rigidity, Pro-rich domains, hydrogen bonding with the CN micelle (Raynes, Day, Augustin, & Carver, 2015), and their presence in folded and unfolded proteins makes the PPII helices a dominant fundamental secondary structure of proteins (Horng & Raines, 2006). Particularly, the peak at 1645 cm^{-1}, referred to as random coil, was found as a short polyproline II (PPII) helix (Dukor & Keiderling, 1991).

Accordingly, the presence of more Pro amino acids in A2 β-CN compared with A1 β-CN, can potentially elevate the formation of PPII structures and alter the protein's function (Farrell, Qi, Wickham, & Unruh, 2002).

The spectra of milk samples containing A2 or I β-CNs (A2 β-CN family) revealed substantial differences in the area between 1681 and 1677 cm^{-1} in comparison with that of the other samples (Fig. 3A; Table 1), which could be a result of the conversion from turns into PPII structures (Farrell, Wickham, Unruh, Qi, & Hoagland, 2001). In the past, the observed peak at 1678 cm^{-1} was related to the *trans* stretching of Pro residues (Lin-Vien, Colthup, Fateley, & Grasselli, 1991), also observed recently in the study of Markoska et al. (2021). The nature of Pro residues, PPI and PPII structures, PP mutarotation, transition from PPI to PPII (Dukor & Keiderling, 1996) appear to likely influence not only the structure of different proteoforms of β-CN, but also the functionality of milk and dairy products, and possibly differing the human digestive properties (Daniloski, McCarthy, & Vasiljevic, 2021b).

The current study determined that milk samples with different genetic variations of β-CN might be classified into different groups using FTIR fingerprinting based on their proteins' secondary structures. However, bigger scale data and thorough investigations with higher sample sizes containing not only diverse proteoforms of β-CN, but also other CNs, are required to further elucidate on the structural influence of these genetic variations on milk. This would also include the impact of α_{S1}-CN, κ-CN or β-Lg levels, their polymorphic variants, degree of κ-CN glycosylation, composite α_{S1}-β-κ-CN variants, which were not assessed in the current study, all of which influence different interactions, mineral levels in milk, protein conformation, and CN micelle size (Bijl et al., 2014b; Bonfatti, Di Martino, Cecchinato, Vicario, & Carnier, 2010).

The protein content of all milk samples included in this study ranged from 32.00 to 37.00 mg mL^{-1}, with no significant difference among them (Supplementary material Tables S1–S6). Although the content of CNs was not significantly different between all β-CN milk proteoforms, the content of κ-CN was higher in milk with A1 β-CN in their structures (data not shown). In comparison, the β-CN concentration, reported on a mass per volume basis (mg mL^{-1}), was 10.02, 9.76 and 11.18 mg mL^{-1} for A1/A1, A1/I and A1/A2 β-CN milk samples, respectively, while A2/A2, A2/I and I/I β-CN milk samples contained 11.89, 11.04, and 10.11 mg mL^{-1} β-CN, respectively ($p > 0.05$). These results were comparable with data reported elsewhere in the literature (Daniloski et al., 2022; Vincent, Elkins, Condina, Ezernieks, & Rochfort, 2016).

Recently, Daniloski et al. (2022) related the path-terminating role of κ-CN and its content on the CN micelle size; decreased fraction of total κ-CN content appeared to correlate well with larger CN micelle size in A2/A2 β-CN milk compared with the other homozygous and heterozygous milk samples. Namely, a few studies showed that not only the proteoforms of β-CN, but also the content and the genetic variants of κ-CN and other CNs might influence the structure (Bijl et al., 2014b; Daniloski et al., 2022; Day et al., 2015). The largest study to date using Fourier transform-mid infrared spectroscopy (FT-MIR) investigated the impact of different proteoforms of β-CN, κ-CN and β-Lg on milk coagulation properties (Cendron et al., 2021). In particular, Cendron et al. (2021) denoted that the clustering of the samples highlighted unexpected relationships among the proteoforms in terms of final milk quality, suggesting it could be possible to select appropriate proteoforms maintaining sufficient diversity. However, as of yet, there is no consensus on the structure of CN micelle governed by the major milk proteins and their genetic variants (Cendron et al., 2021). On the contrary, Joshi et al. (2021) proved that FTIR was a powerful tool for recognition of the presence of His only in isolated CN containing A1/A1, but not A2/A2 β-CN. With a prediction accuracy of 95.2% in PLS-DA, Xiao et al. (2022) presented that the proposed predictive models based on MIR spectroscopy can be used for low-cost, rapid, and large-scale classification of A1/A1 and A2/A2 β-CN milk samples, which might be extremely beneficial in the dairy industry. It will be interesting in the future to combine the effect of a β-CN proteoform as one factor and the proteoforms of other milk proteins, thermal processing, feeding programs, as other factors, thus the present FTIR analysis will be further elaborated and more reliable prediction methodologies using both PCA or PLS-DA could be established.

3.3. Dimension reduction analysis: chemometrics and verification of the classification model

PCA was performed on the whole dataset using all 114 β-CN milk samples. However, the PCA score plot failed to identify any natural clustering of the milk samples between the six groups with 95% confidence (Supplementary material Fig. S1). Namely, this overlap indicated that some groups were very similar leading to difficulties to distinguish among the samples. For this reason, only five randomly chosen samples per proteoform were taken into consideration as described in Section 2.4 and further elaborated with PCA and PLS-DA chemometric tools. Over 50% of the total variance of the spectra was explained by the first two principal components (PC1 and PC2), being 36.6% by PC1 and 16.4% by PC2 (Fig. 3B). Similarly, PLS-DA analysis arranged the samples into different groups. Two primary components (PCs), accounting 28.1 and 18.9%, respectively of the variation in the β-CN milk samples, explained the percent variation.

While it is true that the difference narrows when the number of samples is made very large or the clusters are widely separated (i.e., cleanly separated data), the percentage variations of both PCs although not high, remain significant (Ruiz-Perez, Guan, Madhivanan, Mathee, & Narasimhan, 2020). PLS-DA was able to select the correct hyperplane even with fewer samples and even when the separation between the clusters or percentage variations of PCs was low (even values close to 0) (Ruiz-Perez et al., 2020). While A1/A1 β-CN milk appeared distinct from other β-CN milk genetic variants, A2/A2 and A2/I β-CN milk samples were quite similar to each other, as were A1/I and I/I β-CN milk samples (Fig. 4A).

Although the PLS-DA can be thought to correspond to a good model with a high discriminating power, these values of the presented statistics can be attained purely by chance due to a random choice of samples in the test, validation and training sets. This means that it is not known which value of these diagnostic statistics really corresponds to appropriate discrimination between groups (Szymańska, Saccenti, Smilde, & Westerhuis, 2012). To overcome these problems and to give a measure of the statistical significance of the diagnostic statistics (*p*-value), a permutation test was introduced (Szymańska et al., 2012). The permutation test was applied to validate the model and therefore to investigate the significant levels of the separation of β-CN milk samples of different cows (Fig. 4B). In this regard, R^2 (0.595) and Q^2 (0.42) values, which are close to 0.5, gave an indication that this model may be acceptable for further analysis. However, in this study PLS-DA model was only used as an additional tool to prove the reliability of the PCA chemometric analysis. The FTIR wavenumbers mostly contributing to this separation were determined using variable of importance in projection (variable importance plot: VIP) analysis (Fig. 4C).

Although PC2 (16.4%) explained a smaller proportion of the data variance compared with PC1 (36.6%), it efficiently grouped milk samples and significantly distinguished the samples of both families, governed by the specific β-CN. Nevertheless, a particular

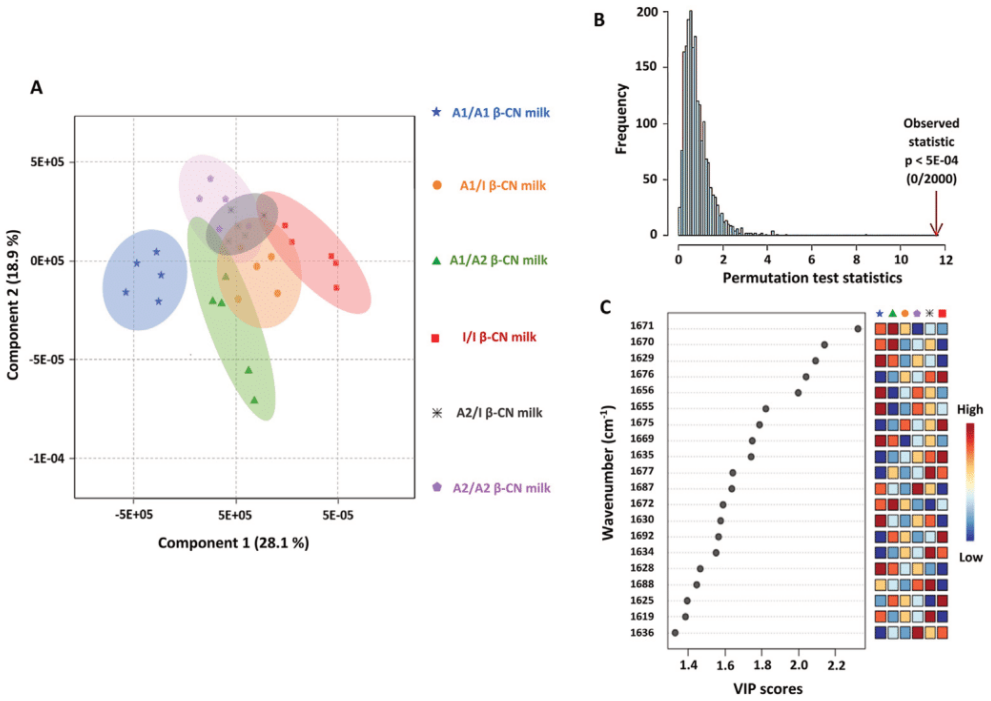

Fig. 4. PLS-DA analysis (A) of FTIR spectra from cows of A1/A1, A1/A2, A1/I, A2, A2/I, and I/I β-CN genotype ($R^2 = 0.595$; $Q^2 = 0.42$) (★, A1/A1 β-CN milk; ●, A1/I β-CN milk; ▲, A1/A2 β-CN milk; ■, I/I β-CN milk; ✳, A2 A2/I β-CN milk; ⬟, A2/A2 β-CN milk) with (B) permutation test $p \leq 5 \times 10^{-4}$ for PLS-DA model for different β-CN milk samples and (C) variable importance in projection plot demonstrating important features for observed separation in PLS-DA.

separation on the PC1 component was also evident. Despite the fact that the impact of both β-CN families was accounted for (either the impact of A1, A2, or I β-CNs), mainly the A2 β-CN family drove the negative groupings (PC1 and PC2). Based on the PC1 loading plot (Fig. 3C) enhanced couplet and intense singlet (PC2) negative loading peaks between 1695 and 1685 cm^{-1}, corresponding to aggregated β-sheets, were evident in both β-CN milk families, but mainly in milk with I β-CN (Fig. 3C; Table 1), which is in agreement with significant changes outlined in Fig. 4B (VIP analysis). The only difference between A2 and I β-CNs is a mutation in the amino acid polypeptide chain at position 93 (Met^{93}–Leu^{93}) (Petrat-Melin et al., 2015).

Formerly, peptides that contained Leu had a tendency to form ordered structures, particularly amphipathic β-sheets (Krantz, Zidovetzki, Kagan, & Zipursky, 1991), which may explain high intensity of this peak in all milk containing I β-CN. Leucine is a unique and flexible amino acid that tends to occur in β-sheet conformations. It prefers short β-sheets and has a negative correlation to coils, yet positive to helices (Malkov, Živković, Beljanski, Hall, & Zarić, 2008), similarly to the present study's results (Table 1). Methionine, on the other hand, is relatively neutral to its appearance in sheets, helices and coils (Huang & Nau, 2003). This amino acid is not rigid, and due its unbranched side chain, provides ample flexibility of the polypeptide chain, thus might influence the formation of α-helices and β-sheets (Aledo, 2019) as in milk containing A1 β-CN in the structure (Table 1). While high levels of Pro might result in a lower content of β-sheets in CNs (Fox et al., 2015), β-sheet conformations predominate (30%) other structural features in β-CN (Farrell et al., 2001, 2013). As it can be visualised from the peak intensity (PC2), the fewer area of β-sheet strands in A2/A2 β-CN milk might be related to the conversion of these structures into PPII helices (Farrell et al., 2002).

Furthermore, greater content of α-helical strands was observed in milk that contained A1 β-CN (≈ 1657 cm^{-1}) (see Section 3.2.). In Fig. 4C two examples are given, i.e., in the amide I region around 1656 cm^{-1} and 1655 cm^{-1}, the VIP scores were rather high. In contrast, sharp negative peaks at around 1643 cm^{-1} and 1623 cm^{-1} presented on PC2, were observed in milk carrying A2 or I β-CNs, which initiates the presence of PPII helices (Dukor & Keiderling, 1991), also supported by the VIP score (Figs. 3C and 4C). Moreover, Markoska et al. (2021) assigned the peak at 1620 cm^{-1} to Pro residues presented in *cis* structures. The β-CN is predicted to contain a significant content (30%) of PPII structures (Farrell et al., 2013), thus it is possible that their presence might be the main reason for distinguishing between these two families, as discussed previously in Section 3.2.

4. Structural assignments: FTIR versus milk profiling

As part of this study, using the randomly chosen set of bovine milk samples with different proteoforms of β-CN, it was found that it may be possible to authenticate the milk families of A1 and A2 β-CNs by means of FTIR fingerprinting. The results obtained by protein profiling with RP-HPLC were superior in terms of sensitivity and specificity (Vincent et al., 2016) compared with those obtained using FTIR described in the current study. Indeed, FTIR can collect many spectra in a relatively short period of time, allowing accurate logging of the secondary structure of proteins (Markoska et al., 2019). Daniloski et al. (2022) critically analysed the structure of A1/A1, A1/A2, and A2/A2 β-CN milk observed by FTIR. Their study found a higher level of β-turn and α-helical structures in A1/A1 β-CN milk, while intermolecular β-sheets were more numerous in A1/A2 β-CN milk. Contrarily, A2/A2 β-CN milk possessed a great deal of polyproline II (PPII) structures (Daniloski et al., 2022), thus proving the

effectiveness of FTIR to differentiate between these three milk phenotypes using their CN secondary structure (Daniloski et al., 2022).

The FTIR instrumentation cost is moderately high but still an order of magnitude lower than that of the chromatographic analytical methods; it requires low operational costs and no need of an experienced operator (Dehaine, Tijsseling, Rollinson, Buxton, & Glass, 2022). Presently, in the analysis of illegal food components or cross-contaminations, such as presence of a small amount of A1 β-CN in A2/A2 β-CN milk found in the market, FTIR analysis has the disadvantage of dealing with a whole sample approach. The use of FTIR served as an additional evidence for the noticeable difference between these two β-CN families (Daniloski et al., 2022). Hence, due to a high-throughput, there is necessity to use first extraction then separation of CNs, chromatographic column or capillary, depending on the chosen chromatographic technique (Vincent et al., 2016). Given the relative ease with which FTIR can be applied, and the large number and type of systems to which it is amenable, it is not surprising that the use of FTIR as an investigative, *in situ* interfacial tool is constantly increasing. Therefore, the efficiency and accuracy rate of FTIR combined with multivariate models should be further improved to promote its application in a large-scale identification of milk categories.

5. Conclusion

As part of this study, using the randomly chosen set of bovine milk samples possessing different β-CN proteoforms, it appears that it may be possible to authenticate the milk samples. This was accomplished due to several structural differences that were initially established by FTIR and then highlighted by chemometric analysis. Milk samples containing A2 β-CN had greater proportions of random coil and β-sheet conformations, with lower amounts of α-helical and β-turn structures compared with the milk containing A1 β-CN. These differences could likely be due to the presence of Pro^{67} in the primary structure of A2 or I β-CNs. Furthermore, the tendency of Pro to create PPII structures can be an additional reason for distinguishing between these two families. However to fully authenticate milk based on its proteoforms, several conditions should be considered. Firstly, most of the milk samples contain different proteoforms of other milk proteins (specifically κ-CN and β-Lg) that have previously been associated with the altered physicochemical and structural properties of bovine milk and dairy products (Bijl, de Vries, van Valenberg, Huppertz, & van Hooijdonk, 2014a; Day et al., 2015; Ketto et al., 2017; Poulsen et al., 2013). Moreover, other factors including cow breed, lactation stage, diet, season, environmental factors, to name a few, affect the milk composition and the structure of milk proteins (Bijl et al., 2020). However, in the present study these contributing factors were neither included nor further discussed. Therefore, to fully develop a predictive model using FTIR for differentiating milk based on its various proteoforms, a large data set needs to be considered that would include all various factors influencing structural behaviour of milk proteins.

Author contributions

Davor Daniloski conceived the study and research question; designed and wrote the original draft, conceptualised, reviewed, edited the manuscript, designed the tables and the figures. Davor Daniloski prepared the methodology, formal analysis and investigation. Todor Vasiljevic and Noel A. McCarthy provided critical feedback and analysis, secured funding, reviewed and edited the manuscript, and supervised the study. Tom F. O'Callaghan provided critical feedback, reviewed and edited the manuscript. All authors have contributed to the manuscript and reviewed the final version.

Conflict of interest and authorship conformation form

All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.

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The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2022.105350.

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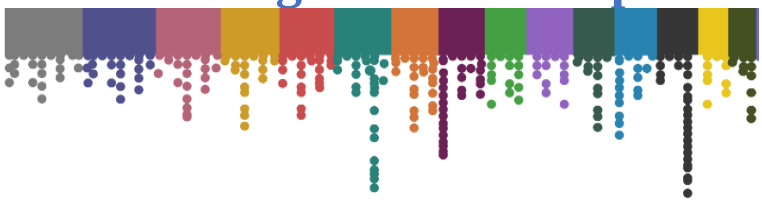
Main findings: Part I. Chapter 7.



Properties of sodium caseinate as affected by the β-casein phenotypes

- Properties of sodium caseinates with various β-caseins were studied by FTIR and NMR
- Dispersions with A1 β-casein exhibited noticeable ordered structural properties
- Sodium caseinates possessing A1 β-casein showed lower solubility
- Increased α-helixes in A2/A2 sodium caseinate's adsorbed interface, led to greater emulsion stability
- A2/A2 dispersions and emulsions possessed high amount of β-turn conformations

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Title of Paper/Journal/Book: **Properties of sodium caseinate as affected by the β-casein phenotypes**

Surname: **Daniloski** First name: **Davor**

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2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

[redacted] **17/03/2024**

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3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the work as follows:

The undersigned certify that:

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Davor Daniloski	85	Designed and wrote the original draft, conceptualised, reviewed, edited, revised the manuscript, designed the experiments, the tables and the figures, and data analysis	[redacted]	17/03/2024
Noel A. McCarthy	5	Concept development, manuscript editing, revision and supervision		17/03/2024
Martin J. Auldist	2	Manuscript editing and revision		17/03/2024
Todor Vasiljevic	8	Concept development, experimental design, manuscript editing, revision, submission and supervision		17/03/2024

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Properties of sodium caseinate as affected by the β-casein phenotypes

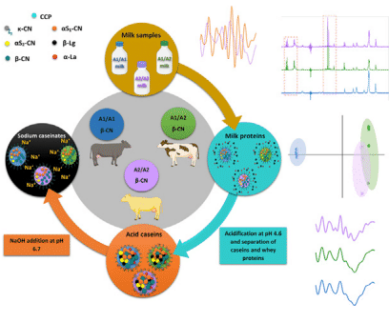
Davor Daniloski [a,b], Noel A. McCarthy [b], Martin J. Auldist [c,d], Todor Vasiljevic [a,*]

[a] Victoria University, Advanced Food Systems Research Unit, Institute for Sustainable Industries and Liveable Cities and College of Health and Biomedicine, Melbourne, Victoria 8001, Australia
[b] Teagasc Food Research Centre, Food Chemistry and Technology Department, Moorepark, Fermoy, P61 C996, Cork, Ireland
[c] Agriculture Victoria, Department of Jobs, Precincts and Regions, Ellinbank, Victoria 3821, Australia
[d] University of Melbourne, Centre for Agricultural Innovation, School of Agriculture and Food, Faculty of Veterinary and Agricultural Sciences, Melbourne, Victoria 3010, Australia

HIGHLIGHTS

- Properties of sodium caseinates with various β-caseins were studied by FTIR and NMR.
- Dispersions with A1 β-casein exhibited noticeable ordered structural properties.
- Sodium caseinates possessing A1 β-casein showed lower solubility.
- Increased α-helixes in A2/A2 sodium caseinate's adsorbed interface, led to greater emulsion stability.
- A2/A2 dispersions and emulsions possessed high amount of β-turn conformations.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of the study was to investigate the properties of sodium caseinate dispersions and oil-in-water emulsions obtained from cows' milk of either A1/A1, A1/A2, or A2/A2 β-casein phenotype. Protein structural characterisation was examined using Fourier Transform Infrared and Nuclear Magnetic Resonance spectroscopies, with physicochemical and interfacial properties assessed by analysing adsorbed protein content, hydrophobicity, solubility, and emulsion stability of the samples. Results showed variations in the secondary structure of all samples dependent of the presence of A1 or A2 β-caseins. The main differences included greater amounts of α-helix and β-sheet in A1/A1 and A1/A2 sodium caseinate dispersions that influenced their lower solubility, while random coils/polyproline II helixes were found only in A2/A2 sodium caseinate dispersion. In contrast, upon adsorption on the interface of A2/A2 sodium caseinate emulsion, the protein adopted ordered conformational motifs. This conformational shift supposedly arose from structural differences between the two β-casein proteoforms, which most likely enhanced the emulsion properties of A2/A2 sodium caseinate compared to either A1/A1 or A1/A2 sodium caseinates. The A2 β-casein in both, A1/A2 and A2/A2 sodium caseinates, appears to be able to more rapidly reach the oil droplet surface and was more efficient as emulsifying agent. The current results demonstrated that the conformational rearrangement of proteins upon adsorption to emulsion interfaces was dependent not only on hydrophobicity and on solubility, but also on the conformational flexibility of A1/A1, A1/A2, and A2/A2 β-casein phenotypes. These findings can assist in predicting the behaviour of sodium caseinates during relevant industrial processing.

* Corresponding author.
E-mail address: todor.vasiljevic@vu.edu.au (T. Vasiljevic).

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1. Introduction

Bovine milk is a complex mixture of minor and major proteins, with caseins (CNs) accounting for approximately 80 %, with the remaining 20 % of milk protein known as whey proteins (WPs). The CN fraction is made up of four different phosphoprotein isoforms, including αs_1-, αs_2-, β-, and κ-CNs (ratio 4.0: 1.0: 3.5: 1.5), 90% of which are colloidal complexes with diameters of 50–500 nm, known as CN micelles [34]. The CN micelle has a high concentration of calcium phosphate in the form of micellar calcium phosphate (MCP) nanoclusters. The hydrophobic interactions and hydrogen bonds between the CN phosphoproteins and the bridging of MCP nanoclusters, stabilises these micellar complexes and inhibits calcium phosphate precipitation and sedimentation [20,37,38]. The acidification of milk to pH 4.6 (CN isoelectric point) leads to the solubilisation of MCP causing CNs' precipitation with a concomitant reduction in the net negative charge of the proteins, yielding a mixture of individual CN proteins. Subsequently, the coagulated CN micelles are washed, leading to removal of minerals, lactose, and WPs [10]. The CN can be dispersed by mixing with water and adjusting the pH back to 6.8 through the introduction of an alkaline solution, such as sodium hydroxide (NaOH), followed by drying, forming a product commonly known as sodium caseinate (NaCN) [32]. Due to its excellent functional, nutritional, and emulsification characteristics, NaCN is widely used in the formation and stabilisation of food emulsions [2], Oliver, 2011 & Hemar, 2011).

Even though the CN phosphoproteins in the NaCN (referred as primary CN particles) appear to be already present at the same ratio as in the CN micelle, the amount of MCP and the hydrodynamic radius of CN particles in NaCN is significantly lower than that of native CN micelles in bovine milk [26,38]. These particles consist of 35–40 % β-CN, found as an intrinsically disordered amphiphilic protein with a short *N*-terminal domain and C-terminal tail, giving β-CN the ability to act as a surface-active molecule [46]. In particular, the amino acids in the *N*-terminal domain of β-casein are hydrophilic and highly negatively charged, leading to their exposure to a polar continuous phase. These amino acids protrude from the surface, and promote the formation of dangling tails that form a thick adsorbed layer providing electrostatic and steric repulsion [23,43]. Their functionality can be altered based on the chemical and structural properties of the protein as well as the genetic factors [17]. Furthermore, the phosphorylated serine (SerP) residues clustered in the *N*-terminus of β-CN confer thickness and steric stability in the adsorbed layer surrounding the oil droplets, reducing interfacial tension in an emulsion, and are important for emulsion formation and stabilisation [17,58]. Two most investigated genetic variants, which account for>95 % of all β-CN types found in common breeds of cattle, include A1 and A2 β-CNs [13]. The only difference between these two genetic variants is the substitution of proline (Pro) in A2 β-CN by the histidine (His) in A1 β-CN at position 67 in the polypeptide chain of this protein [11]. Recently, A1/A2 milk, which is a mixture of both β-CNs and A2/A2 milk, only composed of A2/A2 β-CN, have gained a lot of marketing and research interest due to their possible impact on the structure and functionality of dairy products [11,15]. In this regard, A2/A2 milk was reported to carry larger CN micelles compared to A1/A1 or A1/A2 milks [13,16]. It has also previously been shown that A2/A2 milk has lower gel strength after rennet coagulation [54] and acid gelation [12], but has better emulsion formation capability compared to A1/A1 milks [17,50].

Although the structural properties of CN micelles and the interfacial properties of bovine milks with various β-CN phenotypes have previously been investigated [13,50], the current study aims to determine to what extent β-CN phenotype might influence conformational structure and emulsifying properties in dissociated CN micelles in the form of NaCNs, which could have important implications to the food industry.

2. Materials and methods

2.1. Milk sampling and sodium caseinate preparation

Fresh milk samples obtained from Holstein-Friesian cows with specific β-CN phenotypes were obtained from the Agriculture Victoria Research Centre in Ellinbank (Victoria, Australia). Immediately after milking, milk samples from each phenotype were pooled into individual containers and chilled at 4 °C. The fresh samples were analysed by Lactoscan milk analyser (Lactoscan LS-60, Milkotronic Ltd., Nova Zagora, Bulgaria), which provided an approximate composition of all milk samples (data not shown). Whole milk samples were then skimmed by removing the fat layer after centrifugation (Avanti J-26XP, Beckman instrument Australia Pty. Ltd, Gladesville, NSW, Australia) at 3225 × g for 20 min at 20 °C. The NaCNs were prepared from skim milks following the method of Hemar et al. [32]. Briefly, at ambient temperature, the skim milks were acidified to pH 4.6 using 2 M HCL (Sigma-Aldrich, St. Louis, MO, USA) to achieve CN curd formation and facilitate separation from the serum phase. Subsequently, the curd was poured into a cheesecloth and washed with ultra-pure water until the water ran completely clear. Finally, the curd was mixed with ultra-pure water in a ratio of 1:3 and brought to a neutral pH of 6.8 with 2 M NaOH (Sigma-Aldrich, St. Louis, MO, USA), followed by thorough mixing for 2 days at 20 °C. Sodium azide (0.02 % w/w, Sigma-Aldrich, St. Louis, MO, USA) was added to prevent microbial growth. The prepared NaCNs were then frozen at − 80 °C for 24 h and subsequently lyophilised using a pilot scale freeze dryer (Alpha 1–4 LSC plus, Christ, Osterode, Germany), with a primary and secondary drying step set at − 80 °C for 45 and 5 h, respectively. The NaCN powders were vacuum packed in double-sealed plastic bags at ambient temperature and stored at − 80 °C and transferred to − 20 °C after 24 h until required for further analysis.

2.2. Reconstitution of NaCNs and protein dispersions

The NaCN powders were reconstituted with ultra-pure water at 20 °C to produce protein dispersions at a total protein concentration of 1.0 % (w/w). Initially, the powders were left to hydrate without stirring for 15 min, and then mixed for 1 h, before being adjusted to pH 6.8, if necessary (2 M HCL or 2 M NaOH, Sigma-Aldrich, St. Louis, MO, USA). As previously described, magnetic stirring was continued for an additional 1 h and dispersions left to hydrate overnight at 4 °C. Prior to analysis, the NaCN dispersions were equilibrated to a room temperature. The NaCN dispersions carrying different genetic variants of β-CN were denominated as A1/A1 NaCN - D, A1/A2 NaCN - D, and A2/A2 NaCN - D.

2.3. Physicochemical and interfacial properties

2.3.1. Protein analysis and casein composition

Total nitrogen (TN), non-CN nitrogen (NCN), and non-protein nitrogen (NPN) content of NaCN dispersions was determined using the Kjeldahl method (ISO, 2014 [39]), with a nitrogen conversion factor of 6.38 to convert to total protein content. The protein profile of NaCN dispersions was determined by Reversed Phase-High Performance Liquid Chromatograph, with an analytical C4 column (Phenomenex Aeris WIDEPORE, 150 mm × 4.6 mm, 3.6 μm particle size, 300 Å porosity, Torrance, USA), equipped with a UV detector (RP-HPLC: LC-2030C, Shimadzu Corporation, Kyoto,

Japan). Following the procedure described by Daniloski et al. [13] the NaCNs were diluted at a concentration of 0.8 mL at 3.2 mL solution of 8 M urea, 165 mM Tris, 44 mM sodium citrate, and 0.3% v/v β-mercaptoethanol. After filtration through 0.45 μm pore size filters, 20 μL of the solution was injected. The mobile phase was composed of ultra-pure water and 0.1% trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO, USA) with a gradient of acetonitrile (Sigma-Aldrich, St. Louis, MO, USA). The UV absorption was detected at 240 nm. The positions of different CNs and the phenotypes of only β-CNs have been identified by comparing with purified CNs (Sigma-Aldrich, St. Louis, MO, USA) or with spectra obtained from the literature [55].

2.3.2. Solubility of the sodium caseinates

Protein solubility was determined using a method by Anema, Pinder, Hunter, and Hemar [1]. Ten mL of the prepared protein dispersions were centrifuged at 700 × g for 10 min at 20 °C (Avanti J-26XP, Beckman instrument Australia Pty. Ltd, Gladesville, NSW, Australia). These conditions were chosen to mimic natural deposition of sediments in a common protein beverage. Solubility was given by the protein content of the supernatant expressed as a percentage of the total protein content in the original dispersion [5]. The protein content of each supernatant and dispersion was then quantified as per section 2.3.1.

2.3.3. Surface hydrophobicity of the sodium caseinates

Surface hydrophobicity of the protein in NaCN dispersions was measured using a fluorometric assay method with 1-anilinonaphthalene-8-sulfonic acid (ANS; Sigma Aldrich, St Louis, MO, USA) as a fluorescence probe. The relative fluorescence intensity (RFI) was measured at excitation and emission wavelengths of 390 and 470 nm, respectively, using a Shimadzu fluorescence spectrophotometer (5301-PC, Shimadzu Corp., Kyoto, Japan) [47].

2.3.4. Emulsion preparation and characterisation

Emulsions of protein dispersions were prepared in a ratio of 1:10, by mixing 100 mL of canola oil (10 % w/w) with 900 mL of 1 % (w/w) NaCN dispersions as described by McCarthy et al. [46] with some modifications. This mixture was homogenised initially by a laboratory homogenizer (Polytron, Kinematica AG. Lucerne, Switzerland) at 10000 rpm for 1 min followed by a pilot plant scale homogenizer operating at 25 MPa, at 60 °C for 3 min/L of sample (Pilotech Instrument and Equipment Co. Ltd., Shanghai, China). The samples were abbreviated as A1/A1 NaCN - E, A1/A2 NaCN - E, and A2/A2 NaCN - E (the same names were used for the adsorbed layer of all NaCN samples). The pH of all emulsions was 6.8 before analysis. The methods described by Cameron, Weber, Idziak, Neufeld, and Cooper [7], Nishanthi, Chandrapala, and Vasiljevic [51] and Pearce and Kinsella [53] were used to determine the emulsifying activity index (EAI), emulsion stability index (ESI), and adsorbed proteins to estimate the emulsification capacity and the stability of the emulsions prepared of NaCNs with different β-CN phenotypes. The EAI (m^2/g) was presented as a function of the turbidity of the emulsions and the oil volume fraction [7]. The ESI was calculated by measuring the turbidity of the emulsions after storing at 4 °C for one day [53]. Adsorbed protein content (mg/mL) was measured as the amount of protein associated with the fat layer after centrifugation of the emulsion at 20 °C for 30 min at 12000 × g (Avanti J-26XP, Beckman instrument Australia Pty. Ltd, Gladesville, NSW, Australia) [51]. This was calculated as the difference between the protein content in the stock solution and the protein content in the aqueous layer of emulsion. The Kjeldahl method (ISO, 2014) was used for all protein estimates.

2.4. Conformational characteristics

2.4.1. Attenuated total reflection-Fourier Transform Infrared (ATR-FTIR) spectroscopy

The infrared spectra of NaCN dispersions were measured using an ATR-FTIR spectrometer (PerkinElmer Frontier, MA, USA) [14], with ultra-pure water used as the background; for the NaCN emulsions an *in situ* FTIR subtraction validated method was used to evaluate the changes of the secondary structure of adsorbed proteins at oil/water-interfaces [60]. Each spectrum was an absorbance spectrum measured in transmission and a result of the average of 64 scans recorded with a resolution of 4 cm^{-1} at ambient temperature in the range of 4000–600 cm^{-1}. After baseline corrections, second derivative of the original spectra were smoothed with the 25-point Savitsky-Golay method. The spectra were obtained in the Amide I region 1700–1600 cm^{-1} and edited using a Spectragryph software (v. 1.2.7, Oberstdorf, Germany). The method of Markoska, Daniloski, Vasiljevic, and Huppertz [45] was used to estimate the area of each component representing secondary structures. In the Amide I region several regions were analysed, including 1700–1682 cm^{-1} (intermolecular/aggregated β-sheet); 1681–1665 cm^{-1} (β-turn); 1664–1646 cm^{-1} (α-helix); 1645–1638 cm^{-1} (random coil); 1637–1615 cm^{-1} (intramolecular β-sheet); and 1614–1601 cm^{-1} (side chain) [14].

2.4.2. One dimensional proton Nuclear Magnetic Resonance (1D ^1H NMR) spectroscopy

The ^1H NMR was used to determine the chemical shifts (δ ppm), of the proton signals of the components both in NaCN dispersions and in NaCN emulsions (adsorbed layer) following the methods by Daniloski et al. [13] and Saeed, Gillies, Wagner, and Howell [59], respectively. The samples (2.5 mg/mL protein) were dispersed in 1 mL of a dispersion of ultra-pure water and deuterium oxide to lock the NMR signal (D_2O [99.8 atom percent excess and pH 6.0: Sigma-Aldrich, St. Louis, MO, USA]) in a ratio of 9:1, and mixed in a 1.5 mL Eppendorf tube. The samples were vortexed for 30 s and 0.6 mL of each solution was transferred using a glass pipette into a clean 5 mm standard NMR tube (Sigma-Aldrich, St. Louis, MO, USA). The NMR measurements were acquired on a Bruker Avance spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) at 20 °C, operating at a 600 MHz transmitter frequency using 5 mm TXI probe with Z- and XYZ-gradients. After adjusting the lock level, the shims were optimised, and the probe was tuned for the ^1H nucleus. Chemical shifts for ^1H NMR spectra were referenced to the residual resonances of the deuterated solvent and are reported as parts per million (ppm). The number of scans was 128 (ns = 128), the transform size was 64 K with 90° pulse sequence, 2 s relaxation delay and spectral width of 6003.51 Hz (10.00 ppm), obtained in ten replications. TopSpin (version 4.1.1) software (Bruker BioSpin, Billerica, Massachusetts, USA) was used to convert the time domain signal or free induction decay (FID), in a single step into a frequency domain spectrum by 0 and 1st order correction for pk; the FIDs were corrected with a 0.3 Hz line-broadening parameter. The NMR spectra were analysed using three NMR regions, namely amino/aromatic (H^N: 9.0–5.5 ppm), Amide (H^α: 5.5–3.5), and aliphatic (3.5–1.5 ppm). From the aliphatic region, the methyl peak (CH_3: 1.5–0.0 ppm) was analysed separately due to its high loading and the area of each component was estimated [45]. The bins corresponding with the water region (4.73–4.99 ppm) were eliminated from the analysis. All the spectra were manually phased and baselines corrected.

3. Data analysis

All results were analysed using Minitab statistical software (Version 20; Minitab, Pennsylvania, USA). Statistical analysis was performed by Analysis of variance (one-way ANOVA) with Tukey - HSD's post hoc test. The effect of the fixed factor (genetic variant for both NaCN dispersions and emulsions) was taken into consideration for all data. When $p \leq 0.05$, the differences were considered to be significant. All experiments were performed in triplicate.

4. Results

4.1. Physicochemical and interfacial properties

Protein fractions derived from NaCN - Ds possessing either A1/A1, A1/A2, or A2/A2 β-CNs were characterised by RP-HPLC and their relevant amounts in all samples are shown in Table 1. There were no noticeable differences between the NaCN - Ds based on the protein content ($p > 0.05$), since all NaCN - Ds were reconstituted to the same protein content, nevertheless, from all proteins, the levels of κ-CN were significantly ($p < 0.05$) higher in A1/A1, but especially A1/A2 NaCN – Ds, than that of A2/A2 NaCN - D. On the contrary, the content of β-CN was almost 17 % greater in dispersions containing A2 β-CN compared to A1/A1 NaCN - D ($p < 0.05$). As expected, no peak corresponding to either β-Lactoglobulin or α-Lactalbumin was detected in the NaCN - Ds (data not shown). The chromatograms showed that αs_1- and β-CNs were the main proteins of the colloidal fraction of all three types of NaCN - Ds representing approximately 50 % and 40 % of total bovine NaCNs, respectively ($p < 0.05$, Table 1). Additionally, both A1 and A2 β-CN genetic variants showed different times of elution, with A1 β-CN eluting at ~ 27 min, compared to A2 β-CN which eluted at ~ 28 min (data not shown). A2 β-CN appears to be more hydrophobic protein with a higher hydrophobicity level compared to that of A1 β-CN [14]. Thus, in the present study, the surface hydrophobicity was probed using ANS, a fluorescent surface hydrophobicity probe. The ANS fluorescence intensity was only slightly higher for A2/A2 NaCN - D than for A1/A2 and A1/A1 NaCN - Ds ($p > 0.05$); as shown in Table 2 the surface hydrophobicity was not affected by the β-CN genetic variant ($p > 0.05$). On the other hand, the solubility of NaCN - Ds was significantly ($p < 0.05$) affected by the β-CN phenotype (Table 2). NaCN - Ds containing A2 β-CN had the highest solubility, while the lowest protein solubility (by ~ 7 %) was attributed to A1/A1 NaCN - D.

The β-CN phenotype significantly ($p < 0.05$) influenced both emulsion activity (EAI) and emulsion stability indexes (ESI) of the NaCN - Es (Table 2). Emulsion activity index of the A2/A2 NaCN - E (~137 m^2/g) was 9 % higher than that the EAI of A1/A1 NaCN - E (~125 m^2/g), and 3 % greater compared to A1/A2 NaCN - E (~133 m^2/g). Furthermore, the ESI was high for A1/A2 (~55 h) and A2/A2 (~58 h) NaCN - Es as opposed to that of the A1/A1 NaCN - E (~49 h). As the EAI refers to the capacity of proteins adsorbed at the interface between water and oil droplets during the formation of the emulsion to prevent gravitational separation, flocculation and coalescence [51], the adsorbed proteins of the NaCN - Es with either A1/A1, A1/A2, or A2/A2 β-CNs were estimated and presented in Table 1. The β-CN phenotype significantly ($p < 0.05$) influenced the variation of the amount of adsorbed proteins. Namely, A2/A2 NaCN - E showed a high level of adsorbed proteins (3.76 mg/mL), followed by A1/A2 NaCN - E (2.85 mg/mL), while the A1/A1 NaCN - E (1.70 mg/mL) showed the least. Based on the RP-HPLC data (Table 1), the most prominent differences in the adsorbed proteins among all three types of NaCN - Es was observed for both β- and αs_1-CNs. The β-CN concentration was 2.15 mg/mL out of the adsorbed proteins in the A2/A2 NaCN – E, which was higher by 46 and 135 % than that out of the adsorbed proteins in the A1/A2 or A1/A1 NaCN - Es ($p < 0.05$). Also, in the prior NaCN - E the content of αs_1-CN as part of the adsorbed proteins was 1.34 mg/mL, which was almost 77 % greater compared to the adsorbed proteins of the latter phenotypes of NaCN - Es.

4.2. Protein secondary structure

The changes in the protein secondary structure in both NaCN dispersions (NaCN - Ds) and the adsorbed layer of emulsions (NaCN - Es) was determined by FTIR spectroscopy (Figs. 1 and 3). In addition, the second derivative of the FTIR spectra in the Amide I region (1700–1600 cm^{-1}) was calculated in order to visualise otherwise hidden peaks (quantification of changes in the secondary structure of the proteins) (Table 3). The differences in peaks centred between 1630 and 1620 cm^{-1} detected in the Amide I vibrational region of all three NaCN - Ds were assigned to intramolecular β-sheets. Both NaCN - Ds containing A1 β-CN showed a similar amount of intramolecular β-sheet structures, compared to A2/A2 NaCN - D where their presence was lower by almost 45 % ($p < 0.05$, Table 3). Out of all three types of NaCN – Ds, only the A2/A2 NaCN - D adopted random coil structures, while on the contrary, the α-helices were completely absent in this dispersion ($p < 0.05$). The spectroscopic profile of A1/A1 NaCN - D (Fig. 1) showed an absorption shoulder peak at 1680 cm^{-1} attributed to a significantly higher level of aggregated β-sheet, compared to NaCN - D containing A2 β-CN ($p < 0.05$).

After emulsification, conformational changes in the secondary structure of proteins in the adsorbed layer of all three NaCN - Es were observed ($p < 0.05$, Fig. 3 and Table 3). The content of the structural intramolecular β-sheet elements seemed to increase in all three NaCN - Es ($p < 0.05$), specifically, an increase of 46 % in A1/A1, 36 % in A1/A2, and 29 % in A2/A2 NaCN - Es compared to NaCN - Ds (Table 3). Other significant changes to occur in the secondary structure of proteins after emulsion formation were the appearance of random coils at around 1643 cm^{-1} in A1/A2 NaCN - E and α-helices at 1662 cm^{-1} in A2/A2 NaCN - E; these peaks were not present in the dispersions (Table 3 and Fig. 1A). Additionally, the amount of side chains in all three NaCN - Es decreased sig-

Table 1
Protein composition of A1/A1, A1/A2, and A2/A2 NaCN dispersions (D) and aqueous layer of emulsions (E_{AL}) as determined by the Reversed Phase - High Performance Liquid Chromatography.

	Protein content (mg/mL)				
Sample	κ-CN	αs_2-CN	αs_1-CN	A1 β-CN	A2 β-CN
A1/A1 NaCN - D	0.86 ± 0.02^{b}	0.71 ± 0.02 ^{a}	4.18 ± 0.10^{b}	2.72 ± 0.13 ^{a}	n/d
A1/A2 NaCN - D	1.10 ± 0.20 ^{a}	0.61 ± 0.04^{b}	4.42 ± 0.22^{b}	1.74 ± 0.11^{c}	1.49 ± 0.14^{b}
A2/A2 NaCN - D	0.42 ± 0.01 ^{cd}	0.57 ± 0.02 ^{bc}	4.89 ± 0.08 ^{a}	n/d	3.22 ± 0.06 ^{a}
A1/A1 NaCN - E_{AL}	0.43 ± 0.01 ^{cd}	0.53 ± 0.00^{c}	3.58 ± 0.03^{c}	2.30 ± 0.02^{b}	n/d
A1/A2 NaCN - E_{AL}	0.56 ± 0.01^{c}	0.15 ± 0.05 ^{e}	3.82 ± 0.03^{c}	0.92 ± 0.33 ^{d}	0.97 ± 0.02 ^{d}
A2/A2 NaCN - E_{AL}	0.24 ± 0.00 ^{d}	0.32 ± 0.01 ^{d}	3.55 ± 0.01^{c}	n/d	1.07 ± 0.04^{c}

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$); n/d: not detectable.

Table 2
Physicochemical and interfacial properties of different β-CN phenotypes of NaCN dispersions and NaCN emulsions.

Sample	Dispersions		Emulsions		
	Solubility (%)	Surface hydrophobicity (%)	Emulsion activity index (m^2/g)	Emulsion stability index (h)	Adsorbed protein (mg/mL)
A1/A1 NaCN	87.29 ± 0.37^{b}	49.26 ± 2.46 ^{a}	124.51 ± 4.70^{c}	49.41 ± 2.40^{b}	1.70 ± 0.57^{b}
A1/A2 NaCN	93.78 ± 4.12 ^{a}	49.62 ± 0.29 ^{a}	132.63 ± 6.59^{b}	54.92 ± 0.96 ^{ab}	2.85 ± 0.07 ^{ab}
A2/A2 NaCN	92.88 ± 3.02 ^{a}	49.73 ± 1.56 ^{a}	137.66 ± 5.34 ^{a}	58.09 ± 0.29 ^{a}	3.76 ± 0.08 ^{a}

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$); n/d: not detectable.

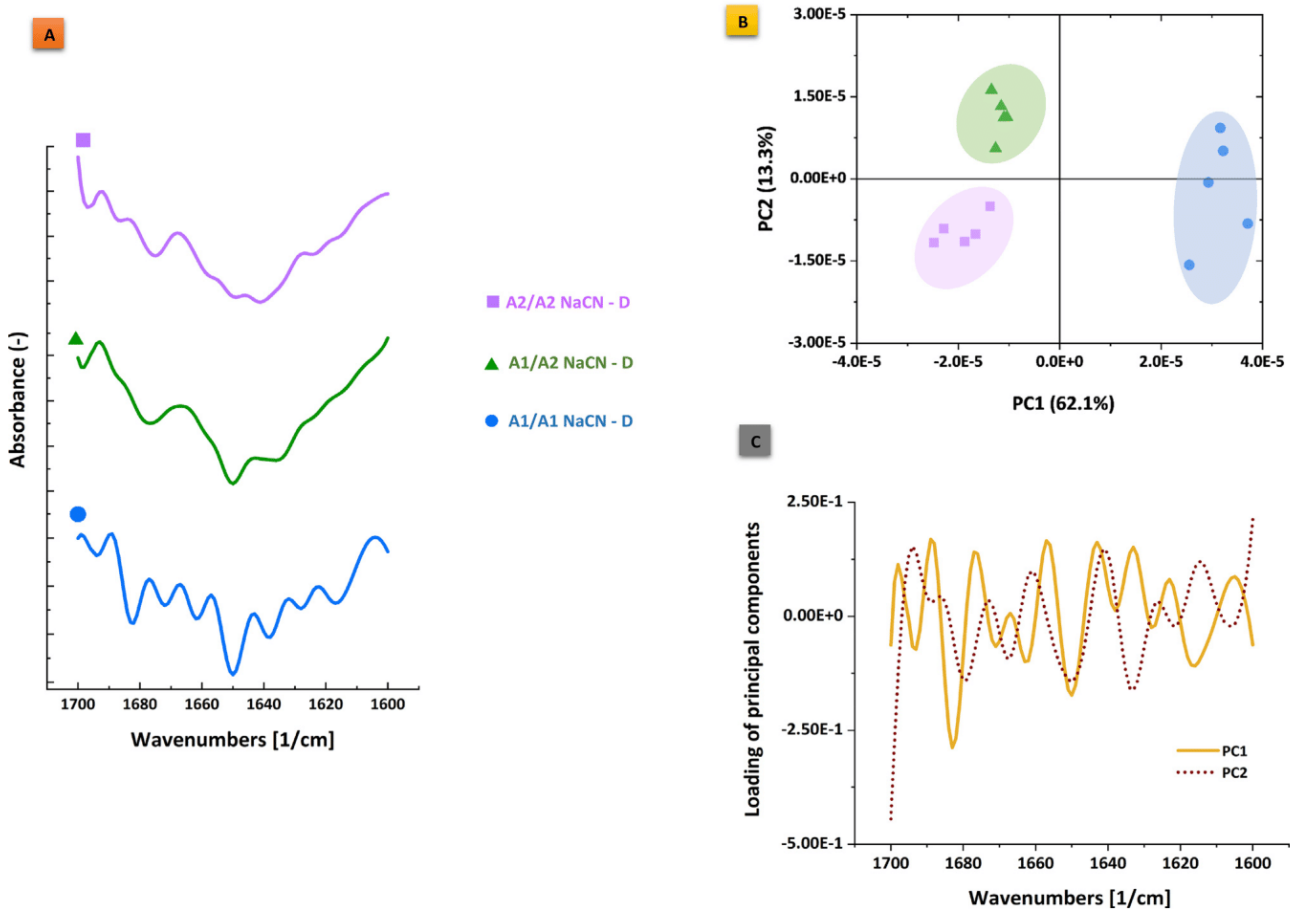

Fig. 1. A) Second derivative spectra of Amide I region of NaCN dispersions. **B)** Scatter plot of the PCA scores of FTIR spectra of NaCN dispersions (the shaded areas are a guide and their positioning is manual). **D)** The plot of the PCA loadings of FTIR spectra of NaCN dispersions.

Table 3
Total percentage areas of different secondary structures in Amide I of NaCN dispersions and adsorbed layer of NaCN emulsions carrying different β-CN phenotypes.

Band Assessment	Band frequency (cm1)	Peak area (%)					
		A1/A1	A1/A2	A2/A2	A1/A1	A1/A2	A2/A2
		NaCN dispersions			NaCN emulsions (adsorbed layer)		
Side chain	1600–1614	7.16 ± 1.06 ^{a}	8.16 ± 0.84 ^{a}	3.99 ± 1.12^{b}	0.65 ± 0.03^{c}	0.73 ± 0.01^{c}	0.99 ± 0.06^{c}
Intramolecular β-sheet	1615–1637	34.41 ± 3.89 ^{ab}	36.17 ± 8.24 ^{ab}	21.55 ± 7.62^{c}	54.00 ± 0.84 ^{a}	51.56 ± 1.67 ^{a}	28.09 ± 0.97^{b}
Random coil	1638–1645	n/d	n/d	50.13 ± 1.08 ^{a}	n/d	20.61 ± 1.38^{b}	28.00 ± 3.03^{b}
α-helix	1646–1664	29.82 ± 2.68 ^{ab}	32.82 ± 9.50 ^{a}	n/d	17.60 ± 1.60 ^{bc}	12.78 ± 1.03^{c}	22.58 ± 1.69^{b}
β-turn	1665–1681	14.06 ± 3.89 ^{ab}	15.21 ± 3.66 ^{a}	20.21 ± 1.78 ^{a}	13.20 ± 0.84 ^{ab}	6.32 ± 0.66^{c}	14.64 ± 2.57 ^{a}
Aggregated β-sheet	1682–1700	14.55 ± 2.05 ^{a}	7.64 ± 0.17^{b}	4.12 ± 0.13^{c}	14.55 ± 0.72 ^{a}	8.00 ± 0.29^{b}	5.70 ± 1.55^{c}

Mean values within a row that do not share a common superscript letter are significantly different ($p \leq 0.05$); n/d = not detectable.

nificantly ($p < 0.05$) compared to levels in their respective dispersions. Despite the emulsification process, β-turn and aggregated β-sheet conformations were significantly higher in the adsorbed layer of A2/A2 and A1/A1 NaCN - Es, shown as an extensive shoulder peak between 1683 and 1697 cm^{-1} and an intensive peak around 1670 cm^{-1}, respectively ($p < 0.05$), compared to the NaCN - E containing A1/A2 β-CNs in its structure (Fig. 3A and Table 1).

Table 2
Physicochemical and interfacial properties of different β-CN phenotypes of NaCN dispersions and NaCN emulsions.

Sample	Dispersions		Emulsions		
	Solubility (%)	Surface hydrophobicity (%)	Emulsion activity index (m^2/g)	Emulsion stability index (h)	Adsorbed protein (mg/mL)
A1/A1 NaCN	87.29 ± 0.37^{b}	49.26 ± 2.46 ^{a}	124.51 ± 4.70^{c}	49.41 ± 2.40^{b}	1.70 ± 0.57^{b}
A1/A2 NaCN	93.78 ± 4.12 ^{a}	49.62 ± 0.29 ^{a}	132.63 ± 6.59^{b}	54.92 ± 0.96 ^{ab}	2.85 ± 0.07 ^{ab}
A2/A2 NaCN	92.88 ± 3.02 ^{a}	49.73 ± 1.56 ^{a}	137.66 ± 5.34 ^{a}	58.09 ± 0.29 ^{a}	3.76 ± 0.08 ^{a}

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$); n/d: not detectable.

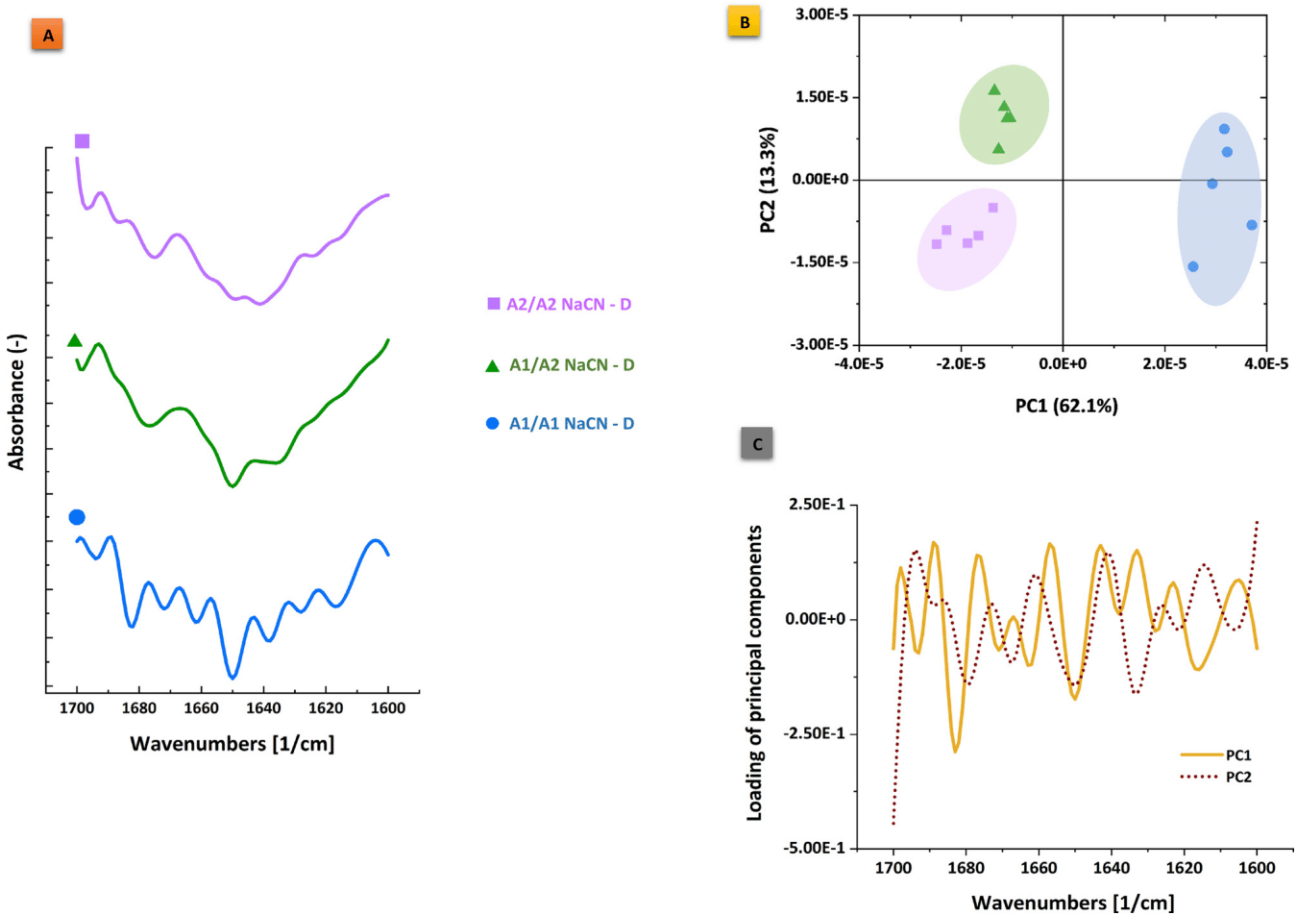

Fig. 1. A) Second derivative spectra of Amide I region of NaCN dispersions. **B)** Scatter plot of the PCA scores of FTIR spectra of NaCN dispersions (the shaded areas are a guide and their positioning is manual). **D)** The plot of the PCA loadings of FTIR spectra of NaCN dispersions.

Table 3
Total percentage areas of different secondary structures in Amide I of NaCN dispersions and adsorbed layer of NaCN emulsions carrying different β-CN phenotypes.

Band Assessment	Band frequency (cm1)	Peak area (%)					
		A1/A1	A1/A2	A2/A2	A1/A1	A1/A2	A2/A2
		NaCN dispersions			NaCN emulsions (adsorbed layer)		
Side chain	1600–1614	7.16 ± 1.06 ^{a}	8.16 ± 0.84 ^{a}	3.99 ± 1.12^{b}	0.65 ± 0.03^{c}	0.73 ± 0.01^{c}	0.99 ± 0.06^{c}
Intramolecular β-sheet	1615–1637	34.41 ± 3.89 ^{ab}	36.17 ± 8.24 ^{ab}	21.55 ± 7.62^{c}	54.00 ± 0.84 ^{a}	51.56 ± 1.67 ^{a}	28.09 ± 0.97^{b}
Random coil	1638–1645	n/d	n/d	50.13 ± 1.08 ^{a}	n/d	20.61 ± 1.38^{b}	28.00 ± 3.03^{b}
α-helix	1646–1664	29.82 ± 2.68 ^{ab}	32.82 ± 9.50 ^{a}	n/d	17.60 ± 1.60 ^{bc}	12.78 ± 1.03^{c}	22.58 ± 1.69^{b}
β-turn	1665–1681	14.06 ± 3.89 ^{ab}	15.21 ± 3.66 ^{a}	20.21 ± 1.78 ^{a}	13.20 ± 0.84 ^{ab}	6.32 ± 0.66^{c}	14.64 ± 2.57 ^{a}
Aggregated β-sheet	1682–1700	14.55 ± 2.05 ^{a}	7.64 ± 0.17^{b}	4.12 ± 0.13^{c}	14.55 ± 0.72 ^{a}	8.00 ± 0.29^{b}	5.70 ± 1.55^{c}

Mean values within a row that do not share a common superscript letter are significantly different ($p \leq 0.05$); n/d = not detectable.

nificantly ($p < 0.05$) compared to levels in their respective dispersions. Despite the emulsification process, β-turn and aggregated β-sheet conformations were significantly higher in the adsorbed layer of A2/A2 and A1/A1 NaCN - Es, shown as an extensive shoulder peak between 1683 and 1697 cm^{-1} and an intensive peak around 1670 cm^{-1}, respectively ($p < 0.05$), compared to the NaCN - E containing A1/A2 β-CNs in its structure (Fig. 3A and Table 1).

4.2.1. Chemometric analysis of combined data

Analysing the FTIR spectra manually via peak area ratiometrics is possible (Table 3) but is time intensive due to the number of peaks and variables involved. Utilising principal component analysis (PCA) to group similar spectra together accomplishes a similar overall goal while being far easier to automate and produces easily interpreted results [14,31]. Therefore, the observed structural differences resulting from the impact of various β-CN phenotypes were also evaluated by PCA analysis (Fig. 1B). The first (PC1, 62.10 %) and the second principal components (PC2, 13.30 %) accounted for 75.40 % of the variation (Fig. 1B); in the score plot along PC1, the samples that carried A2 β-CN proteoform were visually separated from the grouping of A1/A1 NaCN - D (Fig. 1C). In contrast, A1/A1 NaCN - D was assigned to both positive and negative axes of PC2, which might indicate the possibilities of some conformational similarities with NaCN - Ds possessing either A1/A2 (positive axis) or A2/A2 β-CNs (negative axis) along PC2 (Fig. 1C).The loadings on both PC1 and PC2 included an intense positive peaks at 1700 and 1682 cm^{-1} referred to as aggregated β-sheets (A1/A1 NaCN - D), a negative shoulder peak between 1645 and 1638 cm^{-1} (random coils, A2/A2 NaCN - D), and a broad negative peak at ≈ 1659 cm^{-1} (α-helical structures driven by A1/A2 NaCN - D).

Additionally, the PCA scores of PC1 - PC2 visualises the separation of the three types of emulsions possessing either A1/A1, A1/A2, or A2/A2 NaCNs, based on the FTIR secondary spectra information (Fig. 3B). A multivariate analysis demonstrated that for proteins of NaCN - Es, most of the variance of the data (93.00 %) was explained by the PC1, which clearly separated the scores based on the β-CN proteoform. This effect was associated with positive peaks at 1681, 1654, and 1645 cm^{-1} related to A2/A2 NaCN - E and negative peaks at 1690, 1660 and 1640 cm^{-1} associated to A1/A1 and A1/A2 NaCN - Es (Fig. 3B). The PC2 explained only 2.70 % of the variance of the data and it separated the scores of A1/A1 and A1/A2 NaCN - Es, whereas, A2/A2 NaCN - E was clustered in both positive and negative axis along PC2, revealing its similarities to A1/A1 and A1/A2 NaCN - Es. According to the loading plot (Fig. 3C), PC2 included positive peaks at 1632/1619 (A1/A2 NaCN - E), 1650 (A1/A2 and A2/A2 NaCN - Es), and 1698 (A1/A2 NaCN - E) cm^{-1} and negative peaks at 1690 (A1/A1 NaCN - E), 1665 and 1645 cm^{-1} (both homozygous emulsions) (Fig. 3C).

4.3. Determining the structure of NaCN dispersions and emulsions by ^{1}H NMR

To determine the protein structure of NaCN dispersions and emulsions carrying diverse β-CN phenotypes, characteristic signals for each component from their 1D ^{1}H NMR chemical shifts (δ) spectra were identified. Notably, only the specific resonances in the NMR spectra, which could provide a clear differentiation between the protein structure in dispersions and emulsions of all three NaCNs, were further elaborated. The average ^{1}H NMR spectra of A1/A1, A1/A2, and A2/A2 NaCN dispersions (NaCN - D) and NaCN - emulsions (NaCN - E) are shown in Figs. 2 and 4, respectively. The NaCN - Ds containing A2 β-CN in their structures (especially A2/A2 NaCN - D) were characterised with an intense and strong signal at 1.13 ppm. Namely, the lower peak intensity caused the lower percentage area in the methyl region of A1/A1 NaCN - D (44.23 %) compared to A1/A2 (46.64 %) and A2/A2 (47.68 %) NaCN - Ds ($p < 0.05$, Table 4). Interestingly, an absence of a peak at 1.78 ppm was observed only for A1/A1 NaCN - D opposed to the other genetic variants. Despite this difference, the aliphatic region of all three NaCN - Ds was around 30 % ($p > 0.05$). In the Amide region (3.84–3.70 ppm), an up-field shielding of the peaks was observed ≈ 0.02 ppm mainly related to the alkyl groups of proteins or the α-protons (H^{α}) of amino acids. Moreover, in the Amide region the peaks at 3.84, 3.82, and 3.77 only in A1/A1 NaCN - D appeared as singlets, as opposed to A1/A2 and A2/A2 NaCN - Ds, which peaks in the same interval, were doublets. Another difference between the genetic variants of NaCN - Ds was in the amino region. This region was approximately 12 % and 41 % more present in A1/A1 than in A1/A2 and A2/A2 NaCN - Ds, respectively ($p < 0.05$); the peak at ≈ 6.70 ppm was doublet in A1/A1 NaCN - D, while on the contrary, the same peaks in both NaCN - Ds containing Pro^{67} were present as singlets (Fig. 2A). The chemical shifts from the up-fieled to the downfield region of the spectrum revealed the appearance of doublet peak for A1/A1 and A1/A2 NaCN - Ds (8.03 ppm), yet on the other hand, for A2/A2 NaCN - D, at the same region, the peak at 8.03 ppm was presented as singlet (Fig. 2A).

After emulsification of NaCN - Ds chemical shifts and conformational rearrangements in proteins were observed (Fig. 4A). These structural changes can be result of multiple contributions, such as torsion angles coming back from backbone and side chains, electric fields, hydrogen bonding, ring vibrations, steric repulsions, to name a few [45]. In particular, for A1/A1 NaCN - E, the content of both, the methyl and amino regions appeared greatly altered ($p < 0.05$); hence, the interval between 1.5 and 0 ppm increased by ≈ 50 %, whilst the amino region decreased from 12.01 to 0.28 % (Table 4). In the aliphatic region, slight variations were observed; the peaks at around 2.60 ppm in A1/A2 and A2/A2 NaCN - Es seemed to appear as a sextet in comparison with that in A1/A1 NaCN - E, which peak appeared as triplet (Fig. 4A). Noticeably, the characteristic Amide signals in the interval from 3.70 to 3.30 ppm resulted in similar area percentage values for A1/A2 (20 %) and A2/A2 (15 %) NaCN - Es ($p > 0.05$, Table 4A). Hence, this invariant spectral region represented the similarity of the structural features common to the NaCN - Es carrying Pro^{67} in their structure; these peaks were completely absent in A1/A1 NaCN - E (Fig. 4A). Another similarity between the NaCN - Es possessing A2 β-CN was the peaks' assignment between 7.60 and 5.70 ppm ($p > 0.05$); these peaks were not observed in A1/A1 NaCN - E. In this regard, singlets at both 7.55 and 6.90 ppm, sextets at 6.20 ppm and doublets at around 5.70 ppm for A1/A2 and A2/A2 NaCN - Es specified a significantly higher integrated amino region ($p < 0.05$) compared to the same region of A1/A1 NaCN - E (Table 4).

4.3.1. Discrimination of the structure of dispersions and emulsions

The PCA analysis of the NMR conformational fingerprints of all NaCN dispersions and emulsions was performed to explore possible patterns and trends of clustering. From the PCA overview score plot in Fig. 2B, all NaCN dispersions were presented at ≈ 99 % confidence interval. With only two PCs, PC1 and PC2 explained 95.20 % and 3.60 % of variation, respectively, and distinct clusters of A1/A1, A1/A2, and A2/A2 NaCN - Ds were observed. A clear separation was noticed in A1/A1 NaCN - D positioned in the region of negative PC1 values, whereas the A1/A2 and A2/A2 NaCN - Ds appeared nearby along both positive PC1 and PC2 values. Further, the difference in peak intensity between NaCN - Ds with different β-CN phenotypes was related to different structural orientation of the polypeptide chain in proteins particularly between A1 and A2 β-CNs. The difference in the amino region was also confirmed by PCA loading plot (Fig. 2C), where one positive loading was presented for both samples containing A2 β-CN (5.70 ppm) and one negative loading for A1/A1 NaCN - D (6.00 ppm). Fig. 4A shows the PCA scores plot data of the emulsions which together explained approximately 90.00 % of the total variance, being 86.60 % by PC1 and 4.30 % by PC2. A clear separation between the samples was obtained with significant differences between the homozygous A1/A1 NaCN - E and the NaCN - Es with A2 β-CN in their structures. The A1/A2 and A2/A2 NaCN - Es were clustered with positive and negative scores along the first and second component, whereas A1/A1 NaCN - E

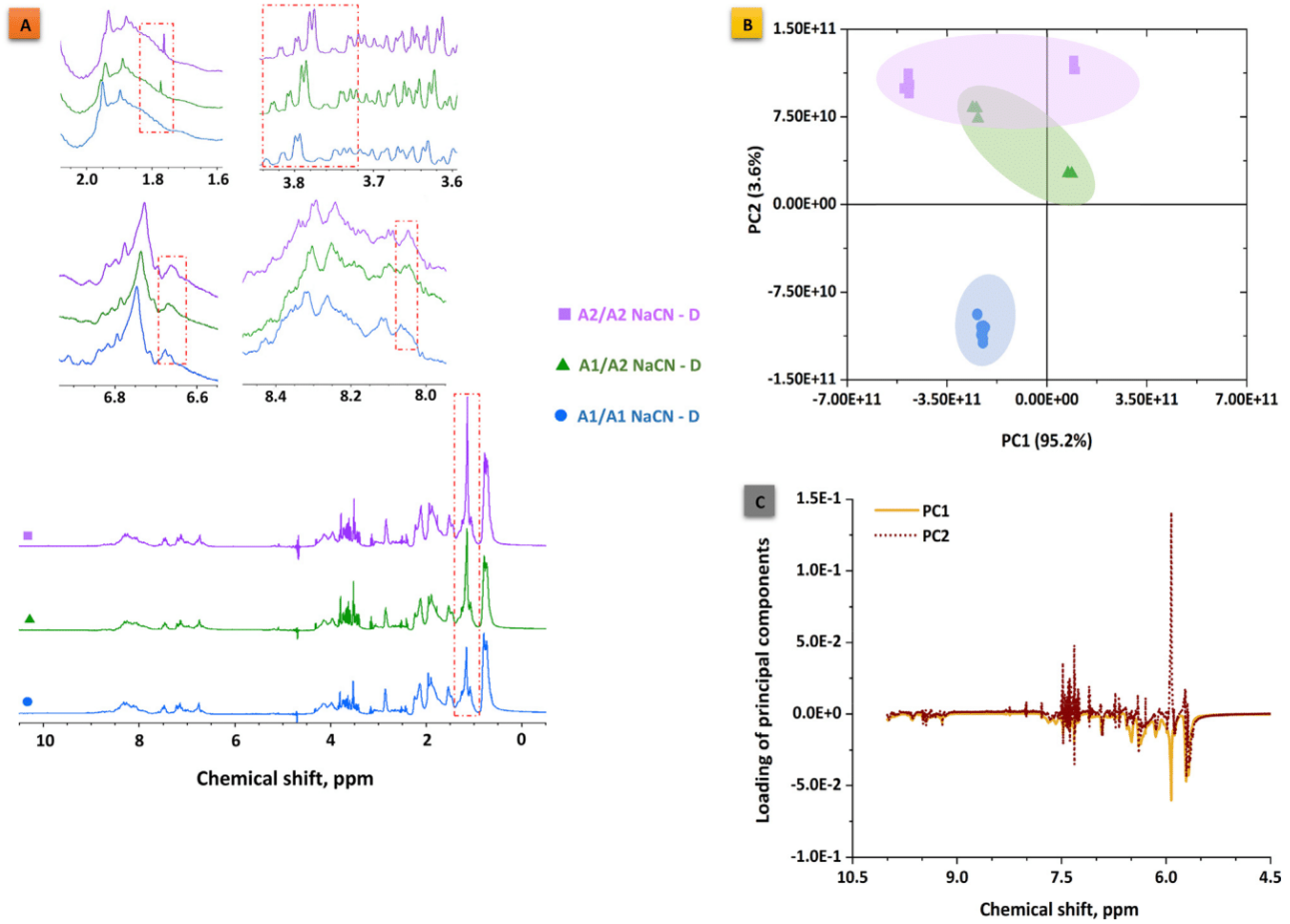

Fig. 2. A) ^{1}H NMR spectra of NaCN dispersions. **B)** Scatter plot of the PCA scores of ^{1}H NMR spectra of NaCN dispersions (the shaded areas are a guide and their positioning is manual). **C)** The plot of the PCA loadings of ^{1}H NMR spectra of NaCN dispersions.

was shown with negative scores along PC1 and both positive and negative scores along PC2. Additionally, in the amino region, broad and intensive positive and negative loadings on PC1 and PC2 were observed around 7.40 and 5.90 ppm for A1/A2 and A2/A2 NaCN - Es; these results were complementary to the data in Table 4 which showed a significantly higher parentage area of the aforementioned NaCN - Es compared to the quantified amino interval of A1/A1 NaCN - E ($p < 0.05$, Table 4).

5. Discussion

The manufacturing characteristics and numerous applications of NaCNs have been well examined over the years for their functional properties [22,42], but relating these properties to specific genetic variants such as A1/A1, A1/A2, and A2/A2 β-CN have received far less attention. Recently, Hemar et al. [32] reported no noticeable differences between the physicochemical and interfacial properties of NaCN - Ds obtained from A1/A2 and A2/A2 milks. Namely, the authors highlighted that even though there is a structural difference at position 67 in the polypeptide chains of A1 and A2 β-CNs, the variation was not large enough to affect the behaviour of NaCN - Ds. In this regard, small angle x-ray and dynamic light scatterings showed that the internal structure of A1/A2 and A2/A2 NaCN - Ds was not homogeneous; both types of NaCN - Ds formed similar monomodal particle distributions and a similar protein layer at the surface of the latex particles [32].

On the contrary, in the current study, a substantial difference in the protein conformational states among all three types of NaCN - Ds and their primary CN particles influencing the NaCN - Es were observed. Namely, the NaCN samples (dispersions and adsorbed layer of emulsions) containing His^{67} possessed greater levels of intramolecular and aggregated β-sheets than those of the A2/A2 NaCN samples (Table 3). In the past, Huppertz [36] explained that 20–35 % of these conformations found in the CN micelle were mainly related to the structural orientation of κ-CN, with both A1/A1 and A1/A2 NaCN samples in the current study containing higher levels of κ-CN (Table 1). The increase of β-sheet structures indicated the exposure of hydrophobic regions, leading to an exposed hydrophobicity of proteins [56]. Raynes, Day, Augustin, and Carver [57] revealed that A2/A2 β-CN had less exposed surface hydrophobicity as opposed to A1/A1 β-CN. On the other hand, the primary structure of bovine A2/A2 β-CN contains more hydrophobic amino acids, such as Pro, thus leading to a possibly higher hydrophobicity of the protein [13]. However, that was not the case in the present study since all three phenotypes of NaCN - Ds had indistinguishable surface hydrophobicity (Table 2). Lucey and Horne [41] described that the surface hydrophobicity refers to the surface of submicellar CN particles in NaCN that would always depend on protein folding and/or protein interactions. This explains the difference of the surface hydrophobicity between the current results of A1/A1, A1/A2, and A2/A2 NaCN - Ds and the results obtained by Raynes et al. [57] where A1 and A2 β-CNs were examined as individual proteins. For example, during RP-HPLC determination, reducing conditions and a chaotropic agent led to complete unfolding and dissociation of the proteins to monomers with the entire amino acid chain exposed. Therefore,

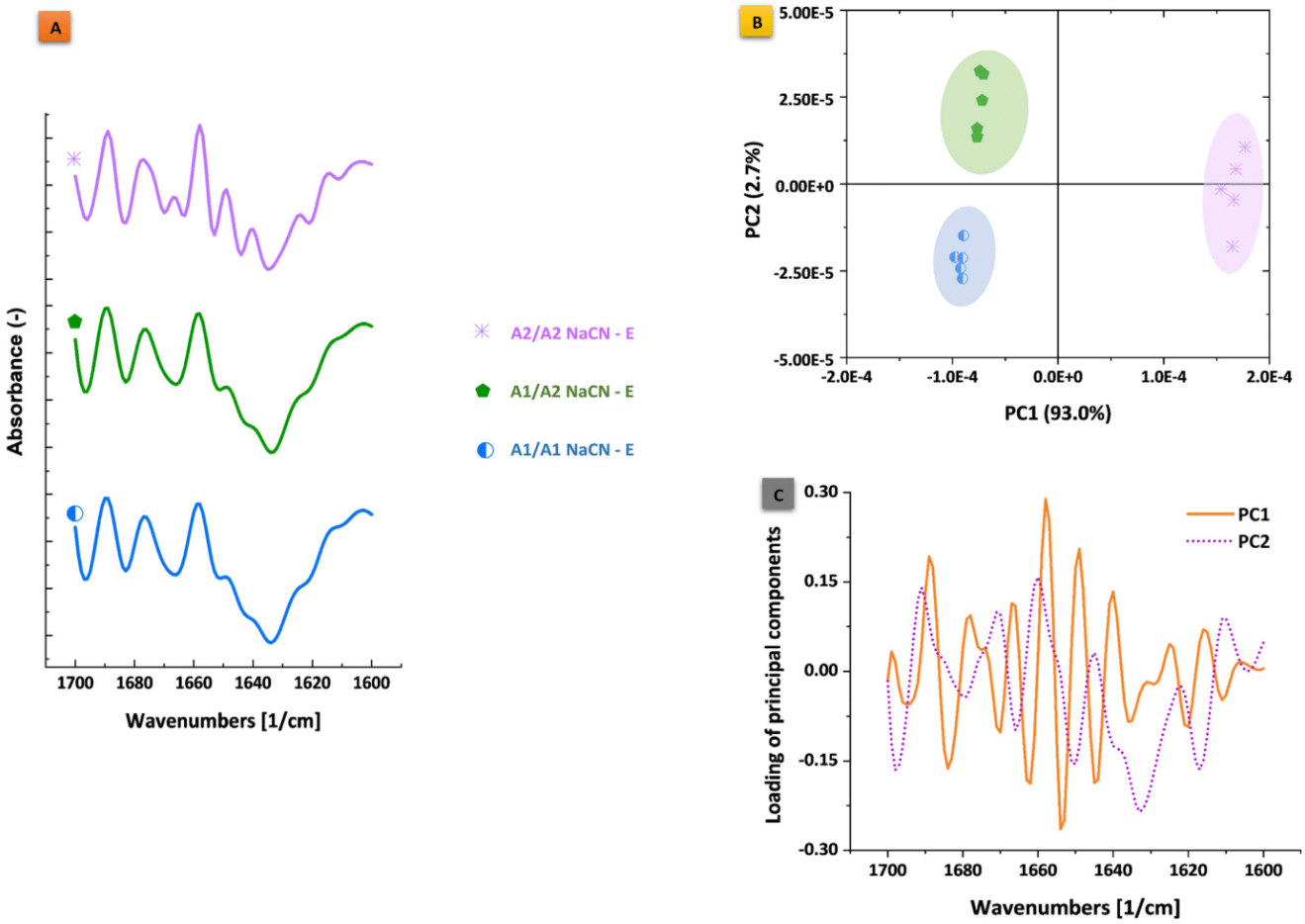

Fig. 3. A) Second derivative spectra of Amide I region of the adsorbed layer of NaCN emulsions. **B)** Scatter plot of the PCA scores of FTIR spectra of the adsorbed layer of NaCN emulsions (the shaded areas are a guide and their positioning is manual). **D)** The plot of the PCA loadings of FTIR spectra of the adsorbed layer of NaCN emulsions.

the exposed protein surface differs compared to that assessed using ANS probe leading to variation in protein hydrophobicity [13,62].

Notably, the genetic variant was found to have a significant effect on the existence of the random coil conformations only in A2/A2 NaCN - D, possibly driven by changes in β-sheets ($p < 0.05$, Table 3), indicating likely loss of the proteins' secondary structure [16,52]. Previously, Fox, Uniacke-Lowe, McSweeney, and O'Mahony [28] assigned the random coil conformational motif to β-CN with an estimated secondary structure between 23 and 70 %. Since A2/A2 NaCN - D had more β-CN compared to the NaCN - Ds carrying A1 β-CN, it is expected that A2 β-CN was the main contributor to the development of random coils in A2/A2 NaCN - D (Table 3). The random coils were classified as short polyproline II helixes (PPII) and were particularly found in A2/A2 untreated and heat treated milks and A2/A2 β-CN [14,16,24]. Further, the presence of Pro^{67} in A2/A2 NaCN - D favours the formation of PPII helixes and appears to play a crucial part in the CN micelle formation and protein–protein binding through PPII mediated interactions [27]. The existence of this amino acid in the hydrophobic part of β-CN may lead to a change in the conformational state of the adsorbed part of β-CN and yet influence the emulsion forming and stabilising characteristics of the NaCN - Es [17]. Namely, a prerequisite of proteins to form emulsions is their adsorption onto the oil/water interfaces [17]. Interestingly, upon emulsification the random coils appeared in A1/A2 NaCN - E and were not significantly lower than that in A2/A2 NaCN - E (Table 3), considering the fact that the secondary structure of protein can change upon adsorption onto an interface [17]. Since in the adsorbed layer of the A1/A2 NaCN - E the presence of the A1/A2 β-CN was higher compared to other CNs in the adsorbed layer of the same emulsion (Table 1, $p < 0.05$), it can be postulated that this protein is the driver of the random coils formation. Furthermore, the appearance of peaks between 5.8 and 5.5 ppm in the NMR spectra were found to be consistent with a random coil structure [40]; the well resolved αCH signals in the adsorbed layer of A1/A2 and A2/A2 NaCN - Es are supposed to be related to the Pro residues as part of β-CN, which apparently initiated the formation of random coil/PPII structures. Additionally, the intense signals observed at around 3.70 ppm only in adsorbed layer of A1/A2 and A2/A2 NaCN - Es might be initiated by the δCH_2 protons of Pro that refers to hydrogen atoms of the residues in a peptide with a random coil conformational motif [63].

The induction of ordered structures in NaCN - Ds was more obvious for A1/A1 and A1/A2 compared to that of the A2/A2 β-CN phenotypes. Nevertheless, in the adsorbed state of the emulsions noticeable changes in the FTIR spectra were induced. This establishes that the secondary structure of proteins differs in the aqueous and adsorbed states. Particularly, the presence of α-helixes (1650 cm^{-1}) in both NaCN - Ds containing His^{67} (~30 % in A1/A1 NaCN - D and ~ 33 % in A1/A2 NaCN - D) might be due to the content of both αs_2- and β-CNs, since Huppertz [36] revealed that both proteins contain the highest amount of α-helical structures, accounted for 54 and 25 %, respectively. In the study of Ghosh, Tucker, and Gai [30], the spectra clearly showed the exact position and line shape of the Amide I vibrational transition of

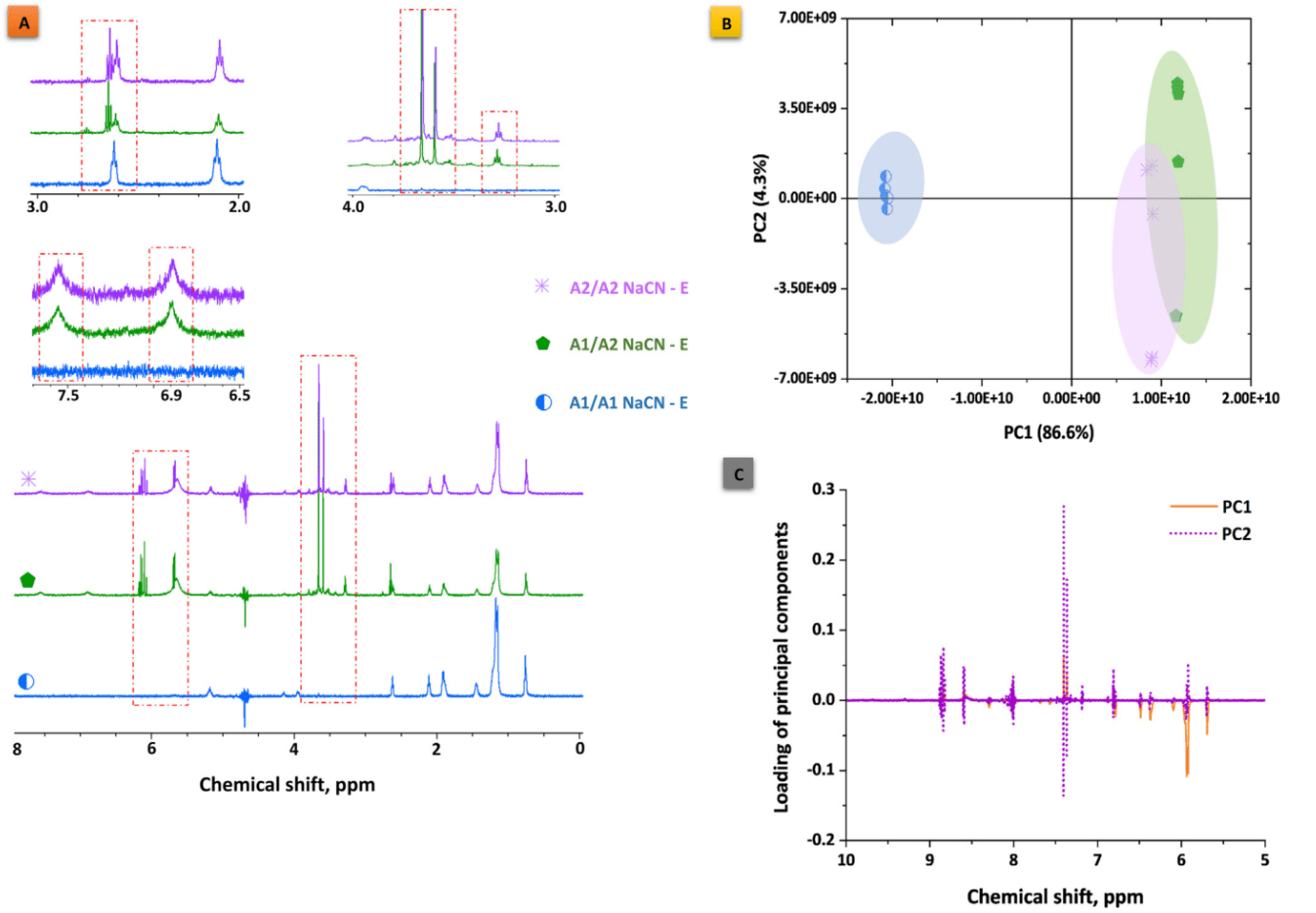

Fig. 4. A) ^{1}H NMR spectra of the adsorbed layer of NaCN emulsions. **B)** Scatter plot of the PCA scores of ^{1}H NMR spectra of the adsorbed layer of NaCN emulsions (the shaded areas are a guide and their positioning is manual). **C)** The plot of the PCA loadings of ^{1}H NMR spectra of the adsorbed layer of NaCN emulsions.

Table 4
Total percentage areas in different regions of NaCN dispersions and adsorbed layer of NaCN emulsions carrying different β-CN phenotypes.

Region/Integral	Band frequency (ppm)	Peak area (%)					
		A1/A1	A1/A2	A2/A2	A1/A1	A1/A2	A2/A2
		NaCN dispersions			NaCN emulsions (adsorbed layer)		
Methyl	1.5–0.0	44.23 ± 0.66^{b}	46.64 ± 1.62 ^{ab}	47.68 ± 2.66^{b}	73.51 ± 0.85 ^{a}	32.02 ± 0.22^{c}	43.83 ± 0.17^{b}
Aliphatic	3.5–1.5	33.85 ± 1.94 ^{a}	33.12 ± 1.84 ^{a}	29.85 ± 1.84 ^{ab}	22.06 ± 0.52 ^{a}	16.28 ± 0.29^{c}	17.69 ± 0.79^{c}
Amide	5.5–3.5	9.91 ± 1.00 ^{bc}	9.64 ± 1.25 ^{bc}	14.51 ± 0.30 ^{ab}	4.17 ± 0.33^{c}	19.88 ± 0.31 ^{a}	15.29 ± 0.52 ^{ab}
Amino	9.0–5.5	12.01 ± 1.59 ^{bc}	10.60 ± 0.88 ^{bc}	7.96 ± 2.06^{c}	0.28 ± 0.09 ^{d}	31.81 ± 0.63 ^{a}	23.18 ± 1.33^{b}

Mean values within a row that do not share a common superscript letter are significantly different ($p \leq 0.05$); n/d = not detectable.

His. Namely, the protonation of the imidazole ring of His was found in the range between 1650 and 1642 cm^{-1} [30]. In the current study, these results might be related to the presence of His also in the aromatic region of the ^{1}H NMR spectrum. Hence, the C_4 and C_2 - protons of His residues are found to be located and subsequently resonated from 8.0 to 6.0 ppm and around 8.1 ppm, respectively, which also indicates high presence of α-helical structures [4,21,48]. Therefore, the spectral changes observed in the current study are possibly connected to the conformational changes of His side chains. As expected, A2/A2 NaCN - D was presented with an absence of α-helical structures, likely due to the presence of the additional Pro^{67} in A2/A2 β-CN polypeptide chain. In fact, this imino acid was considered as a potent breaker of α-helixes [57]. In this context, Huang and Nau [35] postulated that the attenuation of the helical structures might be initiated due to the high rigidity imposed by the Pro cyclic structure which prevents rotations about the N-C$^{\alpha}$ bond. Interestingly, a recent study correlated the decreased amount of α-helixes to the enhanced solubility of proteins [61]. These conformations were found to display a tight structure with no cavities, thus leading to a lower protein solubility and subsequently may be detrimental to the specific conformational change that is required for the emulsifying properties of the proteins [61], as in A1/A1 and A1/A2 NaCN - Ds in the present study. Good solubility allows proteins to migrate to the oil/water interface quickly, and have the flexibility to rearrange at the interfacial film [18]. It appears that the A2 β-CN in A2/A2 NaCN - Es was able to more rapidly reach the oil droplet surface and consequently was more efficient as emulsion forming agent, similarly to the study of Darewicz and Dziuba [17] on isolated A1 and A2 β-CNs.

The modifications in the FTIR spectra of the adsorbed layer of the NaCN - Es provides direct evidence that adsorption to oil/water

interfaces induces a more ordered secondary structure in A2/A2 NaCN - E with a significant increase and appearance of α-helixes accompanied by corresponding decreases in random coil structures (Table 2). Here again, the content of A2/A2 β-CN in the adsorbed layer of the counterpart emulsion was higher compared to the other CNs (Table 1), which explains the governing nature of this protein and the introduction of α-helical structural state [36]. This is in agreement with earlier results obtained by using the far-UV synchrotron radiation circular dichroism for β-CNs in dispersions and emulsions, where the secondary structure calculations confirmed that there was a significant increase in the α-helical content (increasing from 5.5 % in dispersion to 21.5 % in tricaprin/water and hexadecane/water interface), which led to decrease in coil motifs [64]. Similarly, Caessens, De Jongh, Norde, and Gruppen [6] compared the conformation of β-CN in dispersion with that at teflon/water interface and showed a 20 % increase in the amount of α-helixes and a decreased amount of random coils of β-CN upon adsorption, which is in agreement with the current results. The signals at around 6.4 to 6.3 ppm in the ^{1}H NMR spectra that sharpens upon emulsification probably arose from the proton of the γ-methylene group of the Pro residues. Consequently, the Pro ring may be restricted in its molecular freedom possibly by incorporation in a partially ordered structures as in the present study with the appearance of α-helixes and attenuation of random coils (Fig. 3A) [40]. There is a growing evidence from a number of studies of other proteins, which suggested that there is a preference for the formation of α-helical conformations upon adsorption to the adsorbed layer of emulsions and their importance in emulsion stabilisation [6,9,18,29,64,65]. The preference for this structural motif may be governed by the need to form amphipathic moieties, similar to the membrane proteins that also show a high propensity for α-helixes [44,64], which might be the case with the A2/A2 β-CN in the adsorbed layer of A2/A2 NaCN - E.

Another difference between the three NaCN phenotypes either in dispersions or in the adsorbed layer of emulsions was in β-turns. The A2/A2 NaCN - D contained approximately 10–30 % higher amount of these conformations compared to A1/A1 and A1/A2 NaCN - D; A2/A2 NaCN - Es followed the same trend with approximately 80 % greater amount of β-turns as opposed to that of the NaCN - Es carrying A1 β-CN (Table 3). The αs_1- and β-CNs were more present in A2/A2 NaCN - D and in the adsorbed layer of A2/A2 NaCN - E, therefore the involvement of these two CNs in the turn conformational state of the proteins corroborated with the previously reported results [36]. The presence of Pro^{67} favours formation of β-turns, presumably due to their cyclic structures, thus leading to a greater amount of these conformations in the A2/A2 samples as they contain more Pro residues [13]. The presence of β-turns in the polypeptide chains of proteins was also connected to the intense peak around 1.13 ppm (Fig. 2A) in A2/A2 NaCN - D; this intense peak might resulted from cis/trans isomerism of X-Pro bond and can change in the proximity between alkyl fragments (CH_2 - CH_3) of amino acids and H^{α} protons of Pro [25]. Moreover, the peak at around 1.8 ppm refers to the side chains of some amino acids, such as isoleucine (Ile), valine (Val), and leucine (Leu) [19] that are known to increase the proteins turn motifs (Tables 3 and 4). Accordingly, similar to FTIR spectra, ^{1}H NMR spectrum confirmed the presence of more turns in A2/A2 NaCN - D. This can relate to the fact that protons of these amino acids may be involved into hydrophobic bonds with other hydrogens important for numerous functionalities in the CN micelle indicating to different structural organisation compared to the other two types of NaCN - Ds [8,13]. In addition to the prior amino acids, the aromatic rings of phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) residues that are found in the amino region between 8.0 and 6.0 ppm, are important in the formation of amorphous aggregates by mixtures of CNs and the backbone-to-backbone interactions for self-association of individual CNs (Fig. 2A and 4A). These amino acids are some of the main residues of the Pro-glutamine rich (P,Q-rich) sequences [33]. Nevertheless, in this case, since it is known that A2/A2 β-CN contains an additional Pro in its structure (the key residue within the central P,Q-rich region of β-CN) in comparison with the other β-CN phenotypes, this phenomena might affect the interactions and the secondary structures of the NaCN - Ds and Es. One would expect that Pro is not the only or the predominant driving force for the structural variations of the samples, the hydrophobic and hydrogen bonding, electrostatic interactions, as well the other CNs and their genetic variants probably play an equally critical role in this process.

6. Conclusion

The current study was designed to fill the knowledge gaps surrounding the structure of NaCN dispersions and emulsions in a complex and realistic dairy system that arise from selection of either A1/A1, A1/A2, or A2/A2 milk samples. Using a combination of FTIR and ^{1}H NMR techniques for studying the structure of sodium caseinates allowed for a number of new insights, including the increased content of random/PPII structures in A2/A2 NaCN - D and α-helixes and β-sheets in A1/A1 and A1/A2 NaCN - Ds. The NaCN - Ds carrying A1 β-CN led to lower solubility of their counterpart NaCNs and therefore decreased emulsifying properties compared to A2/A2 NaCN. In this regard, A2/A2 NaCN underwent a structure reordering with a significant increase in the protein's α-helical content and a corresponding decrease in the proportion of random coils after it was adsorbed to the emulsion interface. From our results it is clear that the structure of NaCNs and the properties of NaCN emulsions were strongly affected not only by hydrophobicity and solubility of the proteins, but also by the presence of A1/A1, A1/A2, or A2/A2 β-CNs and their different conformational flexibility. Due to its technical usefulness, NaCN is widely used as ingredients in a wide variety of food and non-food applications. In recent years, their supramolecular structure has become an important factor in better understanding their use in value-added products, particularly in sports and nutrition, where high protein products, including beverages and protein bars require physicochemical stability for a relatively long time [3,49]. As it appears from the current results, it is important to understand implications various CN phenotypes may have on techno-functionality of NaCN. For example, A2/A2 NaCN seems that it may be superior in terms of emulsification activity and stability in comparison to A1/A1 or A1/A2 NaCNs.

CRediT authorship contribution statement

Davor Daniloski: Methodology, Formal analysis, Investigation. **Davor Daniloski** conceived the study and research question; designed and wrote the original draft, conceptualised, reviewed, edited the manuscript, designed the tables and the figures. **Todor Vasiljevic** and **Noel A. McCarthy** provided critical feedback and analysis, secured funding, reviewed and edited the manuscript and supervised the study. **Martin J. Auldist** gave critical feedback and analysis, reviewed and edited the manuscript and supplied the milk samples. All authors have contributed to the manuscript and reviewed the final version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Main findings: Part I. Chapter 8.

Rheological and structural properties of acid-induced milk gels as a function of β-casein phenotype

- Effects of acidification on milks with various β-caseins were studied
- Gels carrying A1 β-casein possessed greater water retention and lower permeability
- Increased β-sheets in gels carrying A1 β-casein related to higher storage modulus
- A2/A2 gel possessed a high amount of polyproline II structures
- Gels prepared of A1/A1 and A1/A2 milks possessed a denser microstructure

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Davor Daniloski	83	Designed and wrote the original draft, conceptualised, reviewed, edited, revised the manuscript, designed the experiments, the tables and the figures, and data analysis	[redacted]	17/03/2024
Noel A. McCarthy	5	Concept development, manuscript editing, revision and supervision		17/03/2024
Inge Gazi	5	Concept development, manuscript editing, revision and data analysis		17/03/2024
Todor Vasiljevic	7	Concept development, experimental design, manuscript editing, revision, submission and supervision		17/03/2024

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Rheological and structural properties of acid-induced milk gels as a function of β-casein phenotype

Davor Daniloski [a,b], Noel A. McCarthy [b], Inge Gazi [c,d], Todor Vasiljevic [a,*]

[a] Victoria University, Advanced Food Systems Research Unit, Institute for Sustainable Industries and Liveable Cities and College of Health and Biomedicine, Melbourne, Victoria, 8001, Australia
[b] Teagasc Food Research Centre, Food Chemistry and Technology Department, Moorepark, Fermoy, P61 C996, Cork, Ireland
[c] Utrecht University, Biomolecular Mass Spectrometry and Proteomics, Bijvoet Centre for Biomolecular Research, Utrecht Institute for Pharmaceutical Sciences, Utrecht, the Netherlands
[d] Netherlands Proteomics Centre, Utrecht, the Netherlands

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ABSTRACT

This study aimed to investigate if differences in β-casein polymorphic structure would affect the acid-induced (glucono-δ-lactone) gelation behaviour of corresponding skim milks. Gels obtained from skim milk containing A2/A2 β-casein had significantly lower elastic modulus, water holding capacity, and gel permeability compared to A1/A1 and A1/A2 gels. Microscopy images also showed a denser microstructure and smaller pore size in acid-induced gels prepared with A1/A1 and A1/A2 milks compared to A2/A2 milk. A number of reasons may account for these differences in gelation, specifically, gels with A1 β-casein contained greater amounts of α-helixes and aggregated β-sheets in their secondary structure compared to A2/A2 gel that was comprised mainly of random coils or polyproline II helixes. In addition, compositional differences such as greater total and micellar calcium and higher levels of total κ-casein existed in A1/A1 and A1/A2 milks compared to A2/A2 milk and may have contributed to the difference in gelation and gel structure. Although the role of β-CN phenotype on skim milk gelation may need further investigation, findings from this study clearly indicate that A2/A2 milk was associated with poor acid gelation properties.

1. Introduction

Proteins of bovine milk consist of two major families, namely caseins (CNs) and whey proteins (WPs). Caseins (αs_1-, αs_2-, β-, and κ-CNs) are phosphoproteins and in combination with appreciable quantities of micellar calcium phosphate (MCP) nanoclusters, are synthesised and assembled in form of colloidal particles known as CN micelles (Bijl, Huppertz, van Valenberg, & Holt, 2019; Horne, 2020). Particularly, αs_1-, αs_2-, and β-CNs are located primarily within the interior of the CN micelle, whereas κ-CN is mostly exposed towards the outside, forming a polyelectrolyte layer around the particles that is crucial for the colloidal stability of the micelle (De Kruif, Huppertz, Urban, & Petukhov, 2012; Huppertz et al., 2017). CNs play a major role during the gelation process of fermented dairy products (Lucey, 2020). Acidification of milk leads to destabilisation of the CN micelles at ~ pH 4.6 (isoelectric point of CN), resulting in protein-protein bindings established during the flocculation process, thus in conjunction with main attractive forces it controls the gelation of the milk (Lucey, 2020). Therefore, with the addition of a cyclic ester that hydrolyses when added to water to give the acidic species, such as glucono-δ-lactone (GDL) to milk, protons neutralise the repulsive negative charges on the κ-CN surface collapsing the micelle hairy layer. Simultaneously, solubilisation of MCP and aggregation of the CNs *via* van der Waals, electrostatic and hydrophobic interactions take place, leading to gel formation (Grygorczyk, Alexander, & Corredig, 2013; Lucey, Wilbanks, & Horne, 2022).

Beta-casein (β-CN), an intrinsically disordered protein with a strong amphipathic nature, consists of both a short hydrophilic *N*-terminal domain and hydrophobic *C*-terminal tail (McCarthy, Kelly, O'Mahony, & Fenelon, 2013), and represents ~ 40% of the total CN in bovine milk. It is assumed that it plays a key role in the stabilisation and conformation of CN micelles due to its ability to act as a surfactant and possess chaperone-like activity (Nguyen, Schwendel, Harland, & Day, 2018; Raynes, Day, Augustin, & Carver, 2015). Previously, a number of studies investigated the influence of β-CN concentration on the acid and rennet

* Corresponding author.
E-mail address: todor.vasiljevic@vu.edu.au (T. Vasiljevic).

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gelation behaviour of bovine milk (Holland, Corredig, & Alexander, 2011; Seibel, Molitor, & Lucey, 2015; Zhang et al., 2018). Namely, the association of this protein and its different genetic variants with milk and milk product functionality, including improved or impaired curd consistency and milk gelation properties (depending on which phenotype of β-CN was present in the milk), has garnered the interest of consumers, the scientific community, and dairy industry stakeholders (Nguyen et al., 2018; Poulsen et al., 2013; Poulsen, Glantz, Rosengaard, Paulsson, & Larsen, 2017). Thus, a total of fifteen β-CN genetic variants (A1, A2, A3, B, C, D, E, F, G, H1, H2, I, J, K and L) were identified in the *Bos* genus with notable differences in occurrence and frequency of the variants among species and breeds (Daniloski, McCarthy, & Vasiljevic, 2021). Within β-CN, the genetic variants A1, A2, and B are widely distributed in all breeds, with A2 β-CN showing the highest frequency. Although to a lower extent than the previous three, variants A3, C, and I are also rather common in the *Bos* genus. The remaining genetic variants are rare and described in particular breeds only (Lisson, Lochnit, & Erhardt, 2013; Schettini, Lambert, da Silva Souza, Costa, & de Camargo, 2020). While a number of variants have been established, the most common are A1 and A2 β-CN genetic variants. The main difference between these two genetic variants is the single nucleotide polymorphism in the CSN2 gene which leads to an amino acid mutation in the polypeptide chain of β-CN at position 67 (His^{67}: A1 β-CN into Pro^{67}: A2 β-CN) (Daniloski, Cunha, et al., 2021).

The gelation properties of milk containing these two genetic variants of β-CN have been investigated previously. It has been found that protein from A1/A1 cows is a contributing factor in well-coagulating milks (Hallén, Allmere, Lundén, & Andrén, 2009; Jensen, Poulsen, et al., 2012; Jensen, Holland, Poulsen, & Larsen, 2012). These findings were consistent with the results of later studies, which found that the A1/A1 β-CN phenotype had an beneficial effect on characteristics of milk gels when compared to A2/A2 β-CN across all breeds tested (Danish Jersey, Danish Holstein, and Swedish Red) (Poulsen et al., 2013, 2017). Recently, milks possessing different β-CN phenotypes were used for manufacturing of yoghurt (Nguyen et al., 2018) and the research group revealed that gels produced from A2/A2 milk were more porous, contained thinner protein strands, and had lower gel strength compared to gels from A1/A1 milk; nevertheless, the gelation behaviour of milks carrying either A1 or A2 β-CN genetic variant was not characterised in depth. Therefore, the objective of the current study was to establish how milk behaves during acid-induced gelation as a function of different β-CN phenotypes. This was achieved by analysing structural and physical properties of acid-induced milk gels, providing insight into the effect of β-CN phenotype on the gelation mechanism. These findings are of relevance to the dairy industry, and should be considered in process and product design of fermented dairy products.

2. Materials and methods

2.1. Milk samples

Fresh bovine milk samples carrying different genetic variants of β-CN were gifted by the Agriculture Victoria Research (AVR) institute in Ellinbank, Victoria, Australia. The samples were obtained from 114 cows (calved in late winter or early spring and were between 36 and 271 days in lactation). All cows were healthy, showed no clinical signs of mastitis, and had already been genotyped using capillary electrophoresis (Raynes et al., 2015). Only cows with A1/A1, A1/A2, or A2/A2 β-CN phenotypes were further analysed (n = 52). Lactoscan milk analyser (Lactoscan LS-60, Milkotronic Ltd., Nova Zagora, Bulgaria) provided an approximate composition of the milk samples. Upon receipt, the raw milk samples were pooled into appropriate groups and defatted by centrifugation at 3225×*g* for 20 min at 20 °C (Avanti J-26XP, Beckman instrument Australia Pty. Ltd, Gladesville, NSW, Australia). The defatted milk samples were then frozen at - 80 °C for 24 h (unless otherwise stated, section 2.4.) and subsequently lyophilised in a pilot-scale freeze dryer (Christ Alpha 1–4 LSC plus, Germany), using a primary and secondary drying step at - 80 °C for 45 and 5 h, respectively. The milk powders were vacuum packed in double sealed plastic bags at ambient temperature and stored at - 80 °C.

2.2. Sample preparation

Skim milk powders were reconstituted in Milli-Q water (purified by a Milli-Q apparatus, Millipore Corp., Bedford, MA, USA) at 10% (w/w) to yield reconstituted skim milk of similar overall composition as the raw skim milk. All milk samples were standardised to the same protein content (3.3%, w/w). To ensure complete solubilisation, the reconstituted milk dispersions were gently mixed using a magnetic stirrer bar and left to hydrate overnight at 4 °C prior to testing and gel production. For acid-induced gelation, reconstituted skim milk samples were prewarmed at 42 °C for 30 min. Subsequently, 0.5 g GDL/g protein was added to the samples. Immediately after the addition of GDL, the samples were stirred for 15 s and further analysed. Additionally, in order to obtain supernatants for distribution of CNs and WPs in skim milks (prewarmed at 42 °C) and acid-induced gels, the samples were centrifuged at 100,000×*g* for 1 h at 42 °C in a Beckman Coulter Ultra L - 70 centrifuge (Beckman Instruments, Indianapolis, IN, USA). The clear supernatants were carefully removed and used for protein profiling and mineral determination.

2.3. Physicochemical analyses

2.3.1. Milk protein composition: Genotyping of β-casein genetic variants in milk samples and produced gels

The total protein content of milk and supernatant samples (i.e., obtained from milk or gels) was established by the Kjeldahl method (ISO, 2014, pp. 1–18) and the protein profile was determined using Reversed Phase - High Performance Liquid Chromatography (RP-HPLC), equipped with a Varian 9012 system controller (Shimadzu, LC-2030C Europa GmbH, Duisburg, Germany) coupled with a refractive index (RI) detector (RID - 20A). The samples (0.8 mL) were reduced using a denaturing urea solution (3.2 mL: 8 M urea, 165 mM Tris, 44 mM sodium citrate, and 0.3 mL v/v β-mercaptoethanol, Sigma-Aldrich, St. Louis, MO, USA). Elution was attained with Milli-Q water (mobile phase [Eluent] A), and acetonitrile (ACN: Sigma-Aldrich, MO, USA) (mobile phase [Eluent] B), both containing 0.1% trifluoroacetic acid (TFA; Sigma-Aldrich, MO, USA). Proteins were separated with a Jupiter C4 column (Phenomenex Aeris WIDEPORE, 150 mm × 4.6 mm, 3.6 μm particle size, 300 Å porosity, Torrance, USA), that operated at 42 °C, UV detection at 240 nm, and a gradient flow rate of 800 μL/min (Daniloski, McCarthy, Markoska, Auldist, & Vasiljevic, 2022). The relative protein content of the major milk proteins (αs_1-CN, αs_2-CN, β-CN, κ-CN, β-Lg, and α-La) was estimated and calculated as the integrated peak area of a certain compound compared with total integrated peak area within each RP-HPLC chromatogram. For calibration purposes, bovine αs-CN, β-CN, κ-CN, α-La, and β-Lg standard proteins were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Despite the individual content of every milk protein having been estimated and presented, only the genetic variants of β-CN, κ-CN, and β-Lg were further analysed, since it was found that they have an effect on the gelation properties of bovine milks (Bonfatti, Di Martino, Cecchinato, Vicario, & Carnier, 2010; Poulsen et al., 2013). Accordingly, the above mentioned HPLC station coupled with a reversed-phase analytical column C8 (Zorbax 300SB-C8 RP, Agilent Technologies, Santa Clara, SA, USA) with a silica-based packing (150 mm × 4.6 mm, 3.5 μm particle size, 300 Å) was used. In this regard, C8 column was chosen due to the fact that the aforementioned proteins possess high net hydrophobicity and would be able to participate in hydrophobic interactions with the stationary phase of the C8 column; these proteins have the ability to bind a variety of hydrophobic ligands (Creamer, Plowman, Liddell, Smith, & Hill, 1998; Huppertz, 2013). Further, Vincent, Elkins, Condina, Ezernieks, and Rochfort (2016) found

that the separation of intact milk proteins and recognition of their phenotypes was possible mainly by using the C8 column (Vincent et al., 2016). The samples were prepared by denaturing, reducing and acidifying in an aqueous solution of guanidine (Gdn) HCl (6 M GdnHCl, 0.1 M bisTris buffer, 5.37 mM sodium citrate, and 19.5 mM DTT, Sigma-Aldrich, St. Louis, MO, USA) in a 1:1 ratio (v/v). Each sample was vortexed for 10 s, incubated at room temperature for 1 h to promote proteins solubilisation, and diluted in the proportion 1:3 (v/v) with a solution containing 4.5 M GdnHCl in Milli-Q water, ACN, and TFA (100:900:1, Sigma-Aldrich, St. Louis, MO, USA). The column operated at 45 °C, UV detection at 214 nm, and a gradient flow rate of 500 μL/min (Vigolo, Franzoi, Penasa, & De Marchi, 2022).

2.3.2. Minerals and pH measurements of milks, supernatants and gels

The total and soluble mineral content (Ca) in both, reconstituted skim milk (before adding GDL) and milk serum (before GDL addition and after gelation process) were determined using an Inductively Coupled Plasma - Atomic Emission Spectrometer (ICP - AES Multitype, Shimadzu Corporation, Kyoto, Japan). The samples were ashed and dissolved in 1 M nitric acid (Sigma-Aldrich, St. Louis, MO, USA) before analysing the mineral content (Daniloski, McCarthy, Markoska, et al., 2022). The pH of skim milk samples was measured before and continuously during the entire gelation process (after GDL addition) using a calibrated pH meter equipped with a combined pH electrode with temperature sensor and fixed cable (Metrohm AG, Oberdorfatrasse, Herisau, Switzerland). The pH measurements were recorded in parallel with the rheological measurements (section 2.4.1.) (Sah, Vasiljevic, McKechnie, & Donkor, 2016).

2.4. Gelation experiments

2.4.1. Rheological measurements

The rheological properties of acid-induced gels were characterised using a controlled-stress rheometer (Physica MCR 301, AntonPaar GmbH, Ostfildern-Scharnhausen, Germany) equipped with a cup (27.11 mm diameter) and bob (25 mm diameter) geometry (CC 25/PR-SN, Anton Paar) with 1.055 mm measuring gap and 37.5 mm gap length, maintained at 42 °C (Sah et al., 2016). Formation of gels was monitored by measuring the elastic modulus *(G′)*, the viscous modulus *(G″)*, and the loss tangent *(tan δ)* for 120 min (0.5% strain and 1 Hz frequency). Subsequently, the flow behaviour of the acid-induced milk gels, which differed in the β-CN phenotype, was evaluated by subjecting a shear rate sweep to the same sample after 10 s of equilibration. The shear rate was increased from 0.1 to 100 s^{-1} over a 15 min period before decreasing from 100 to 0.1 s^{-1} over another 15 min period. Gelation was defined as the point at which the G' of the gel was ≥1 Pa (Meletharayil, Patel, Metzger, & Huppertz, 2016).

2.4.2. Gel water retention

Water holding capacity (WHC) of acid gels was assessed using an established method by Meletharayil, Patel, and Huppertz (2015) with slight variations. Namely, 20 mL of skim milk was transferred into a 50 mL Falcon tube (Falcon, Blue Max; Becton Dickinson and Co., Franklin Lakes, NJ, USA); the appropriate amount (0.5 g) of GDL was added to the milk samples to acidify them to pH of 4.6. The tubes were incubated at 42 °C for 120 min, and kept for 12 h at 4 °C before further analysis. The tubes were then centrifuged at 3000×*g* for 15 min at 4 °C (Avanti J-26XP, Beckman instrument Australia Pty. Ltd, Gladesville, NSW, Australia). The supernatant (whey) was carefully decanted, collected, and weighed. The WHC of the gels was expressed as a percentage, considering the weight of the gel (pellet) after supernatant was expelled, relative to the weight of the total sample.

2.4.3. Gel permeability

The permeability coefficient of acid-induced milk gels was measured as described previously with minor modifications (Dissanayake, Kelly, & Vasiljevic, 2010). The milk samples were loaded in 50 mL Falcon tubes (Falcon, Blue Max; Becton Dickinson and Co., Franklin Lakes, NJ) and tempered at 42 °C before the addition of GDL. Open-ended glass tubes with inner diameter 3.7 mm, length 25.0 cm, and with the aid of rubber stoppers were further submerged in the acidified milks. Additionally, the tubes were sealed with Parafilm (Parafilm M, Sigma-Aldrich, St. Louis, MO, USA) to prevent solvent evaporation, and gelation was induced by warming the milk samples at 42 °C for 120 min. Following that, the glass tubes containing the gels were removed from the Falcon tubes, and the gel heights were measured. The gel-filled glass tubes were then immersed in SMUF at 42 °C. Due to the osmotic pressure gradient between the top of gels and the surface of SMUF in the Falcon tubes, the SMUF diffuses through the gels and collects on the surface. The height of the SMUF on the gel surface was measured after two different time intervals (60 and 120 min after the experiment). The permeability coefficient *(B)* was calculated using the following equation (1):

$$B = \left[ln\left(\frac{h\infty - ht_2}{h\infty - ht_1}\right)\right] \frac{\eta H}{\rho g(t_2 - t_1)} \quad (1)$$

Where B is the permeability coefficient (m^2), $h\infty$ is the height of the SMUF in the reference tube (m), and ht_1 and ht_2 are the heights of SMUF (m) in the gel tube at time t_1 (s) and t_2 (s), respectively. The value η is the viscosity of SMUF (Pa x s), ρ is the density of SMUF (kg/m^3), g is acceleration due to gravity (ms^{-2}), and H is the length of the gel (m). The reference glass tube was included with the absence of gels but subjected to all other experimental conditions.

2.5. Structural fingerprinting

The reconstituted skim milks (incubated at 42 °C) and acid-induced gels (section 2.2.), both kept in individual copper cups (7 cm diameter and 7 cm length) were rapidly immersed into liquid nitrogen (- 196 °C) for 15 s. The liquid nitrogen used was freshly filled to prevent the presence of particulates that may provide nucleation for the growth of ice crystals. The frozen blocks were immediately stored at - 80 °C overnight to prevent ice re-crystallisation and thus minimise any changes in microstructure. After that, the samples were lyophilised in a pilot scale freeze dryer (Christ Alpha 1–4 LSC plus, Germany) under the same conditions as previously described (section 2.1.). The prepared powders were used for conformational characteristics of these milks and gels.

2.5.1. Microstructure of milk and gel samples as a function of β-CN phenotype

Microstructures of the milks and gels were studied using a Scanning Electron Microscope (SEM) as described by Sah et al. (2016) with some modifications. In brief, the sample powders were mounted on an aluminium SEM stub with a double-sided adhesive carbon tape, sputtered with gold (up to 15 nm) using a JEOL NeoCoater (model MP-19020NCTR, Peabody, MA, USA), and subjected to observation. Fields of the specimen were examined under a high-vacuum Neo-Scope JCM-5000 benchtop SEM (JEOL Ltd., Tokyo, Japan) and micrographs were recorded.

2.5.2. Fourier transform infrared (FTIR) spectral profiling

FTIR spectra of the milks and gels were acquired using a FTIR spectrometer (PerkinElmer, Boston, MA, USA) according to the methods reported by Mudgil, Jumah, Ahmad, Hamed, and Maqsood (2018), slightly modified. The FTIR spectra were recorded in the region of 4000 - 650 cm^{-1}, at 42 °C, a resolution of 4 cm^{-1}, and by averaging 16 scans of each spectrum. At the beginning of the measurements, the background spectrum was scanned with a blank diamond attenuated total reflectance (ATR) cell using the same instrumental conditions as for the sample spectra acquisition. Spectra were transferred into absorbance to examine the nature of molecular interactions, particularly the secondary

protein structure (Amide I region: between 1700 and 1600 cm^{-1}). Therefore, intermolecular (aggregated) β-sheet at 1700 cm^{-1} - 1682 cm^{-1}; β-turn at 1681 cm^{-1} - 1665 cm^{-1}; α-helix at 1664 cm^{-1} - 1646 cm^{-1}; random coil at 1645 cm^{-1} - 1638 cm^{-1}; intramolecular β-sheet at 1637 cm^{-1} - 1615 cm^{-1}; and side chain at 1614 cm^{-1} - 1600 cm^{-1} structures were included in the examination (Daniloski, McCarthy, O'Callaghan, & Vasiljevic, 2022). The spectra of ten sub samples of each sample were taken by refilling the ATR cell.

2.6. Spectral data and statistical analyses

A one-way ANOVA (A1/A1, A1/A2, or A2/A2 phenotypes) was used to test the experimental data, and the significance was indicated by $p \leq 0.05$. Tukey's test was used to determine significant difference between means. Data were analysed by Minitab version 19 software (Minitab Inc., Pennsylvania, USA). All measurements were carried out in triplicate unless otherwise stated. Daniloski, McCarthy, Markoska, et al. (2022) earlier described a technique for data processing of the chosen spectrum measurements. Mean centring was performed using Spectragryph software (version 1.2.7, Oberstdorf, Germany), followed by a second-order Savitzky-Golay derivative form of all spectra inside the broad Amide I region (C=O stretching of proteins: 1700 - 1600 cm^{-1}) before creating the classification model. To estimate the area of each component representing secondary structures, a curve-fitting technique was utilised. The FTIR's second derivative spectra were subjected to Principal Component Analysis (PCA) using Origin software (Origin Pro 2021, v. 95E, OriginLab Corporation, Northampton, MA, USA) to characterise the behaviour, and thus distinguish and classify the samples. The multivariate analysis was performed with 95% significance.

3. Results

3.1. Milk protein composition and physicochemical determination

Skim milks, milk serums, and supernatants from acid gels were analysed using RP-HPLC and the genetic variants of β-CN in A1/A1, A1/A2, and A2/A2 samples were confirmed (data not shown). Table 1 shows the protein fractions and their relevant concentrations in all samples. There were no noticeable differences between the milks based on protein content ($p > 0.05$), since all milks were reconstituted to the same protein content (section 2.2.). However, from all milk proteins, the levels of κ- and αs_2-CNs were significantly ($p < 0.05$) lower and the content of αs_1-CN was moderately greater in A2/A2 milk compared to that of A1/A1 or A1/A2 milk indicating that the higher αs_1-CN concentration might have been achieved at the expense of κ-CN. No significant differences between all three milk groups were observed in the ratios of CNs to WPs concentrations (Table 1, $p > 0.05$). The A1/A1 and A1/A2 milks were comprised of ~12–18% and 6–25% greater amounts of both total β-Lg and α-La, respectively, than A2/A2 milk ($p < 0.05$). Interestingly, after acidification there was a higher level of soluble β-CN and lower amount of soluble κ-CN in A1/A1 gel relative to the other phenotypes ($p < 0.05$); A1/A2 gel contained the highest content of β-CN in its structure (7.34% and 4.34% greater than that in A1/A1 gel and A2/A2 gel, respectively). This may indicate that the non-micellar β- and κ-CNs of all three milk types possessed a different ability to permeate out of the micelle structure into the serum phase during acidification. Nevertheless, following their counterpart milks, A1/A1 and A1/A2 gels carried greater levels of αs_2-and κ-CNs, and lower amount of αs_1-CN as opposed to A2/A2 gel ($p < 0.05$). Proportion of CNs to WPs was approximately ~1 to 0.04 in all three groups of gels, with approximately 80–87% of WPs solubilised in the serum; thus, indicating that the WPs might had a minor and rather insignificant influence on the gelation properties of these three milk types (Table 1, $p > 0.05$). There were no significant differences in pH (~6.45) and total Ca content (27–29 mM) between all three types of milk ($p > 0.05$). Nonetheless, after acidification, A1/A2 but mainly A2/A2 gels contained significantly greater levels of soluble Ca compared to that of A1/A1 gel ($p < 0.05$), suggesting a lower proportion of CN bound Ca in gels containing A2 β-CN (Table 2).

Table 1
Milk protein composition between skim milk and serum before and after gelation.

	Protein content (mg/mL)						
Sample	κ-CN	αs_2-CN	αs_1-CN	A1 β-CN	A2 β-CN	β-Lg	α-La
A1/A1 milk	4.01 ± 0.03 ^{a}	1.75 ± 0.06 ^{a}	9.70 ± 0.06 ^{b}	10.58 ± 0.15 ^{a}	n/d	3.90 ± 0.01 ^{a}	1.66 ± 0.06 ^{b}
A1/A2 milk	4.07 ± 0.03 ^{a}	1.85 ± 0.02 ^{a}	9.41 ± 0.02 ^{c}	5.87 ± 0.03 ^{b}	5.51 ± 0.02 ^{b}	3.67 ± 0.04 ^{a}	1.81 ± 0.02 ^{a}
A2/A2 milk	2.02 ± 0.02 ^{b}	1.56 ± 0.04 ^{b}	11.42 ± 0.01 ^{a}	n/d	10.86 ± 0.25 ^{a}	3.42 ± 0.02 ^{b}	1.35 ± 0.02 ^{c}
A1/A1 serum (milk)	0.19 ± 0.01 ^{c}	0.50 ± 0.02 ^{a}	0.07 ± 0.01 ^{b}	0.92 ± 0.02 ^{a}	n/d	3.46 ± 0.05 ^{a}	1.19 ± 0.06 ^{b}
A1/A2 serum (milk)	0.26 ± 0.02 ^{b}	0.32 ± 0.02 ^{b}	0.04 ± 0.01 ^{b}	0.29 ± 0.03 ^{b}	0.57 ± 0.02 ^{b}	3.37 ± 0.05 ^{a}	1.57 ± 0.02 ^{a}
A2/A2 serum (milk)	0.59 ± 0.02 ^{a}	0.26 ± 0.02 ^{c}	0.29 ± 0.03 ^{a}	n/d	0.93 ± 0.02 ^{c}	3.46 ± 0.05 ^{a}	1.31 ± 0.02 ^{a}
A1/A1 serum (gel)	0.25 ± 0.02 ^{a}	0.21 ± 0.02 ^{b}	0.10 ± 0.01 ^{a}	0.15 ± 0.01 ^{a}	n/d	3.32 ± 0.06 ^{a}	1.50 ± 0.02 ^{a}
A1/A2 serum (gel)	0.26 ± 0.01 ^{a}	0.26 ± 0.02 ^{a}	0.01 ± 0.00 ^{b}	0.04 ± 0.01 ^{b}	0.06 ± 0.00 ^{a}	2.95 ± 0.58 ^{c}	1.28 ± 0.02 ^{b}
A2/A2 serum (gel)	0.14 ± 0.02 ^{b}	0.24 ± 0.01 ^{ab}	0.01 ± 0.00 ^{b}	n/d	0.07 ± 0.01 ^{b}	3.00 ± 0.01 ^{b}	1.00 ± 0.04 ^{c}

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$). The analysis of all three sample types [milk, serum (milk), and serum (gel)] was performed separately; n/d: not detectable.

3.2. Acid gelation and physicochemical properties of skim milk gels

Fig. 1 shows the elastic modulus (G') of skim milk samples measured as a function of pH. The G' in all skim milk samples was comparably low above pH ~5.4, where the electrostatic repulsion between CN particles was strong enough to prevent gel formation. As shown in Fig. 1A, gelation occurred with a decrease in pH, where the G' began to increase between pH ~5.3 - 5.1, indicating initial gel structure formation. The G' profiles for A1/A1 and A1/A2 acid-induced gels were both similar and significantly firmer than A2/A2 gel (Fig. 1A; Table 3). Interestingly, the G' for A1/A1 and A1/A2 acid-induced gels continued to increase with decreasing pH; however, the G' for A2/A2 milk reached a temporary plateau between pH 4.8 and 4.7, where the G' value did not increase

Table 2
Physicochemical characteristics of milks (Ca content) and acid-induced milk gels (water holding capacity and permeability) as a function of β-CN phenotype.

Phenotype	Ca (mM)			WHC (%)	Permeability coefficient (x 10^{-15} m^2)
	Milk	Serum (milk)	Serum (gel)	Gels	
A1/A1	29.11 ± 0.06 ^{a}	6.39 ± 0.05 ^{e}	20.68 ± 0.03 ^{d}	83.91 ± 1.38 ^{a}	0.52 ± 0.04 ^{c}
A1/A2	28.38 ± 1.91 ^{ab}	7.55 ± 0.03 ^{e}	26.85 ± 0.05 ^{bc}	83.12 ± 2.07 ^{a}	3.42 ± 1.45 ^{b}
A2/A2	27.36 ± 0.13 ^{abc}	6.24 ± 0.05 ^{e}	26.53 ± 0.03 ^{c}	69.27 ± 3.28 ^{b}	4.76 ± 1.46 ^{a}

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$).

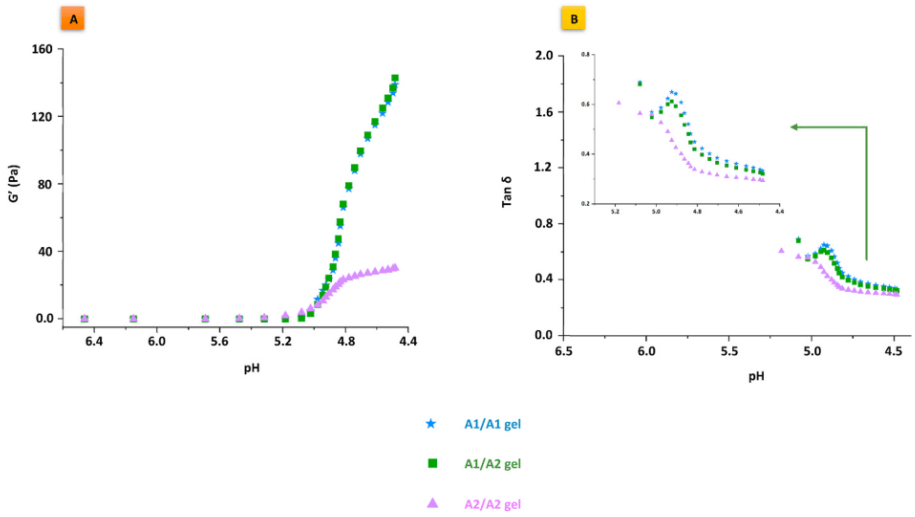

Fig. 1. A) Elastic modulus (*G′)* as a function of pH during acidification with GDL at 42 °C of A1/A1 (blue), A1/A2 (green), and A2/A2 (purple) milks. **B)** Damping factor *(tan δ)* as a function of pH during the acidification with GDL at 42 °C of A1/A1 (blue), A1/A2 (green), and A2/A2 (purple) milks. Values are the means of data from triplicate analyses. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Rheological parameters measured during acid gelation of the milk samples.

Sample	Gelation point pH (G' = 1 Pa)	G' (Pa)			Gelation pH			Tan δ		
		Time (min)								
		30	60	120	30	60	120	30	60	120
A1/A1 milk	5.08 ± 0.01 ^{b}	4.83 ± 1.40 ^{a}	36.00 ± 0.42 ^{a}	139.00 ± 1.41 ^{a}	5.07 ± 0.01 ^{b}	4.78 ± 0.01 ^{b}	4.50 ± 0.01 ^{a}	0.56 ± 0.01 ^{a}	0.56 ± 0.00 ^{a}	0.33 ± 0.00 ^{a}
A1/A2 milk	5.06 ± 0.01 ^{b}	3.14 ± 1.18 ^{a}	38.50 ± 1.84 ^{a}	143.00 ± 8.49 ^{a}	5.02 ± 0.02 ^{b}	4.86 ± 0.03 ^{a}	4.48 ± 0.01 ^{a}	0.55 ± 0.01 ^{a}	0.54 ± 0.03 ^{a}	0.33 ± 0.01 ^{a}
A2/A2 milk	5.27 ± 0.01 ^{a}	6.04 ± 0.23 ^{a}	18.90 ± 0.48 ^{a}	30.15 ± 2.81 ^{b}	5.15 ± 0.02 ^{a}	4.88 ± 0.01 ^{a}	4.53 ± 0.03 ^{a}	0.49 ± 0.10 ^{a}	0.34 ± 0.05 ^{b}	0.28 ± 0.02 ^{b}

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$).

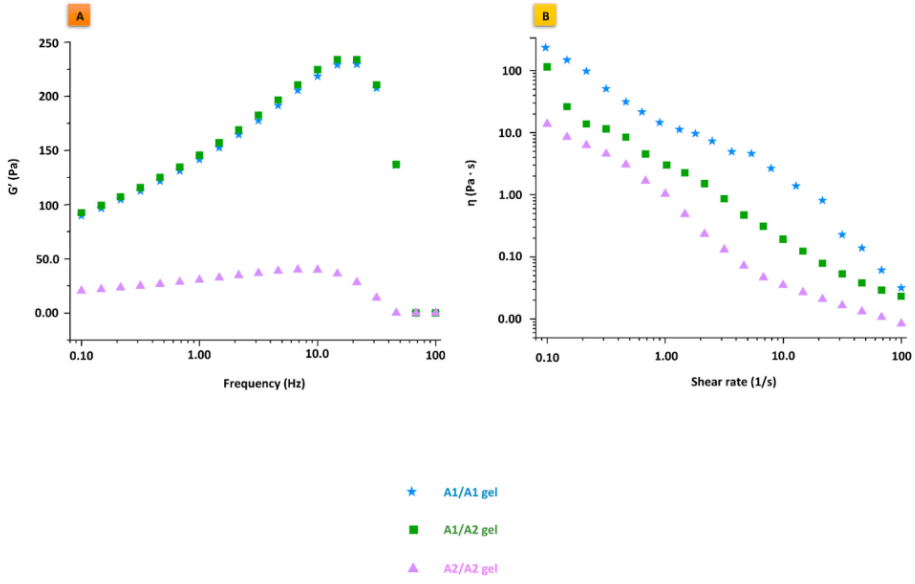

Fig. 2. A) Elastic modulus (*G′)* A1/A1 (blue), A1/A2 (green), and A2/A2 (purple) milks obtained from small strain oscillation frequency sweep of the gels. **B)** Apparent viscosity as a function of shear rate for A1/A1 (blue), A1/A2 (green), and A2/A2 (purple) gels. Values are the means of data from triplicate analyses. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

substantially despite a further reduction of pH (Fig. 1A). Additionally, the *tan δ* profiles measured as a function of pH (Fig. 1B) showed significant difference between A1 β-CN containing milks and the A2/A2 milk throughout acidification. The changes in *tan δ* observed for A1/A1 and A1/A2 milks were very similar, namely they decreased sharply at the start of the gelation and increased with a maximum *tan δ* at pH 4.9 (0.65 for A1/A1 milk and 0.61 for A1/A2 milk) before decreasing again. The *tan δ* at its maximum (pH 5.02) was significantly lower for A2/A2 gel (0.49), compared to milk gels carrying A1 β-CN ($p < 0.05$) (Fig. 1B). It is also worth noting that A1/A1 and A1/A2 gels showed moderately higher *tan δ* (0.33 for both gels) at pH 4.6 ± 0.1 compared to that of A2/A2 gel (0.28). Frequency sweep profiles of the acid-induced skim milk gels are presented in Fig. 2A and show that for all samples the G' values of A1/A1 and A1/A2 gels were significantly higher than that of A2/A2 gel at a frequency range of 1–100 Hz; in all gels a decrease in G' at ~10 Hz was observed.

The apparent viscosity (η) of stirred gels for a range of constant shear rates is shown in Fig. 2B. The gels displayed a typical shear-thinning behaviour, with apparent viscosity decreasing upon increasing shear rate. Dissimilar flow curves were observed for the gels made of either A1/A1, A1/A2, or A2/A2 milks. Initially, the η of the gels differed greatly. The highest η was assigned to A1/A1 gel (244 Pa s), followed by A1/A2 gel (114 Pa s), and A2/A2 gel (~14 Pa s). Accordingly, A2/A2 milk gels displayed lower η across the shear rate ramp, compared to the other gels made from milk samples containing the other two phenotypes (A1/A1 or A1/A2 β-CN phenotype). Flow curve of gel made from A1/A2 milk (Fig. 2B) were between the curve for A1/A1 and the curve for the gels made from A2/A2 milks. At the highest measured shear rates, as expected, the apparent viscosities became comparable 0.03, 0.02, 0.01 Pa s for A1/A1, A1/A2, and A2/A2 gels, respectively, since the viscosity was governed by the protein concentration (particle volume fraction) and all gels contained the same amount of protein.

The ability of the acid-induced gels to retain water was affected by the genetic variant of β-CN ($p < 0.05$) as shown in Table 2. Namely, after maintaining the samples at low temperature over-night, the WHC of the A2/A2 gel decreased, thus the gel exuded more water. Water holding capacity of A1/A1 and A1/A2 gels was approximately 85% (30–35% higher than the A2/A2 gel), indicating greater structural stability and water retention ($p < 0.05$). This difference in WHC can be explained by the permeability coefficients of these gels. Permeability coefficients of acid-set gels containing A1 β-CN in their structure (A1/A1 and A1/A2 gels) were significantly ($p < 0.05$) reduced compared to the permeability coefficient of A2/A2 gel (Table 2), thus reflecting their relative compactness. Moreover, a relationship between WHC, gel permeability, and the final G′ of the gels can be observed, wherein increased final G' and viscosity positively correlate with increased WHC and permeability coefficients for the gels prepared from milk samples containing variant A1, when compared to those containing the A2/A2 phenotype.

3.3. Structural analyses of skim milk gels

3.3.1. Conformational analysis by Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of milks measured at 42 °C are shown in Fig. 3. The differences in peaks centred at ~1630 cm^{-1} detected in the Amide I vibrational region of all three milk types were assigned to intramolecular β-sheets. Namely, both milks containing A2 β-CN had a similar level of intramolecular β-sheet structures, compared to A1/A1 milk where their presence was lower by almost 50% (Table 4) ($p < 0.05$). Interestingly, A2/A2 milk contained the highest amount of random coils; furthermore, compared to the other milks carrying A1 β-CN in their structures, an absence of α-helical conformations was seen in this milk ($p < 0.05$). A peak shoulder at 1698 cm^{-1} revealing exposure of some aggregated β-sheets was noted mainly in A1/A1 milk. This

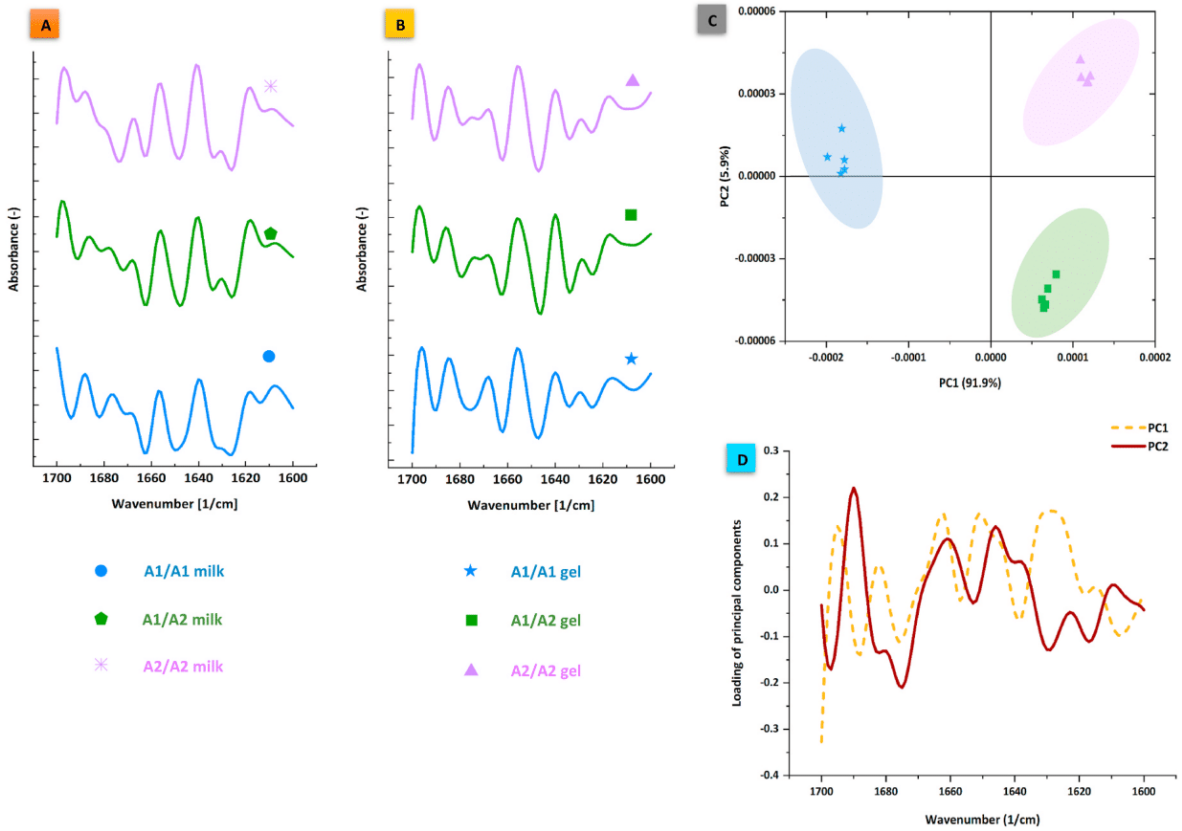

Fig. 3. A) Second derivative spectra of Amide I region of milk samples. **B)** Second derivative spectra of Amide I region of gel samples. **C)** Scatter plot of the PCA scores of FTIR spectra of gel samples. **D)** The plot of the PCA loadings of FTIR spectra of gel samples.

Table 4
Total percentage areas of different secondary structures in Amide I in genetic β-CN variants in milk and gel samples.

Band Assessment	Band frequency (cm^{-1})	Peak area (%)					
		A1/A1	A1/A2	A2/A2	A1/A1	A1/A2	A2/A2
		Milks			Gels		
Side chain	1614–1601	2.99 ± 1.63 ^{c}	4.61 ± 3.07 ^{b}	2.95 ± 1.56 ^{c}	3.76 ± 2.30 ^{bc}	5.04 ± 0.54 ^{a}	5.10 ± 0.87 ^{a}
Intramolecular β-sheet	1637–1615	13.67 ± 4.06 ^{c}	25.58 ± 3.36 ^{b}	20.51 ± 3.13 ^{bc}	30.71 ± 1.99 ^{a}	32.75 ± 1.29 ^{a}	31.81 ± 1.74 ^{a}
Random coil	1645–1638	7.86 ± 3.06 ^{d}	10.26 ± 3.50 ^{c}	42.34 ± 2.14 ^{a}	n/d	10.58 ± 2.14 ^{c}	33.79 ± 1.50 ^{b}
α-helix	1664–1646	35.07 ± 3.09 ^{c}	46.74 ± 5.04 ^{a}	n/d	36.71 ± 2.26 ^{c}	41.05 ± 2.42 ^{b}	n/d
β-turn	1681–1665	19.91 ± 1.84 ^{a}	4.66 ± 1.53 ^{d}	15.94 ± 4.43 ^{b}	10.51 ± 0.90 ^{c}	n/d	20.58 ± 3.55 ^{a}
Aggregated β-sheet	1700–1682	20.50 ± 0.46 ^{a}	8.15 ± 2.52 ^{d}	18.53 ± 2.43 ^{b}	18.31 ± 1.15 ^{b}	10.58 ± 0.78 ^{c}	8.72 ± 1.38 ^{d}

Mean values within a row that do not share a common superscript letter are significantly different ($p \leq 0.05$); n/d = not detectable.

change was confirmed with the area percentage in Table 4, where the presence of these structures was distinguished in both homozygous milks (particularly A1/A1 milk) compared to A1/A2 milk ($p < 0.05$). After the acidification of the skim milks there was a significant conformational change in the secondary structure of proteins (Fig. 3B). The level of structural intramolecular β-sheet elements seemed to increase in all three gel types during acidification ($p < 0.05$), specifically, by 124% in A1/A1, 28% in A1/A2, and 55% in A2/A2 gels compared to their counterpart un-acidified skim milks (Table 2). Furthermore, the amount of the aggregated β-sheets slightly decreased in the process of gel making for all three β-CN phenotypes, however, their amount was considerably higher in A1/A1 gel than A1/A2 and A2/A2 gels ($p < 0.05$), possibly due to the higher aggregation of some protein aggregates and new molecular rearrangements during acidification stages (Fig. 3B and Table 4). Interestingly, despite the acidification process, for several protein structure's modifications all three types of gels followed the similar pattern as their milk precursors ($p < 0.05$). Hence, random coil and β-turn conformations were significantly higher in the A2/A2 gel (an extensive shoulder between 1675 and 1670 cm^{-1} and an intensive peak around 1640 cm^{-1}, respectively), yet lack of α-helixes was noted ($p < 0.05$), compared to both gels containing A1 β-CN in their structures (Fig. 3B and Table 4).

The identification and grouping of A1/A1, A1/A2, and A2/A2 gels was performed by PCA. According to Fig. 3C, samples belonging to the same β-CN phenotype were grouped together, indicating the efficient discrimination of the phenotypes. With a diversity of ~98% (PC1 = 92%, PC2 = 6%), the PCA scatter plot showed reasonably good classification of the samples into three groups, namely A1/A1 gel (blue group), A1/A2 gel (green group), and A2/A2 gel (purple group) (Fig. 3C). Particularly, PC1 was positively correlated with A1/A2 and A2/A2 gels and negatively associated to A1/A1 gel; thus, PC1 was assigned to an increased response of A2 β-CN as the distinguishable driver for the separation. The loading on PC1 (Fig. 3D) included intense positive peaks at 1640 cm^{-1} and 1690 cm^{-1}, refereed as random coils (A2/A2 gel) and aggregated β-sheet (A1/A2 gel), respectively; a negative peak between 1660 cm^{-1} and 1650 cm^{-1} (a-helix conformation), and a broad negative peak at ~ 1630 cm^{-1} (intramolecular β-sheet) driven by A1/A1 gel. In contrast, PC2 separated the scores of A1/A2 gels (negative), from the scores of both homozygous gels (positive). Therefore, the governing nature of both β-CN genetic variants was observed. According to the loading plot, PC2 component presented an inclusive positive doublet peaks from 1681 cm^{-1} to 1675 cm^{-1} (β-turn, mainly due to the presence of A2/A2 β-CN), and 1630 - 1615 cm^{-1} (intramolecular β-sheet, both homozygous β-CNs). Moreover, a singlet positive peak at 1650 cm^{-1} referring to α-helix; (govern by A1/A1 gel), and a sharp negative peak at 1690 cm^{-1} (aggregated β-sheet), which is probably associated to A1/A2 gel. Notably, the results from PCA loading plot (Fig. 3D) and those distinguishing the second derivative data of all gels (Fig. 3B and Table 4) were similar and the influence of either A1/A1, A1/A2, or A2/A2 β-CN in all gels was noticeable. Overall, the results indicated that the variance of the data was correlated to the effect of β-CN phenotype, which might be the main reason of the separation in PC1 and PC2 components.

3.3.2. *Microstructural analyses of skim milk gels*

The microstructure of skim milk gels is shown in Fig. 4. Generally, the gel structure comprised of a three-dimensional network of aggregated CN, interspaced by the zones where the serum is trapped or immobilised. Substantial differences were noticed between the microstructures of gel originating from A1/A1 and A2/A2 milks. Hence, A2/A2 gel was characterised by a discontinuous branching network, with coarse aggregates surrounding bigger water holding voids (Fig. 4C), an outcome which is congruent with the lower *G'* values shown in Fig. 1A. In contrast, both gels that carried A1 β-CN in their structure exhibited a more compact, dense and homogenous microstructure with visibly fewer and smaller voids noticed (Fig. 4A and B), especially in the A1/A1 gel. More densely packed CN networks can be seen in the micrographs in A1/A1 and A1/A2 gels, and they manifested a normal homogenous microstructure where aggregates can be clearly seen. This compact protein network observed in the microstructure might have resulted in increased WHC and permeability of these gels, higher values of shear rate and *G'*, which demonstrates that A1/A1 and A1/A2 milks generated stronger gels.

4. Discussion

Several attributes of an acid-induced milk gel are an essential functional property for a number of products (i.e., yogurts, cottage/fresh cheese, acid CN) with characteristics such as gel strength, curd formation, water holding capacity, and syneresis, all important in defining end-product functionality (Lucey, 2020). The relationship between pH, solubilisation of calcium phosphate and dissociation of CN micelles is multifaceted with many factors, including protein concentration, level of native WP, mineral content and distribution, all affecting the rate of gelation and final gel strength (Lucey, 2002). However, the results shown in the current study have indicated that β-CN phenotype also plays a significant role in creation of acid induced milk gels. Specifically, the effect of β-CN phenotype on milk gelation traits found that A1/A1 and A1/A2 milks were characterised by both shorter gelation times and higher *G'* values in comparison to those of A2/A2 milk. A number of previous studies have examined the effect of bovine milk phenotype on acid- or chymosin-induced gelation of milk (Hallén et al., 2009; Nguyen et al., 2018; Poulsen et al., 2013). Previously, Nguyen et al. (2018) demonstrated a correlation between β-CN phenotype and the properties of acid-induced gels produced during yogurt fermentation, with A2/A2 milk having a significantly lower storage modulus compared to that of the A1/A1 milk and suggested that the differences in gelation are linked to the primary amino acid structure of the proteins. However, while this may be the case there could be other factors affecting gelation which might be indirectly associated with β-CN phenotype, for example,

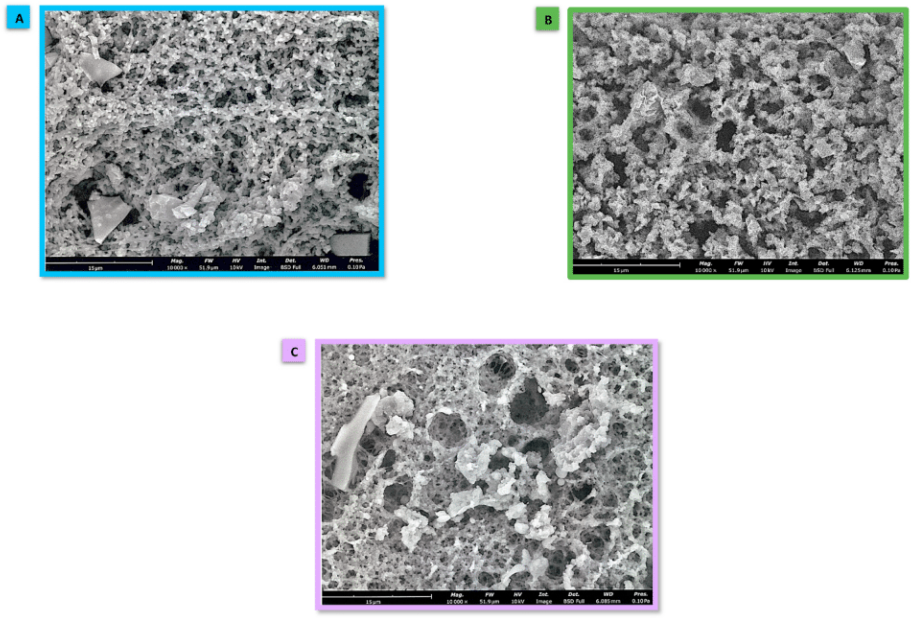

Fig. 4. Representative scanning electron micrographs of A1/A1 (**A:** blue), A1/A2 (**B:** green), and A2/A2 (**C:** purple) gels. Bar scale = 15 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

A1/A1 and A1/A2 milks gave the higher G' values (Fig. 1) but also contained greater levels of micellar κ-CN (3.76 and 3.81 g) and β-Lg (0.72 and 0.58 g) compared to A2/A2 milk (1.88 g and 0.42 g for κ-CN and β-Lg, respectively) (Table 1). Similarly, Poulsen et al. (2013) suggested that lower levels of total κ-CN, similar to that found here in A2/A2 milk, were associated with poor rennet-induced coagulation of milk. Also what is worth mentioning in the current study is that all three types of milk samples contained different amounts of the AB κ-CN phenotype, with a 3 and 5-fold increase of A κ-CN in A1/A1 (1.95 g out of 4.01 g total κ-CN) and A1/A2 milks (1.56 g out of 4.07 g total κ-CN), compared to the ratio of κ-CN variants in A2/A2 milks which was predominantly B κ-CN (1.72 g out of 2.02 g total κ-CN). Additionally, the isoelectric point of κ-CN is fairly high, thus the average CN isoelectric point would also be high in a sample rich in κ-CN (Gazi, Johansen, & Huppertz, 2022). This means that a sample with a greater amount of κ-CN would also coagulate faster and become firmer earlier during acidification compared to the other samples, i.e. it coagulates at a higher pH on account of the higher isoelectric point (De Kruif, 1997). That appears to be the case for A1/A1 and A1/A2 gels in the present study. Conversely, A2/A2 gel is low in κ-CN and simultaneously high in αs_1-CN. Due to both of these factors (Gazi et al., 2022), the average CN isoelectric point of the A2/A2 sample would be low; acidification needs to be performed to a fairly low pH in order for coagulation to take place and before curd firming initiation (Lucey, 2020). Based on the CN abundances from Table 1, the estimated CN's isoelectric points for A1/A1, A1/A2, and A2/A2 milks are 4.80, 4.79, and 4.66, respectively.

Furthermore, β-Lg was also found to be heterozygous in all three types of skim milks. Notably, β-Lg A was dominant in A1/A1 (3.24 g out of 3.90 g total β-Lg) and A1/A2 milks (3.50 g out of 3.67 g total β-Lg), whereas A2/A2 milk contained similar levels of both β-Lg A and B (1.50 g for B variant and 1.92 g for A variant out of 3.42 g total β-Lg). However, in relation to the effect that β-Lg phenotype may have had on gelation properties it must be noted that the skim milk samples in the current study were not heat treated and so contained a high level of native WP. Therefore, the difference in gelation may not have been influenced by β-Lg phenotype as shown in Table 1, where the majority of β-Lg was present in the serum phase of gels and not associated with the coagulum, indicating that the contribution of β-Lg towards the differences observed in gel strength between A1 and A2 milks may have been negligible. Nevertheless, its effect should not be completely disregarded, since in the past, Donato, Alexander, and Dalgleish (2007) revealed that the early coagulation of the unheated CN micelles was caused by the binding of the serum protein complexes to the acidified structurally modified CN micelles, possibly *via* electrostatic interactions, at pH around 5.4. Namely, the same phenomena might happen to the A2/A2 milk in the present study since it showed that G'_{30} for A2/A2 milk was greater at a higher pH compared to those of the other milk phenotypes (Table 3). Previously, Hallén et al. (2009) stated that β-Lg genotype had a significant effect on acid-induced gelation of milk but that this was more a result of differences in concentration associated with genotype rather than directly due to differences in the primary structure. This correlates to the results in the current study where the overall β-Lg concentration was similar in the serum phase of all raw skim milk samples (Table 1). In the past, in milk with good coagulation ability, a high prevalence of the AA of both β-Lg (Hallén et al., 2009) and κ-CN were identified (Ketto, Abdelghani, Johansen, Øyaas, & Skeie, 2019), whereas poorly coagulating milk was associated with the A2/A2 β-CN variant (Jensen, Holland, et al., 2012).

The decrease in pH during dissolution of GDL leads to lessened electrostatic repulsions between the CN molecules, which in turn aggregate *via* various interactions, but particularly hydrophobic when close to the isoelectric pH of 4.6 (Liu & Guo, 2008). Raynes et al. (2015) explained that the hydrophobic interactions predominate when β-CN form a micelle; A1 β-CN had more exposed hydrophobicity than A2 β-CN. As a result, in the present study in gels with A1 β-CN, a greater number of hydrophobic groups was likely exposed on the surface of the protein and may be correlated to greater WHC (Table 3), higher G' and *tan* δ values, denser tightly knit-gel structure (Fig. 4) and lower permeability coefficient (B) of A1/A1 and A1/A2 milk gels, compared to these of the A2/A2 gel. Similarly, Nguyen et al. (2018) explained that the substitution from Pro^{67} to His^{67} might be the main reason for the observed properties of A2/A2 gel which were characterised with larger pores, less dense protein network, especially a lower *tan* δ in comparison to A1/A1 gel. In the current study, the weaker gels obtained from A2/A2 milk possessed a lower *tan* δ, which implies a longer relaxation time (Lucey, 2020). During creation of milk gels, the system relies on re-arrangement of the bonds among individual CNs making up the original CN micelles. Thus, lower gel firmness is rather associated with a

lower number of such bonds (Lucey, 2002; Lucey, Tamehana, Singh, & Munro, 2000; Van Vliet, Van Dijk, Zoon, & Walstra, 1991). Walstra (1993) explained that the rearrangements of the CN particles into a more compact structure would increase the number of bonds (more protein-protein bonds are formed gradually at each junction between the CN particles), and thus leading to firmer gels and decreased total free energy of the system (Lucey et al., 2022), as in the gels made of A1/A1 and A1/A2 milks. The reason behind this phenomena can be related to the difference in κ-CN contents in all three gel types if we assume that all κ-CN is located on the surface of the CN micelles (Huppertz & Gazi, 2022). Accordingly, lower amount of κ-CN indeed translates into fewer interactions, at least at the surface of the CN micelles during coagulation (see above) (Lucey, 2020). In accordance with the data of the fractal nature of CN gels' structure (Bremer, van Vliet, & Walstra, 1989; Day, Williams, Otter, & Augustin, 2015), in this study A1/A1 and A1/A2 milks contained more κ-CN, that theoretically led to a much larger number of particles and higher surface area, also observed in the study of Poulsen et al. (2013). Therefore, in these milks a higher number of interactions were created, compared to A2/A2 milk, which had fewer interactions and hence, softer gels.

In addition, the WHC and hydration of proteins can be affected by several intrinsic factors in its solution environment, such as protein conformation (shape and size), steric factors, and most importantly the polarity (hydrophilic - hydrophobic balance) (Chen et al., 2017). The polar amino groups of protein molecules are the major sites responsible for protein - water interactions (Chavan, McKenzie, & Shahidi, 2001), greater water binding in A1/A1 and A1/A2 gels might be caused by better accessibility of polar amino acids to the aqueous phase. Therefore, the difference in WHC of proteins can be due to variations in conformational characteristics. The existence of His^{67} (polar amino acid) led to an increased WHC of A1/A1 and A1/A2 gels compared to A2/A2 gel (Vigolo et al., 2022), which is known to contain Pro^{67} (non-polar amino acid) (Damodaran & Parkin, 2017) resulting in lower WHC of its produced gels.

As discussed previously the fundamental difference between the two β-CN proteoforms is an amino acid residue in position 67, i.e., His in A1 and Pro in A2 β-CN. During glucono-δ-lactone dissolution (Lucey et al., 2000) hydrogen ions are released and are accepted by polar and charged amino acids, including His, leading to the formation of ionic and hydrogen-bonds (Scheiner, Kar, & Pattanayak, 2002) and thus likely fine and stable structure. Namely, the hydrogen bonds can be expected to represent the strongest type of interactions, one that proteins will strive to take advantage of as they adopt their optimal conformational state (Scheiner et al., 2002). Particularly, the versatility of His in molecular interactions arises from its unique molecular structure and the presence of imidazole ring, which provides His with multiple roles in the protein interactions (Liao, Du, Meng, Pang, & Huang, 2013). The presence of hydrogen atoms connected to either or both of the two nitrogen atoms (Nδ and Nε) of the imidazole moiety in a His residue was found to affect the geometry of the five-membered ring (Malinska, Dauter, Kowiel, Jaskolski, & Dauter, 2015). Namely, at low pH, both imidazole nitrogens can be protonated and can create a hydrogen bond with a carbonyl oxygen atom of a main-chain peptide group of any residue. At low pH, the proton affinity of both His imidazole nitrogen atoms is fairly high and ranging between - 250 and - 350 kcal/mol (Li & Hong, 2011). While involvement of the hydrogen bonds in this specific example is complicated (Herschlag & Pinney, 2018), the trends seen here and taking into account the entire complex milk system could not be simply be assigned to a single interaction like hydrogen bonding, but include all other interaction as well, such as hydrophobic bonding, electrostatic interactions, and van der Waals attractions (De Kruif et al., 2012). Proline, on the other hand, is generally nonpolar and has properties opposite to those of His, it provides rigidity to the polypeptide chain by imposing certain torsion angles on the segment of the structure and has no hydrogen donor or acceptor atoms in its side chains (Morgan & Rubenstein, 2013). Although the data indicated only a minor relationship between hydrogen ion and His/Pro, Higgins and Fraser (1954), on the basis of titration data and differences between the spectra of azo-derivatives of CN, suggested likely participation of His in the gel formation of rennet curd. To date this effect has not been observed in acid milk gels, probably due to a larger focus on the effect of calcium phosphate nanoclusters on the structure of CN micelles and its influence on acid-induced gels (Bijl et al., 2014). To confirm this hypothesis and to explain to what extent the CN micelle interactions were influenced by the presence of A1/A1, A1/A2, or A2/A2 β-CNs, including the presence of His or Pro, a significantly larger set of milk proteins and shorter standalone peptides carrying known phenotype exposed under various environmental factors would need to be analysed.

Calcium (Ca) also plays a significant role in milk coagulation in part because coagulating milk samples contain higher levels of total and micellar Ca than non-coagulating samples (Poulsen et al., 2017), which is also observed in the current study ($p < 0.05$, Table 2). The relative distribution between micellar and total Ca appeared to be similar between A1/A1 and A1/A2 gels; this association mainly reflects the relation between the phosphoserine residues of all CNs and Ca ions. McSweeney and Fox (2013) explained that CNs bind Ca in their phosphoserine clusters in the following order: $\alpha s_2 > \alpha s_1 > \beta >$ κ-CNs, resulting from their different levels of phosphorylation. In the current research, A1/A1 and A1/A2 gels in their colloidal phase contained greater amount of αs_2-, β-, and κ-CNs, which might account for the observed high amounts of Ca in these gels. Moreover, Parker and Dalgleish (1981) in the past presented that the binding of Ca ions to β-CN increases with a concomitant decline in pH, as depicted in the present results in case of A1/A2 gel and its high amount of micellar β-CN (Table 1). In contrast, an extensive dissociation of Ca upon acidification in A2/A2 gel resulted in a weaker gel that confirms a previously reported observation (Nguyen et al., 2018). In this regard, the decreased amount of micellar Ca or its greater serum concentration, as in A2/A2 gel (Table 2), can both influence the properties of an acid milk gel at pH < 5 (4.5–4.9). Ozcan-Yilsay, Lee, Horne, and Lucey (2007) demonstrated that the greater rearrangements of the CN micelle structure was initiated by higher levels of micellar Ca resulting in gels with high G' values and a pronounced elasticity, as in both gels that carried A1 β-CN. Notably, as the gelation of all milk types started between pH 5.1 and 5.3, it is expected that CCP be still not completely solubilised (Lucey, 2020). Respectively, it may cause different rearrangements during the formation of the acid gels, providing lower G'_{30} values for A1/A1 and A1/A2 gels compared to that of the A2/A2 gel (Table 3) even though the concentration of micellar Ca is higher in the prior gels. Similar results were found elsewhere in the literature on gels optimised at 40 °C (Koutina, Knudsen, Andersen, & Skibsted, 2014).

Interestingly, upon acidification β-turns were absent in A1/A2 gels, however, both homozygous gels contained 10% (A1/A1 gel) and 30% (A2/A2 gel) of these structures. Huppertz (2013) explained that 20–30% of turns in milk can be formed by β-CN and can be defined as Pro or non-Pro based. Due to the cyclic structure of Pro residues the formation of β-turns can occur, hence it might be expected that a greater level of β-turns would be present in A2/A2 gels as it contains more Pro residues (McSweeney & Fox, 2013), which is in line with the current results. However, β-turns containing Pro residues may also result in van der Waals attractions with surrounding residues, leading to assignment of the Pro residue initially to a β-turn conformation; the turn structures appeared at f67-70 and were observed only in the A1 β-CN genetic variant (Graham, Malcolm, & McKenzie, 1984; Kumosinski, Brown, & Farrell Jr, 1993). This might lead to the presence of β-turns also in A1/A1 gel. Nevertheless, the loss of β-turns in A1/A2 gel upon gelation process may reflect the slow growth of helixes from neighbouring turns (α-helixes slightly increased only in this gel), a phenomenon that was also observed in the study of Prystupa and Donald (1996), who examined gelation of gelatine.

The gels comprised of either A1/A1 or A1/A2 milks were characterised with a high proportion of aggregated β-sheets ($p < 0.05$, A1/A1

and A1/A2 milks, Table 4). Previously, it was discovered that their increasing concentration influenced the conformational rearrangements of β-Lg (Qi, Ren, Xiao, & Tomasula, 2015), which possesses eight β-sheet structures in its monomeric form with 43% of the total secondary structure in this protein (McSweeney & Fox, 2013). Nonetheless, because the samples were not heated and approximately 20% of the total β-Lg was present in the micelle upon acidification (Table 1), it could be assumed that the current findings were mainly related to the features of CN micelles in milk, namely the structural orientation of β-CN (A1 or A2 variants) and κ-CNs (A or B variants). Both gels containing A1 β-CN in their structures contained greater amount of micellar κ-CN, where a great amount of micellar β-CNs were observed in all three milk types, thus governing the observed behaviour. Following the published data, the various secondary orientations of β- and κ-CNs are responsible for about 15–33% and 20–35% of β-sheets detected in bovine milk, respectively (McSweeney & Fox, 2013). Other studies presented that proteins with approximately 40% α-helical conformation (as in A1/A1 and A1/A2 milk, Table 4) tend to form β-sheet structures during the aggregation due to the gelation process occurring at lower temperatures (Cabra, Arreguin, Vazquez-Duhalt, & Farres, 2006; Fink, 1998; Kunjithapatham et al., 2005; Wei et al., 2018). Thus, the aggregated β-sheet formation seems to be involved in protein aggregates and gel network formation ($p < 0.05$) which confirmed a previously reported observation (Nishinari, Zhang, & Ikeda, 2000). Recently, the increase of β-sheet structures indicated the exposure of hydrophobic regions, and thus an exposed surface hydrophobicity of the protein (Ragab et al., 2020), as in the case of A1 β-CN (Raynes et al., 2015). Histidine^{67} a buried polar amino acid was found to result in a significant increase in the rate of protein folding (Dyson, Wright, & Scheraga, 2006) indicating the presence of β-sheet structures, similar to the current data in which both gels with A1 β-CN showed an increased amount of β-sheets.

Upon acidification, pH was found to have a significant impact on the existence of random coils that might be initiated by the changes in β-sheets and α-helixes in all three types of gels, navigating the possibility for losing the proteins' secondary structure ($p < 0.05$, Table 3) (Nishinari et al., 2000). This was observed by high level of random conformations only for samples comprised of A2 β-CN (A1/A1 gel showed an absence of these structures, Table 3), which were around 3-fold more present in A2/A2 than in A1/A2 gel ($p < 0.05$). Hence, if the secondary structures are totally diminished, proteins start to behave as random coils and their gel formation is hindered as well (Nishinari et al., 2000); similarly to the A2/A2 gels. The random coil structural motif has been assigned to β-CN with an estimated secondary structure from 23 to 70% (Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015). Since A1/A2 and A2/A2 gels had more micellar β-CN compared to A1/A1 gel, therefore it is expected that A2 β-CN is the driver for the formation of random coils in its counterpart gel (Tables 1 and 4). Dukor and Keiderling (1991) stated that random conformations are short polyproline II helixes (PPII) and were particularly found in A2/A2 milk (Daniloski, McCarthy, Markoska, et al., 2022). Therefore, in the A2/A2 acid gel the presence of an additional Pro^{67} may govern the existence of PPII helixes (Table 4) and disrupt the formation of α-helixes (Daniloski, McCarthy, Markoska, et al., 2022). This Pro^{67} is part of two successive proline-rich motif (Pro-X-Pro) sequences and theoretically these sequences can be part of PPII structure (Daniloski, McCarthy, Markoska, Auldist, & Vasiljevic, 2022). Evidence of the presence of these conformations was found in A2/A2 milk and A2 β-CN by utilising FTIR and Raman measurements, respectively (Daniloski, McCarthy, Markoska, et al., 2022; Syme et al., 2002). It has been suggested that the PPII structure supports low-affinity binding that generally is a feature of the intrinsically disordered regions of proteins (the binding motifs are conserved in intrinsically disordered regions) (Adzhubei, Sternberg, & Makarov, 2013), which might be the reason for the less dense network in A2/A2 gel. Contrarily, the random coil structures were not observed in A1/A1 gel, indicating less unfolding of the protein secondary structure (Markoska, Daniloski, Vasiljevic, & Huppertz, 2021; Nishinari et al., 2000) and therefore a firmer gel.

5. The impact of other casein phenotypes and composite genotypes on the acid-induced gelation properties

The physicochemical and rheological properties of acid-induced gels as a function of the genetic variants of diverse CNs has been reported in the literature (Poulsen & Larsen, 2021), however, the knowledge on their structural differences is scarce and new insights are still warranted. Despite the fact that more than 39 phenotypes have been assigned to all 4 CNs (Huppertz, Fox, & Kelly, 2018), in the present study the three milk types were comprised of B/B αs_1-CN; A/A αs_2-CN; A1/A1, A1/A2, and A2/A2 β-CNs; and A/B κ-CN (data not shown), which all could potentially influence milk gelation (Gai, Uniacke-Lowe, O'Regan, Faulkner, & Kelly, 2021). In particular, κ-CN and its various phenotypes have been most discussed and related to milk coagulation characteristics (Bijl et al., 2014; Bonfatti et al., 2010; Poulsen et al., 2013). Bisutti et al. (2022) and Gambra et al. (2013) explained that the genetic polymorphisms of κ-CN might substantially influence the rennet-induced coagulation properties. Namely, the presence of B κ-CN in bovine milks was correlated to smaller CN micelles, higher content of total κ-CN, and decreased curd-firming time compared to milks containing A κ-CN (Bisutti et al., 2022; Gambra et al., 2013). Almost a decade ago, Poulsen et al. (2013), Jensen, Holland, Poulsen, and Larsen (2012) and Vallas et al. (2012) correlated the lower levels of total κ-CN and the presence of A κ-CN with bigger size of the CN micelle and poor rennet-induced coagulation of bovine milk. Bijl et al. (2014) stated that A and B phenotypes of κ-CN and its glycosylation were associated with the average size of CN micelle; nevertheless, in the same study, B κ-CN was predominately present in CN micelles with smaller size (Bijl et al., 2014). Contrarily, in the present study, a greater content of A κ-CN and the highest levels of micellar κ-CN in A1/A1 and A1/A2 milks were connected with good acid-induced gelation and firmer gels. These results are parallel to the study of Ketto et al. (2017) and Ketto et al. (2019). In these studies, the authors revealed that, compared to all CNs, only the κ-CN genotypes influenced the properties of acid gels (Ketto et al., 2017, 2019).

Furthermore, the relation between the κ-CN and its phenotypes with the CN micelle size of individual bovine milk samples was presented in a few studies (Bijl et al., 2014; Bonfatti, Chiarot, & Carnier, 2014; Day et al., 2015; Hallén, Wedholm, Andrén, & Lundén, 2008; Vallas et al., 2012). Recently, Daniloski, McCarthy, Markoska, et al. (2022) and Daniloski, McCarthy, and Vasiljevic (2022) also related the total amount of micellar κ-CN and its path-terminating function with the CN micelle size in un- and heat-treated A1/A1, A1/A2, and A2/A2 milks. Namely, a lower proportion of total κ-CN content was found to correspond well with greater CN micelle size in A2/A2 milk compared to that of A1/A1 or A1/A2 milks (Daniloski, McCarthy, Markoska, et al., 2022), however, the authors did not find the reason behind that behaviour and the possible connection between the β-CN phenotypes and κ-CN.

The β-CN has been known as a potent adsorbent onto hydrophobic surfaces mainly by hydrophobic interactions between its *C*-terminal tail and the surface (Huppertz et al., 2018). Even though A2 β-CN was found to have lower surface hydrophobicity compared to A1 β-CN (Raynes et al., 2015), the tripeptide (only difference between A1 and A2 β-CNs) Ile-Pro-Asn is more hydrophobic compared to the tripeptide Ile-His-Asn (Damodaran & Parkin, 2017), thus providing the β-CN with more localised hydrophobicity and possible connection to κ-CN. In this context, it is noteworthy to consider if actually Pro residues contribute to the pH collapse of the κ-CN brush border and thus alter behaviour during acid-induced gelation. Compared to other peptide bonds that can be found exclusively in *trans* position, the X-Pro peptide bonds that are pH dependent are shown in either *cis* or *trans* isomerism (Daniloski, McCarthy, O'Callaghan, & Vasiljevic, 2022; Ivanova, Yakimova, Angelova, Stoineva, & Enchev, 2010). Given the position of all 35 Pro residues in A2 β-CN (34 in A1 β-CN) (Huppertz et al., 2018) it is worthwhile considering whether *cis-trans* isomerism of the peptide bonds involving these Pro-residues, particularly an additional Pro^{67}, affect the structure and the stability of the CN micelle during acid gelation of bovine milk.

Numerous studies found that A2/A2 milks possessed bigger CN micelle sizes compared to A1/A2 or A1/A1 milks (Daniloski et al., 2022a, 2022b; Day et al., 2015); greater CN micelle sizes have been related to poor gelation properties (Poulsen et al., 2013). More importantly, the hydrophobic environments of β-CNs were observed in the presence of polyproline II (PPII) structures predominately found in samples containing A2 β-CN and additional Pro in the polypeptide chain (Farrell, Wickham, Unruh, Qi, & Hoagland, 2001). The PPII conformation favours to be conserved, leading to open and extended supramolecular structures and hence a larger CN micelle size (Daniloski, McCarthy, Markoska, et al., 2022).

These differences among the studies and their contradicting results may explain which genetic variant provides better gelation properties (Grygorczyk et al., 2013; Lucey et al., 2022). For example the difference between A and B κ-CN variants appear at the amino acid residues in positions 136 and 148 (A: Threonine [Thr] and Asparagine [Asp]; B: Isoleucine [Ile] and Alanine [Ala]); these differences in the amino acid composition were found to affect the post transitional modifications of κ-CN (Bijl, Holland, & Boland, 2020; Gazi et al., 2022). Isoleucine and Ala are hydrophobic and neutral amino acids, respectively, hence, one would expect that B κ-CN would be less soluble and more hydrophobic at pH 6.5–6.7 compared to A κ-CN (Thr and Ile: hydrophilic amino acids), and might be the reason for good milk rennet-induced coagulation (Gai et al., 2021). In contrast, Thr and Asp found in A κ-CN, at pH lower than 5.0 possess a tendency to occur in β-turn and β-sheet conformational motifs (Malkov, Živković, Beljanski, Hall, & Zarić, 2008). As explained in section 4 above, these conformational states are navigating the possibility of retaining the proteins' secondary structure, thus leading to acid-gels with improved properties (Prystupa & Donald, 1996).

The composite genotype of αs_1-β-κ-CN was found to have a stronger relationship with acid-induced gelation than only a single protein phenotype. In this regard, an improved acid gel firming rate (3.2 mm/min), firmness at 30 and 60 min (41 mm), and gelation time were associated to B/B-A2/A2-A/A haplotypes compared to the other proteins' genotypes (Ketto et al., 2017); nevertheless, Jensen, Poulsen, et al. (2012) stated that the same composite haplotype was predominant in poorly coagulating milks. In the present study, milks with good acid gelation properties carried the composite haplotypes B/B-A1/A1-A/B and B/B-A1/A2-A/B, but milk with B/B-A2/A2-A/B genotype showed poorly gelation properties. Interestingly, the composite β-κ-CN genotype (A1/A1-AB; A1/A2-AB; and A2/A2-AB) was associated with better firmness and coagulation time (Comin et al., 2008), which is completely contrasted to our results. Frederiksen et al. (2011) provided a similar outcome to the current results that actually related the composite A/B-A1/A2 haplotype which was considered as a sufficient factor for good milk coagulation properties with higher content of κ-CN in the gels (Table 1).

6. Conclusion

This study showed how genetic variants of β-CN in skim milk influenced acid-induced gelation, with A1/A1 and A1/A2 milks having significantly higher gel strength and lower gel porosity, compared to A2/A2 milk. The associated findings of A2/A2 milk gels may be related to the increased content of random/PPII structures due to the fact that Pro possesses a tendency to create this conformations. However, it is important to note that there may be other individual genetic variants contributing to gel strength, such as α_s-CN, κ-CN, and β-Lg (Poulsen & Larsen, 2021). While the lower gel strength observed in A2/A2 skim milk may negatively influence yogurt functionality, it may also have consequences on curd formation properties during gastric phase of digestion. For almost a century, the physiological importance of curd firmness formed through the gastric coagulation of CNs (which might reflect their acid-induced coagulation) has been focused on the functionality of the so called 'soft and hard curd milks' (Brennemann, 1911; Doan, 1938; Huppertz & Chia, 2021; Ye et al., 2019). Consequently, it has been suggested that the consumption of the soft curd milks might lead to reduced gut difficulties in comparison with the hard curd milks (Huppertz & Chia, 2021); in the current study the A2/A2 gel was softer compared to A1/A1 and A1/A2 gels which might indicate the proposed easier digestibility of A2/A2 milk, and thus a digestive comfort (Daniloski, Cunha, et al., 2021), which may need to be confirmed further in future studies.

Conflict of interest and authorship conformation form

Please check the following as appropriate:

- ✓ All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.
- ✓ This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.
- ✓ The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript
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CRediT authorship contribution statement

Davor Daniloski: Methodology, Formal analysis, Investigation. **Davor Daniloski** conceived the study and research question; designed and wrote the original draft, conceptualised, reviewed, edited the manuscript, designed the tables and the figures. **Todor Vasiljevic** and **Noel A. McCarthy** provided critical feedback and analysis, secured funding, reviewed and edited the manuscript, and supervised the study. **Inge Gazi** provided critical feedback, reviewed and edited the manuscript, and giving insight into clarification of the genetic variants of different milk proteins. All authors have contributed to the manuscript and reviewed the final version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare no conflict of interest.

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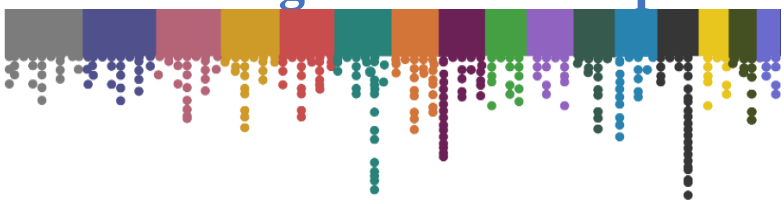

Health-related outcomes of genetic polymorphism of bovine β-casein variants: A systematic review of randomised controlled trials

- Impact of β-casein genetic variants on human health from animal and human *in vivo* studies was systematically analysed
- Both β-casein A1 and β-casein A2 release β-casomorhin7 during the *in vivo* digestion
- Consumption of β-casein A2 may decline in perseverance of gut problems, however, not clinically admissible
- Neither β-casein A1 nor β-casein A2 showed an effect on the other health statuses

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Health-related outcomes of genetic polymorphism of bovine β-casein variants: A systematic review of randomised controlled trials

Davor Daniloski [a,b], Nathan M.D. Cunha [c], Noel A. McCarthy [b], Tom F. O'Callaghan [d], Sinéad McParland [e], Todor Vasiljevic [a,*]

[a] Victoria University, Advanced Food Systems Research Unit, Institute for Sustainable Industries and Liveable Cities and College of Health and Biomedicine, Melbourne, Victoria, 8001, Australia
[b] Teagasc Food Research Centre, Food Chemistry and Technology Department, Moorepark, Fermoy, P61 C996, Cork, Ireland
[c] University of Canberra, Faculty of Health, School of Rehabilitation and Exercise Sciences, Department of Food Science and Human Nutrition, Bruce, Australian Capital Territory, 2617, Australia
[d] University College Cork, School of Food and Nutritional Sciences, T12 YT20, Cork, Ireland
[e] Teagasc Animal and Grassland Research Centre, Moorepark, Fermoy, P61 C996, Cork, Ireland

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ABSTRACT

Background: A number of randomised *in vivo* trials have to date investigated the health impacts of the genetic variants A1 and A2 of bovine β-casein. The primary difference between these two genetic variants is the mutation leading to an amino acid exchange at a position 67 in the peptide chain. This systematic review evaluated the effects of bovine milk, β-casein and pure β-casomorphin7, in the form of orally administered nutritional ingredients, on possible incidence and risk for chronic digestive discomfort and development of incurable conditions and diseases in human and animal randomised controlled trials.

Scope and approach: Following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist, searches were performed for publications across 7 electronic databases (Scopus, Embase, Web of Science, Medline, EBSCO, PubMed, and Cochrane) up to and until July 2020, to identify randomised controlled trials. The subsequent search results were screened for relevance firstly by title, then abstract, and the chosen ones by full text, with additional screening of included articles reference lists.

Key findings and conclusions: In total 2006 peer-reviewed journal articles were identified and after applying exclusion criteria, 19 studies were deemed suitable for inclusion. Human-based and animal-based results from the clinical *in vivo* studies demonstrated that consumption of A2 β-casein milk can lead to improved tolerance of milk via decline in the ubiquity of gut related discomfort. However, the exact mechanism for these effects or specific individuals that may benefit from A2 β-casein milk as opposed to A1 β-casein milk is still poorly understood. Notably, consumption of A2 β-casein milk had very low to completely no effect on the other health statuses investigated, particularly non-communicable diseases, such as cardiovascular diseases, neurological disorders, and diabetes. Based on current data, there is not sufficient evidence to merit public health authority recommendations related to the consumption and health associations of A1 β-casein milk or A2 β-casein milk. Interestingly, regardless of the scientific evidence between A2 β-casein milk and health, this milk continues to gain prominence on the market, thus further functional research is required to understand the mechanisms of action of these identified peptides and gene variants and any implications A1 or A2 β-casein milk may have on human health and techno-functional properties of milks.

1. Introduction

Milk is known as one of the most nutritious food products, in which the gross milk constituents (proteins, lipids, lactose and minerals) are present in appropriate proportions and can have a significant impact on human health over consumers' life-time (Bogahawaththa, Ashraf, Chandrapala, Donkor, & Vasiljevic, 2018). In recent decades, consumers have been increasingly interested in milk and dairy products for their

* Corresponding author.
E-mail address: todor.vasiljevic@vu.edu.au (T. Vasiljevic).

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high-quality attributes including sensory appeal and their ability to meet the daily intake of essential nutrients (O'Callaghan, Sugrue, Hill, Ross, & Stanton, 2019). Bovine milk typically contains 3.5–4% protein, which varies considerably due to several different factors (Goulding, Fox, & O'Mahony, 2020). Almost 80% of these proteins consist of caseins (CNs - originate from the family of phosphoproteins), which are present in milk in form of complex aggregates or CN micelles. The remaining 20% of total milk proteins are termed whey proteins (WPs), predominantly consisting of α-lactalbumin (α-La) and β-lactoglobulin (β-Lg) (Ali, Amin, Asif, & Mansoor, 2019; Farrell et al., 2004).

The CN class of proteins can be categorized into four different types, α_{S1}-CN (CSN1S1, 39–46% of the total CNs), α_{S2}-CN (CSN1S2, 8–11% of the total CNs), β-CN (CSN2, 25–35% of the total CNs), and κ-CN (CSN3, 8–15% of the total CNs) (Bijl, Holland, & Boland, 2020; Huppertz, Fox, & Kelly, 2018). Heterogeneity of CNs further include polymorphisms related to genetic forms and frequently show different extents of post-translational modification (Farrell et al., 2004). For example, twelve different genetic variants of β-CN including A1, A2, A3, B, C, D, E, F, G, H, I, and J have been identified (Nguyen, Busetti, Johnson, & Solah, 2015). Additionally, in the literature it has been shown that the β-CN gene actually contains thirteen variants (instead of H, there are two isoforms H1 and H2) (Patel et al., 2020); while a fourteenth allele, A4, has been detected in Korean native cattle, however, clinical investigations of this genetic sub-type have not been acknowledged to date (Nguyen et al., 2019; Şahin, Boztepe, & Aytekin, 2018).

Variations in CN form stem from DNA mutations, which are primarily nucleotide substitutions governing changes in the proteins' amino acid sequence, which predominantly include amino acid substitutions and, in some instances, deletions (Summer, Di Frangia, Ajmone Marsan, De Noni, & Malacarne, 2020). While raw milk quality is assessed by several criteria, namely microbial content, somatic cell count, gross composition and the absence of contaminants; in terms of genetics, the quality of milk may also be related to the single nucleotide polymorphism in the gene coding for β-CN (Ali et al., 2019). The type of β-CN present in milk depends on hereditary mutations, some forms of which have been hypothesized to be related to the development of several non-communicable diseases in humans, particularly cardiovascular diseases, metabolic and neurological conditions (Brooke-Taylor, Dwyer, Woodford, & Kost, 2017). β-CN constitutes on average 25–30% of proteins in bovine milk and consists of 209 amino acid residues. While a number of proteoforms have been identified, the most common are the A1 β-CN and A2 β-CN genetic variants (Rashidinejad, Bremer, Birch, & Oey, 2017). Initially, all cattle possessed the A2 β-CN genetic variant (the oldest variant), however, the A1 β-CN genetic variant currently prevails due to the single amino acid mutation in European dairy herds a few thousand years ago (Sebastiani et al., 2020). The defining feature of these two types of β-CN is the inclusion of either histidine (His^{67}) in A1 β-CN milk or proline (Pro^{67}) in A2 β-CN milk, located at position 67 in the polypeptide chain (Jianqin et al., 2015). The presence of a particular amino acid residue induces conformational changes, which in turn affect patterns of enzymatic cleavage during digestion. For example, His^{67} in A1 β-CN milk allows for easier proteolytic cleavage releasing a range of different peptides including β-casomorphins (BCMs), while Pro^{67} in A2 β-CN milk hinders cleavage by digestive enzymes due to structural features (Summer et al., 2020). Owing to the fact that the genetic variation between these two β-CN proteoforms has been clarified by the replacement of a single amino acid in this position, it is more likely that this mechanism results in alternation of the protein functions and functionalities, enzymatic and acidic hydrolysis and liberation of bioactive peptides (BCMs); thus, it may have an influence on further milk processing and human nutrition (Cieślińska et al., 2012; Poulsen et al., 2017). Recently, some studies have indicated that even A2 β-CN milk structurally can allow for release of β-casomorphin7 (BCM7) just less readily and less abundantly compared to A1 β-CN milk (Asledottir et al., 2017, 2018; Cieślińska et al., 2012). Notably, the other genetic variants, including F β-CN and B β-CN from the A1 β-CN family, and A3 β-CN and I β-CN from the A2 β-CN family have been found to liberate BCM7 during digestion. Therefore, upon proteolysis of A1 β-CN family or A2 β-CN family, short BCM opioid peptides are released (Asledottir et al., 2018). These peptides are recognised for their μ-opioid features and a high opioid receptor affinity (Haq, Kapila, & Kapila, 2015). In general, BCMs are comprised of 4–11 amino acid peptides stored in an inactive state within the native protein sequence and are released during either *in vivo*, *in vitro* or *ex vivo* gastrointestinal digestion. The most prominent of these are BCM5 and BCM7, which represent fragments f60–64 and f60–66 of β-CN, respectively (De Noni, Stuknytė, & Cattaneo, 2015).

Moreover, in humans, consumption of A1 β-CN milk has been hypothesized to be related to a greater likelihood of certain allergies, as well as eczema and asthma, and also some non-communicable diseases (Summer et al., 2020). It is for these reasons, recent research studies have been performed to examine possible implications of increased frequency of the A2 β-CN allele in dairy cattle breeds, since it may have possible technological uses and allegedly increased milk digestibility (Cieślińska et al., 2019; Massella et al., 2017; Sebastiani et al., 2020). Dairy food intolerance is a frequently documented gastrointestinal condition, typically associated with lactose intolerance (Brüssow, 2013), often by association without clinical confirmation. Nonetheless, depending on the gastrointestinal fate of BCM7 (and therefore the family of A1 β-CN milk), it is conceivable that, in some situations, intolerance of dairy products could be due to difficulties associated with digestion of A1 β-CN rather than lactose, per se (Jianqin et al., 2015). This may contribute to several symptoms, including analgesia, sedation, mildly lowered blood pressure, fatigue, increased pacing, and diminished bowel motility, among others (Brooke-Taylor et al., 2017). Moreover, BCM7 has been suggested to be responsible for elevated risk of diabetes (Küllenberg de Gaudry et al., 2019). Notably, before making any judgement on the effects of the presence of BCM7 in the human gut, problems related to increased permeability of the intestinal membrane should be taken into consideration and the potential for a BCM7 peptide to cross this membrane and enter circulation. As a result of increased permeability of the intestine (so called "*leaky gut*") caused by several conditions, including stress, stomach ulcers, ulcerative colitis, autism, some individuals may be predisposed to the physiological effects of these peptides liberated from specific genetic variants of β-CN (Asledottir et al., 2017; Halverson & Alagiakrishnan, 2020). Therefore, the question remains - can BCM7 pass the gut-blood barrier and be transferred to other organs? It may be possible in some individuals suffering from increased permeability of the gut membrane due to underlying conditions or an undeveloped intestinal barrier in the case of infants or older patients (Shani-Levi et al., 2017). In fact, autistic children suffering from a "*leaky* gut" showed the presence of BCM7 in their urine (Sokolov et al., 2014). In the literature, a few *in vivo* studies indicate the presence of BCM7 in the blood serum of human infants (Kost et al., 2009; Wasilewska et al., 2011). These findings are contradictive to the putative mechanism of "*leaky gut*" syndrome and for a conclusive confirmation, this hypothesis needs to be tested under clinical conditions.

This is the first systematic review, to our knowledge, of published *in vivo* studies investigating the health effects of A1 β-CN and A2 β-CN genetic variants with naturally released BCM7 from bovine milk (after consumption of bovine milk and digestion of β-CN). At present, the evidence to substantiate the claims related to the negative impact of A1 β-CN milk or beneficial effects of milk other than the family of A1 β-CN milk, is relatively weak and, in many instances, contradictory. The criteria for this systematic review was the hypothesis that the ingestion of A1 β-CN contributes to exposure of tissue to BCM7, which has a number of pro-inflammatory consequences and modified epigenetic control of gene expression (Summer et al., 2020; Trivedi, Zhang, Lopez-Toledano, Clarke, & Deth, 2016). Consequently, the aim of this systematic literature review was to critically analyse the existing data from well-controlled human clinical trials and animal studies on the impact of ingestion of A1 β-CN milk and A2 β-CN milk on any health-related outcomes.

2. Materials and methods

This systematic literature review was conducted following the PRISMA 2009 guidelines (Moher et al., 2015) and was prospectively registered in an international registry of systematic reviews (PROSPERO registration no. CRD42020178056). The systematic review strategy was guided by the PICOS (population, intervention, comparator, outcomes and setting) approach. The criteria within each of these categories were as follows:

- Population: Human and animal populations of any age or health status;
- Intervention: Original studies investigating the effects of A1 β-CN milk and A2 β-CN milk on human and/or animal health;
- Comparator: Human and/or animal comparators, using a placebo or control intervention;
- Outcomes: Data analysed according to the influence of their health statuses (e.g. an increased risk of non-transmissible diseases, such as cardiovascular disease, cancer, or diabetes);
- Setting: Participants must have been consuming bovine milk, particularly A1 β-CN milk, A1/A2 β-CN milk (conventional), and/or A2 β-CN milk; if BCM7 had been liberated during the *in vivo* digestion the presence and bioavailability of BCM7 should have been explained.

2.1. Systematic literature search

Several different electronic databases were systematically searched from inception: Scopus, Embase, Web of Science, Medline, Food Science and Technology Abstracts (EBSCO), PubMed, and Cochrane databases, until July 2020. The search results were screened for relevance firstly by title and abstract. In the case of any publications where that was not clear, the paper was referred to a full-text analysis and checked for final inclusion (Fig. 1). Finally, an additional screening of included articles reference lists has been performed for clarification. Results were limited to human and animal quantitative intervention trials based on the consumption of A1 β-CN milk and A2 β-CN milk, investigating their effects on human and animal health, published in English, in peer-reviewed journals. The search terms used were: (A1 AND milk OR Non-A1 milk; OR A1_betacasein OR A2_betacasein) and (beta-casomorphin; or betacasomorphin7; or beta-casomorphin-7; or beta-casomorphin).

2.2. Study selection and eligibility criteria

Information was retrieved manually and separately from each database. Studies that assessed outcomes related to the direct effects of A1 β-CN proteoform compared to non-A1 β-CN proteoform (A2 β-CN proteoform) in the human or animal trials were chosen. Studies which

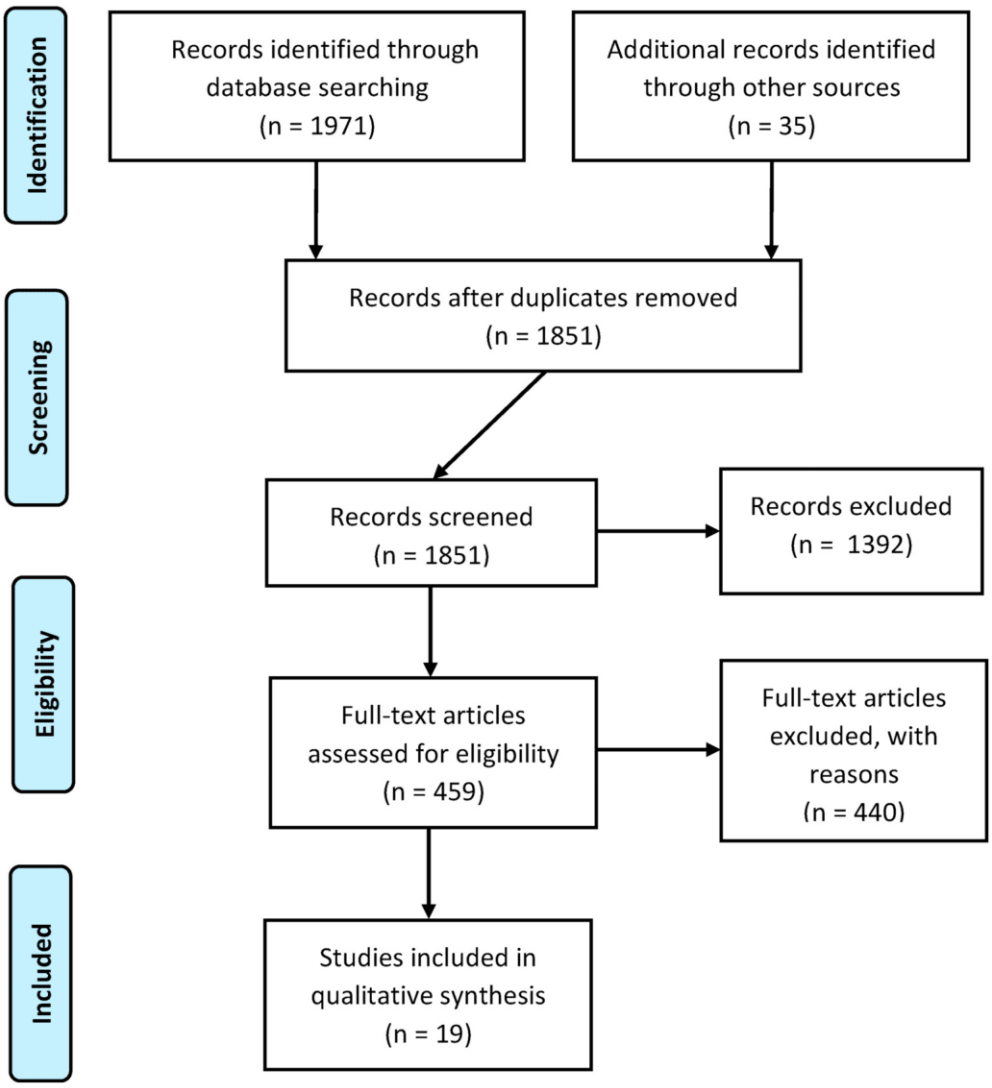

Fig. 1. PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) flow diagram for eliminated and included search literature.

did not include appropriate outcome variables, or which were only accessible as abstracts from conference presentations, observational studies, reviews, study protocols or studies that did not report relevant outcome measures were excluded. Two authors (D.D. and T.V.) independently performed article screening using the Covidence Systematic Analysis Tool (Veritas Clinical Research, Melbourne, Australia). Both authors evaluated the search results against the selection criteria on the basis of the title, abstract, and full-text analysis (as previously explained). Any disagreement was resolved through consensus or by discussion with a third author (N.M.D.). The authors excluded all studies in which the intervention was not related to bovine milk, CN, β-CN or BCM7 and the study was not *in vivo*. Moreover, all conference papers, letters to editors, abstracts, patents, original papers that were not available in English, were excluded.

2.3. Data extraction and risk of bias assessment

Two authors (D.D. and T.V.) carried out data extraction and risk of bias assessment separately, and any dispute was settled through consent or debate with one co-author (N.M.D.) until a consensus was reached. The corresponding details were extracted into tables for each specific human and animal sample: study design, the characteristics of the participants, the characteristics of both experiment/exposure and control operation, and the outcome results. The methods used for the risk of bias assessment differed based on the scope of the analysis. The Cochrane risk of bias assessment was used to determine the likelihood of bias in randomised controlled trials (RCTs), including the unique possible risk of bias (RoB) related to crossover trials (Higgins et al., 2019). This tool includes criteria for assessing sequence generation, allocation concealment, blinding of participants, personnel, and outcome assessors, incomplete outcome data, and selective outcome reporting. For each criterion, studies were assessed for risk of bias as low, unclear, or high.

3. Results

3.1. Study selection

A total of 2006 articles were identified during the systematic search of electronic databases. The secondary search identified 35 new documents from other sources. Following elimination of duplicates, 1851 articles were screened for eligibility. Of these, 1392 records were excluded after title and abstract screening, and another 440 were excluded after full text screening. Finally, 19 documents (original research studies) met the requirements for inclusion and were incorporated in this systematic review (Fig. 1). The vast majority of the articles were excluded due to three main factors: 1) not clarifying the bovine milk and which CN forms have been examined (n = 144); 2) not classifying milk that contain A1 β-CN or A2 β-CN genetic variants (n = 981); and 3) not presenting the direct extraction of the BCM7 peptide from A1 β-CN and A2 β-CN milks (i.e., naturally released from milk; n = 267). Instead, these studies contained BCM7 synthetically created in a laboratory or provided by the pharmaceutical industry. Other reasons for exclusion were: non-interventional studies (n = 232); review papers (n = 42); not including oral exposure to test materials for the *in vivo* studies (n = 85); and not including an appropriate outcome for determining gastrointestinal, cardiovascular, metabolic, and nervous conditions (n = 81).

3.2. Study characteristics

All 19 studies included in this review were randomised controlled studies (Barnett, McNabb, Roy, Woodford, & Clarke, 2014; Boutrou et al., 2013; Chabance et al., 1998; Chia et al., 2018; Chin-Dusting et al., 2006; Crowley, Williams, Roberts, Dunstan, & Jones, 2013; Deth, Clarke, Ni, & Trivedi, 2015; González-Domenech et al., 2020; Guantario et al., 2020; Haq, Kapila, Sharma, Saliganti, & Kapila, 2014; He, Sun, Jiang, & Yang, 2017; Ho, Woodford, Kukuljan, & Pal, 2014; Jianqin et al., 2015; Kamiński, Cieslinska, & Fiedorowicz, 2012; Kirk et al., 2017; Milan et al., 2020; Sheng, Li, Ni, & Yelland, 2019; Venn, Skeaff, Brown, Mann, & Green, 2006; Yadav et al., 2020). Thirteen trials described the digestion of bovine milk, fresh milk products, CN (β-CN) or BCM7 within the scope of *in vivo* human trials, including adults (Boutrou et al., 2013; Chabance et al., 1998; Chin-Dusting et al., 2006; Deth et al., 2015; He et al., 2017; Ho et al., 2014; Jianqin et al., 2015; Kirk et al., 2017; Milan et al., 2020; Venn et al., 2006); children and adolescents (Crowley et al., 2013; González-Domenech et al., 2020; Sheng et al., 2019). Additionally, 6 studies reported bovine CN, β-CN and/or BCM7 activity *in vivo* in animals (Barnett et al., 2014; Chia et al., 2018; Guantario et al., 2020; Haq et al., 2014; Kamiński et al., 2012; Yadav et al., 2020) (Table 1). Table 1 outlines the included participants, type of study (trial), examined population, country conducting the examination, the study support (funding agencies), and the final outcomes from all trials. Some studies were allocated to more than one category depending on the relevant content. Additionally, Supplemental Tables 2, 3, and 4 summarise the effect of bovine milk, CNs, β-CN genetic variants, and/or BCM7 on the gastrointestinal system (GIT), cardiovascular diseases (CVD), and diabetes mellitus (DM), respectively. Supplemental Table 5 presents the association with neurological disorders (ND) and Supplemental Table 6 provides an overview of the influence from different β-CN genetic variants on athletic performance, pulmonary inflammation, and provides a summary of other health-related conditions, affected by consuming A1 β-CN milk or A2 β-CN milk as part of the diets (see Supplemental Tables 2–6). Fig. 2 illustrates the hypothesized health effects of bovine BCM7 liberated from specific variants of β-CN, described in this study.

3.3. Risk of bias and methodological assessment

The risk of bias (RoB) and methodologies were assessed by two researchers (D.D. and N.M.D.) for all included studies according to the Cochrane RoB tool (Figs. 3 and 4 [a - human trials; and b - animal trials]) (Higgins et al., 2019). The overall RoB was categorized as "Low" for both human and animal trials because blinding was likely adequate, and the outcome data was complete and unlikely to have resulted in bias. All RCTs (n = 19) presented clear and well-defined objectives and data analysis methods. However, there was considerable heterogeneity across the studies in study design, type of intervention, control group, exposure to the bovine milk, CN or BCM7, outcomes, and included participants. Therefore, the data could not be agglomerated nor could be considered as conclusive evidence. It is for this reason, the methodological limitations within their approaches and results occurred.

3.3.1. Study design

In both human and animal *in vivo* trials, the study designs were not comprehensively described in all trials and sample sizes varied considerably (human: n = 6–600; and animal: n = 6–48). Four human RCTs, including Boutrou et al. (2013) (n = 16) (Chabance et al., 1998); (n = 6) (Chin-Dusting et al., 2006); (n = 15); and (Kirk et al., 2017) (n = 21); and one animal RCT (Kamiński et al., 2012) (n = 6) had small sample sizes. In contrast, one human RCT (He et al., 2017) (n = 600) and one animal RCT (Barnett et al., 2014) (n = 48) included the highest number of participants within their groups. Whilst these were appropriate sample sizes for the study designs and within-subject comparisons, larger scale, longer and sufficiently powered studies are needed to have further confidence about the impact of the interventions; it can lead to a decreased generalisability of the results (Ho et al., 2014). The majority of studies had "Low" risk of selection bias, and some of them showed "Unclear" risk of selection bias. However, the risk of selection bias was "High" for the human study of González-Domenech et al. (2020) due to being single-blinded. Three human trials with a crossover design (Chin-Dusting et al., 2006; González-Domenech et al., 2020; Venn et al., 2006) did not include a washout period. Instead of introducing a

Table 1
Characteristics of the included randomised controlled trials.

Study	Type of Study/Trial	Examined Population	Country and study funding support	Outcomes
Chabance et al. (1998)	*In vivo* (Human) - *Gastrointestinal tract problems*	6 Healthy Volunteers (3 males and 3 females). Age: 24–49 years. Weight: 55–92 kg.	**France** Candia Institute and Ministère de l'Enseignement Supérieur et de la Recherche de la France entitled 'L'Aliment' demain (tomorrow's food).	The number and size of the peptides derived from CN, decreased between the stomach and the end of the duodenum. These results supported the concept that food-born peptides could induce physiological activities in human.
Boutrou et al. (2013)	*In vivo* (Human): Single-blinded parallel study. - *Gastrointestinal tract problems*	16 Healthy Subjects (10 males and 6 females). Age: 18–40 years. BMI (mean - kg/m^2): <30; 28.3 ± 1.8.	**France** No information on funding	*In vivo* presence of a wide range of bioactive peptides in jejunal effluents of humans fed with milk proteins. The study did not give any information of the role of these peptides in the intestinal tract or if they modulate physiologic functions.
Barnett et al. (2014)	*In vivo* (male Wistar Rats): Controlled animal study. - *Gastrointestinal tract problems*	48 Animals (48 males). Age: 4 weeks. Weight: N/A.	**New Zealand** The A2 Milk Company Limited	Consumption of A1 β-CN milk in rats caused increased total gastrointestinal transit time (as measured by titanium dioxide), together with an increased colonic myeloperoxidase activity in the colon and DPP-4 activity in the jejunum.
Haq et al. (2014)	*In vivo* (male Swiss albino mice): Controlled animal study. - *Gastrointestinal tract problems*	24 Animals (24 males). Age: N/A. Weight: 20–25 g.	**India** Director ofNational Dairy Research Institute (ICAR), Karnal.	The consumption of A1/A1 β-CN milk induced inflammatory response in gut by activating Th_2 pathway as compared to A2/A2 β-CN genetic variant.
Ho et al. (2014)	*In vivo* (Human): Cross - over study. - *Gastrointestinal tract problems*	41 Healthy Participants: 12 males and 29 females (79 initially recruited, 37 excluded, 1 declined consent). Age: 19–68 years. Weight: N/A	**Australia** The A2 Milk Company Limited	In comparison with consuming A2 β-CN milk, A1 β-CN milk presented significantly higher BSS stool consistency values, digestive discomfort, greater abdominal pain (related to softer stool), associated intolerance measures.
Jianqin et al. (2015)	*In vivo* (Human): Cross - over study. - *Gastrointestinal tract problems* - *Neurological disorders*	45 Healthy Participants (males or females). Age: 25–68. Weight: N/A.	**China** The A2 Milk Company Limited	Consumption of milk containing A1 β-CN was related with enlarged gastrointestinal inflammation, worsening of gastrointestinal symptoms of post-dairy digestive discomfort (PD3) symptoms, delayed transit, and decreased cognitive processing speed and accuracy.
Crowley et al. (2013)	*In vivo* (Human): Cross - over study. - *Gastrointestinal tract problems*	39 Children diagnosed with CFC (25 boys and 14 girls). Age: 21 months - 12 years. Weight: N/A	**Australia** University of Newcastle	The trial failed to show an effect of from type of CN. Some other components in bovine milk common to both A1 β-CN milk and A2 β-CN milk may be causing a problem in these susceptible children.
Kirk et al. (2017)	*In vivo* (Human): Parallel study. - *Health - related outcomes*	21 Healthy Males (regularly competed in team-sports). Age: Placebo - mean (22 ± 1 y); Regular Milk - mean (23 ± 1 y); and A2 milk – mean (23 ± 1 y). Weight: Placebo - mean (77.1 ± 7.8); Regular Milk - mean (81.4 ± 13.1); and A2 milk – mean (79.4 ± 10.1).	**United Kingdom** No finance was provided for conducting the study	Significant differences between the types of milk were not found. Consumption of 500 ml of A2 β-CN milk or regular milk following repeated sprint exercise, prevented decrements in muscle function in team sports athletes, therefore, reducing recovery time. The study supported the utilisation of A2 β-CN milk for athlete recovery in sports nutrition settings due to the possible benefit of A2 β-CN milk to athletes who suffer from intolerance/ allergies of A1 β-CN milk.
Chin-Dusting et al. (2006)	*In vivo* (Human): Cross - over study - *Cardiovascular diseases* - *Diabetes mellitus*	15 asymptomatic participants (6 males and 9 females) at high risk of developing CVD. Age (f): 40–67 years; Age (m): 32–66 years. Weight: N/A.	**Australia** The A2 Milk Company Limited, New Zealand	The study showed no indication that supplementation with A1 β-CN had any cardiovascular health negative effect in comparison to A2 β-CN. Nothing substantially improved after dietary treatment of A1 β-CN relative to A2 β-CN. Plasma insulin levels were not altered by either intervention. Given as a dairy shake supplement, A2 β-CN has no diabetes protective effects over A1 β-CN.
Venn et al. (2006)	*In vivo* (Human): Cross - over study - *Cardiovascular diseases*	55 Healthy Adults (24 females and 31 males). Age (mean): 43 years. Weight (mean): 77.0 ± 13.9 kg. BMI (mean): 26.6 kg/m^2.	**New Zealand** University of Otago	The findings of the analysis concerning the impact of β-CN variants on plasma cholesterol in humans suggested that A1 β-CN and A2 β-CN did not have a specific influence on cardiovascular risk and did not show any differences.
He et al. (2017)	*In vivo* (Human): Cross - over study - *Gastrointestinal tract problems*	600 Participants self-reported with lactose-intolerance (males and females). Age: 20–50 years. Weight: N/A.	**China** The A2 Milk Company Limited	Milk containing A2/A2 β-CN reduced acute gastrointestinal symptoms of milk intolerance, while conventional milk containing A1/A2 β-CN decreased lactase activity and increased gastrointestinal symptoms.
Deth et al. (2015)				

(*continued on next page*)

Table 1 (*continued*)

Study	Type of Study/Trial	Examined Population	Country and study funding support	Outcomes
	In vivo (Human): Cross-over study. - *Health - related outcomes*	45 Participants self-reported with mild to moderate digestive discomfort (21 males and 24 females). Age: 25–68 years. Weight: N/A.	**China** The A2 Milk Company Limited	Daily consumption of commercially available conventional milk (both genetic variants) was associated with an increase in GSH concentrations, especially milk that contained A2/A2 β-CN. The A2 β-CN milk offered greater antioxidant capacity then A1 β-CN milk.
Sheng et al. (2019)	*In vivo* (Human): Cross-over study. - *Gastrointestinal tract problems*	75 Preschool Children (males and females) mild-to-moderate milk intolerance. Age: 5–6 years. Weight: N/A.	**China and Australia** The A2 Milk Company Limited	Negative influence of conventional milk (A1/A2 β-CN) on inflammation associated with lactose Intolerance in Chinese preschool children, with corresponding improvements in aspects of cognitive performance.
Milan et al. (2020)	*In vivo* (Human) - *Gastrointestinal tract problems*	40 Healthy females. Age: 20–30 years. Weight: N/A.	**Sweden and New Zealand** New Zealand Ministry of Business, Innovation, and Employment (MBIE) through the High-Value Nutrition National Science Challenge (HVN); A2 Milk Company Limited.	In self-reported intolerant, diagnosed lactose intolerant individuals, lactose malabsorption the digestive comfort with lactose-containing milks was ameliorated with milk containing exclusively A2 β-CN.
Kamiński et al. (2012)	*In vivo* (Gilts) - *Cardiovascular diseases*	6 Sibling Gilts (females). Age: 83 days. Weight (mean): 33 kg.	**Poland** University of Warmia and Mazury (project no. 0105–0804).	Based on the findings of this study it was suggested that there had not been any blood problems among pigs which were fed with A1/A1 β-CN or A2/A2 β-CN milk, and the correlation between A1/A1 β-CN milk and CVD was not observed.
Chia et al. (2018)	*In vivo* (Mice) - *Diabetes mellitus*	The number of mice (males and females) included dependend on the procedure and generation. Age (F0 – F4 generation): 3–30 weeks. Weight: N/A.	**Australia** Innovation Connections Grant (ICG number: RC54051) of the Department of Industry, Innovation and Science, Australia; A2 Infant Nutrition Australia Private Limited.	The results from this study demonstrated that A1 β-CN diet can directly influence the homeostasis and T1D incidence, however, this process requires generations in order to be indicated.
González-Domenech et al. (2020)	*In vivo* (Human): Cross-over study. - *Neurological disorders*	37 Patients Diagnosed with Neurogogical Disorder(s). Initially: Group A: 20 Pateints (13 males and 7 females). Age (A): 3–16 years. Group B: 17 Participants (16 males and 1 female). Age (B): 2–18 years Weight: N/A.	**Spain** No information on funding.	This study showed that Gluten-free and Casein-free diets did not influence or change BCM7 concentration in urine nor correlate with behavioural symptoms of autism.
Yadav et al. (2020)	*In vivo* (Balb-c Mice) - *Pulmonary inflammation*	No information on the number of male Balb-c Mice. Age: 3–4 weeks. Weight: N/A.	**India** No information on funding	Long-term feeding of A1/A1 β-CN milk induced significant Th2-driven allergic airway inflammation. A2/A2 β-CN milk did not induce inflammation but rather seemed to have a protective effect for allergies and asthma.
Guantario et al. (2020)	*In vivo* (Balb-c Mice) - *Gastrointestinal tract problems*	24 Balb-c ageing healthy Mice (males and females). Age: 20 months. Weight (mean): Control = 29.1 ± 3.1 g; A1/A2 = 28.3 ± 4.1 g; A2/A2 = 28.4 ± 3.8 g.	**Italy** Centrale del Latte di Italia S.p.A. and CNR project NUTR-AGE (FOE-2019, DSB.004 CE.271).	The results suggested a positive role of milk, particularly when the gastrointestinal system has been exposed to A2 β-CN, on the aging mice model's gut immunology and morphology.

washout period where the bovine milk from particular β-CN genetic variant has been washed out of the patients' system, the authors immediately stopped and then started the new treatment. Therefore, these studies and were rated "High" for risk of other bias (Fig. 3 a and 3 b).

3.3.2. Sampling strategy, participants' characteristics, and outcome measures

All included human and animal RCTs described the human participants and animal subjects in sufficient detail, excluding the study by Yadav et al. (2020) which did not provide information on the number of animal subjects; however, it did mention their age. Hence, the overall RoB for this study was "Low". Three human RCTs (Crowley et al., 2013; González-Domenech et al., 2020; Ho et al., 2014) included participants from four different age groups (toddlers, adolescents, adults and older people) and one animal RCT included subjects from four generations (Chia et al., 2018), which increased the generalisability of findings.

The included human studies were performed in ten countries, only with domestic participants and only minimal demographic information. Hence, these findings may not be applicable to other countries with different cultural behaviour and socio-economic conditions. Out of nineteen RCTs, two human trials (Boutrou et al., 2013; Crowley et al., 2013) and one animal trial (Chia et al., 2018) received a "High" risk classification because these studies did not have complete description of the examined groups (age and gender). The human trials conducted by He et al. (2017); Jianqin et al. (2015); Sheng et al. (2019), did not describe the participants' gender. Moreover, only one gender was assessed in two human trials: Kirk et al. (2017) had only male participants, and Milan et al. (2020) recruited only female participants. Three out of four animal trials (Barnett et al., 2014; Haq et al., 2014; Yadav et al., 2020) examined only male participants, and Kamiński et al. (2012) involved only female participants. Not including both sexes can minimise the replicability and generalisability of the study outcomes. Most RCTs included healthy participants (human and animal trials) with only six human trials including participants with or at risk to develop severe health conditions (Chin-Dusting et al., 2006; Crowley et al., 2013; Deth et al., 2015; González-Domenech et al., 2020; He et al., 2017; Sheng et al., 2019). Nonetheless, out of these six human trails, two

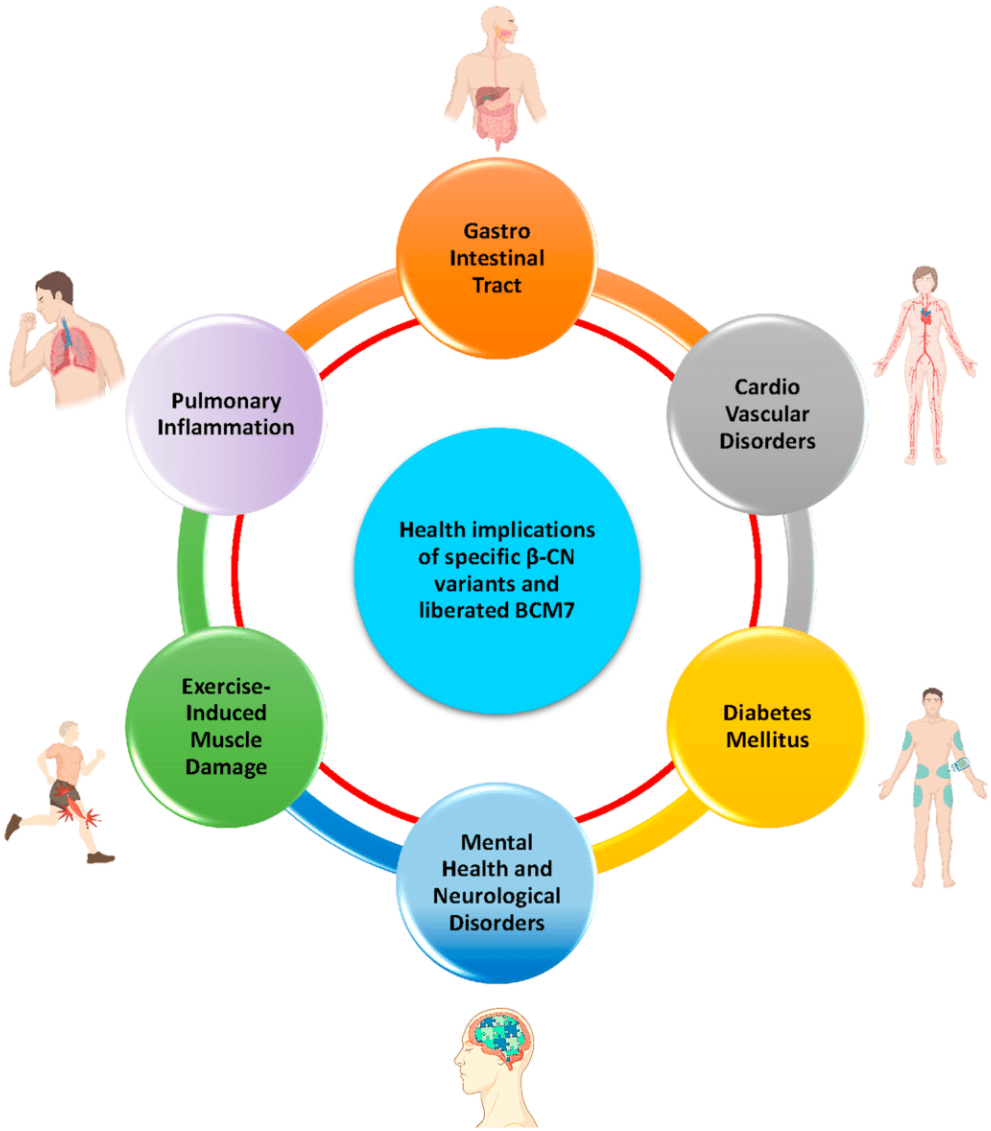

Fig. 2. Suggested disadvantageous health effects of specific β-CN genetic variants and BCM7 from bovine milk assessed in this study.

human trials (Deth et al., 2015; He et al., 2017) involved self-reported mild to moderate lactose-intolerant participants, making direct comparisons difficult.

3.3.3. Data collection and studies' verification

Most studies included narrative synthesis of the results on the influence of bovine milk, CN, different β-CN proteoforms and BCM7. Only two human trials (Chin-Dusting et al., 2006; Venn et al., 2006) presented quantitative results that could be directly compared with regard to the study design, intervention, and outcome (Küllenberg de Gaudry et al., 2019). Although, He et al. (2017) included an adequate description of data analysis, and was the largest study within the scope of this systematic review, the participants were self-reported lactose intolerant and the results may have potential bias. Contrastingly, Sheng et al. (2019) examined the acute effects of both, A1 β-CN milk and A2 β-CN milk on clinically-proven lactose intolerant participants (urinary galactose tests representing lactase deficiency). Some studies received an "Unclear (Some concerns)" RoB classification due to insufficient information provided in the articles. Studies including the "Unclear" risk were observed across all domains (mainly oriented towards "detection bias" and "other bias") because of missing information and lack of detail to make an evaluation (RoB assessment for the included human trials: Fig. 3 a and Fig. 4 a; and RoB assessment for the included animal trials: Fig. 3 b and Fig. 4 b).

3.4. Study results

3.4.1. Gastrointestinal system conditions (GIT)

The results for *in vivo* gastrointestinal conditions within animal (n = 2) and human (n = 9) studies are summarised in Supplemental Table 2. In humans, Chabance et al. (1998) examined the number and size of β-CN peptides, which were released in the stomach and duodenum after ingestion of bovine skimmed milk (no information on the genetic proteoforms were provided). The β-CN peptide sequences liberated in the stomach were: f1-12, f33-44, f107-114, f29-41, f30-41, f106-120 (20 min); f6-17, f29-40, f164-175 (1 h); and f164-175 (4 h). Moreover, β-CN fractions, including f7-18, f114-119, f84-92, f83-93; f7-16, f145-156, f1-12, f155-165, f1-12; and f69-80, were liberated in the duodenum at 20 min, 40 min, and 4 h, respectively. Similarly, Boutrou et al. (2013) showed a significant liberation of numerous β-CN peptides, including the BCMs in the jejunal effluent. The sequence (f60-66) and highest concentration (3.60 ± 0.35 mg) of BCM7 were shown 30 min after ingestion. The presence of BCM7 precursors (f57-66, f58-66 and f59-66) were also shown, with the total amount of BCM7 measured in jejunal

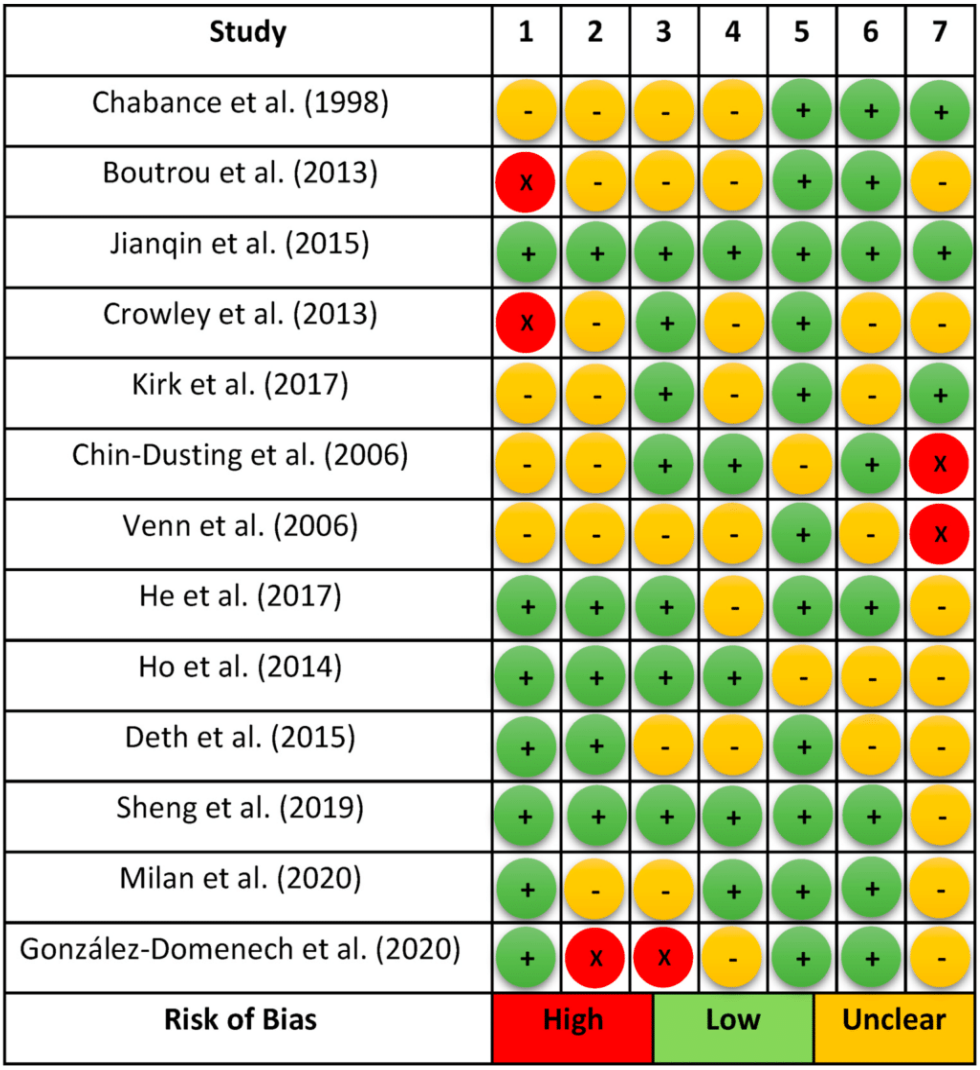

Fig. 3 a. Risk of bias assessment for the included human studies (Traffic Light Plot): Risk of bias for randomised controlled trials (Cochare risk of bias tool). *1. Random sequence generation (selection bias); 2. Allocation concealment (selection bias); 3. Blinding of participant and personnel (performance bias); 4. Binding of outcome assessment (detection bias); 5. Incomplete bias; 6. Selective reporting (reporting bias); 7. Other bias* (McGuinness & Higgins, 2020). Namely, the "[+]: unpredictable data", "[−]: predictable data", and "[x]: some concerns regarding the data" tabulates the judgement for each study in each domain. This presents every risk of bias judgement level in a matrix, with domains along the horizontal and results/-studies down the vertical, similar to the data set (McGuinness & Higgins, 2020).

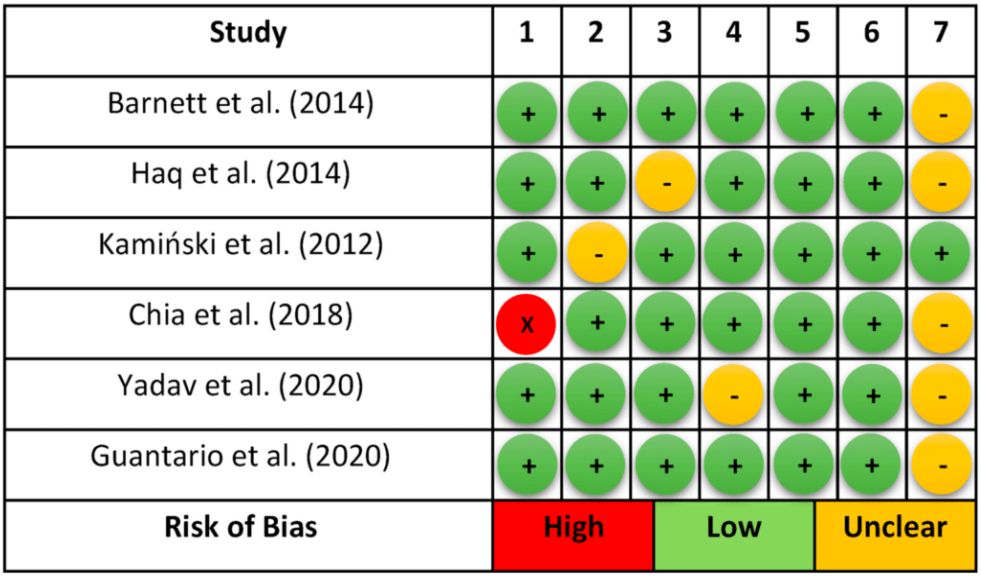

Fig. 3 b. Risk of bias assessment for the included animal studies (Traffic Light Plot): Risk of bias for randomised controlled trials (Cochare risk of bias tool). *1. Random sequence generation (selection bias); 2. Allocation concealment (selection bias); 3. Blinding of participant and personnel (performance bias); 4. Binding of outcome assessment (detection bias); 5. Incomplete bias; 6. Selective reporting (reporting bias); 7. Other bias* (McGuinness & Higgins, 2020). Namely, the "[+]: unpredictable data", "[−]: predictable data", and "[x]: some concerns regarding the data" tabulates the judgement for each study in each domain. This presents every risk of bias judgement level in a matrix, with domains along the horizontal and results/-studies down the vertical, similar to the data set (McGuinness & Higgins, 2020).

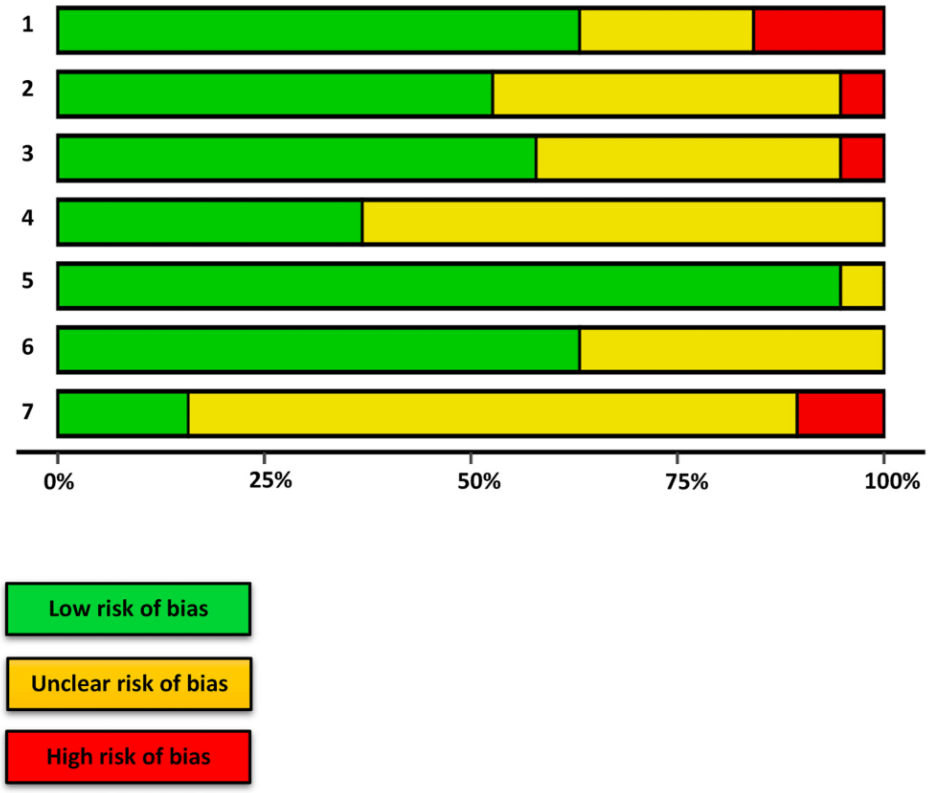

Fig. 4 a. Risk of bias assessment for the included human studies (Weighted Summary Plot): Risk of bias for randomised controlled trials (Cochare risk of bias tool). *1. Random sequence generation (selection bias):* "Low = 63.31%", "Unclear = 20.86%", and "High = 15.83%"; 2. *Allocation concealment (selection bias):* "Low = 52.52%", "Unclear = 42.08%", and "High = 5.40%"; *3. Blinding of participant and personnel (performance bias):* "Low = 57.91%", "Unclear = 36.69%", and "High = 5.40%"; *4. Binding of outcome assessment (detection bias):* "Low = 37.05%", "Unclear = 63.31%", and "High = N/A"; *5. Incomplete bias:* "Low = 94.96%", "Unclear = 5.04%", and "High = N/A"; *6. Selective reporting (reporting bias):* "Low = 63.31%", "Unclear = 36.69%", and "High = N/A"; *7. Other bias:* "Low = 15.83%", "Unclear = 73.74%", and "High = 10.43%" (McGuinness & Higgins, 2020).

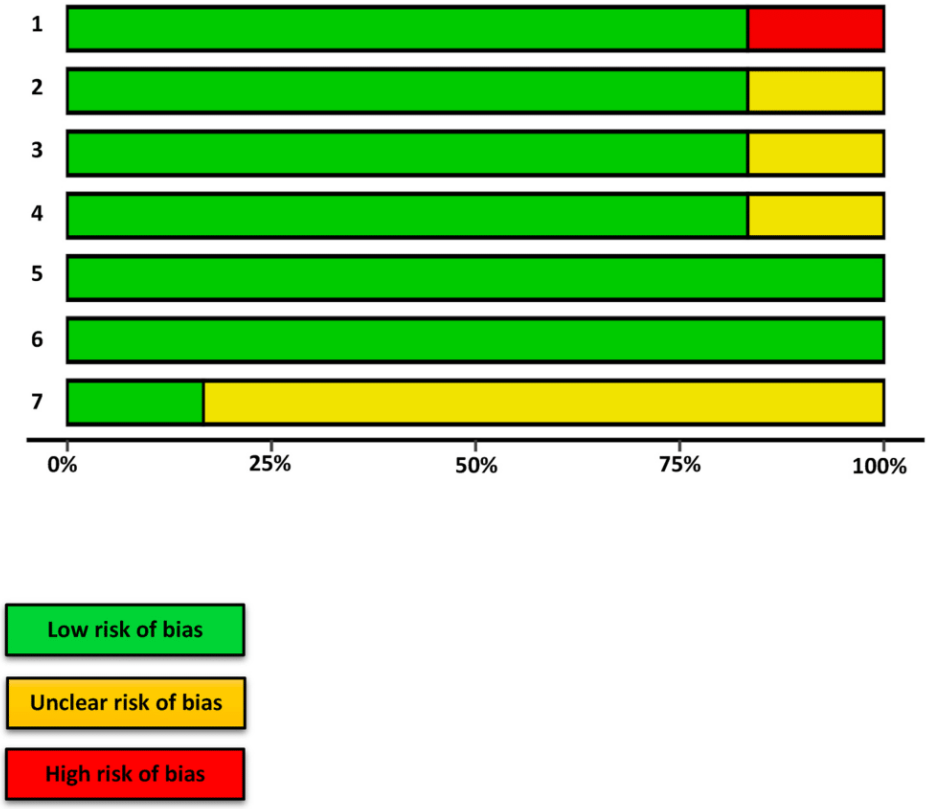

Fig. 4 b. Risk of bias assessment for the included animal studies (Weighted Summary Plot): Risk of bias for randomised controlled trials (Cochare risk of bias tool). *1. Random sequence generation (selection bias):* "Low = 82.98%", "Unclear = N/A″, and "High = 17.02%"; 2. *Allocation concealment (selection bias):* "Low = 82.98%", "Unclear = 17.02%", and "High = N/A"; *3. Blinding of participant and personnel (performance bias):* "Low = 82.98%", "Unclear = 17.02%", and "High = N/A"; *4. Binding of outcome assessment (detection bias):* "Low = 82.98%", "Unclear = 17.02%", and "High = N/A"; *5. Incomplete bias:* "Low = 100%", "Unclear = N/A″, and "High = N/A"; *6. Selective reporting (reporting bias):* "Low = 100%", "Unclear = N/A %", and "High = N/A"; *7. Other bias:* "Low = 17.02%", "Unclear = 82.98%", and "High = N/A" (McGuinness & Higgins, 2020).

effluents was 4 mg after 2 h of digestion.

Five different crossover RCT's structured in two periods (one washout period) showed a significant difference between the diets consisting of either A1 β-CN, A1/A2 β-CN (conventional), or A2 β-CN milk in subsequent stool consistency (Bristol Stool Scale - BSS) and its influence on digestive discomfort and abdominal pain in adults (He et al., 2017; Ho et al., 2014; Jianqin et al., 2015) and children (Crowley et al., 2013; Sheng et al., 2019). Stool consistency (1 = separate hard lumps like nuts; 2 = sausage-shaped but lumpy; 3 = like a sausage or snake but with cracks on its surface; 4 = like a sausage or snake, smooth and soft; 5 = soft blobs with clear-cut edges; 6 = fluffy pieces with ragged edges, a mushy stool; 7 = watery, no solid pieces) measured after milk ingestion was 3.87 ± 0.02 (A1 β-CN) and 3.56 ± 0.02 (A2 β-CN) in the first period, and 3.82 ± 0.02 (A1 β-CN) and 3.47 ± 0.02 (A2 β-CN) in the second period (Ho et al., 2014). The results were assessed through the utilisation of a food frequency questionnaire, urinary galactose examination, subtle cognitive impairment test (computer-based), GIT transit time and inflammation using a smart pill, and GIT symptom scores. The difference in the correlation between the A1 β-CN milk and A2 β-CN milk consumption was significant for both, the abdominal pain (0.46 vs 0.03; P = 0.02) and bloating (0.36 vs − 0.02; P = 0.05) (Ho et al., 2014). Moreover, Jianqin et al. (2015) presented results of a stool consistency of 4.42 ± 0.74 and 4.05 ± 0.25 in participants consuming A1 β-CN milk and A2 β-CN milk in the first trial period, respectively, and 4.35 ± 1.11 and 4.08 ± 0.61 in participants consuming A1 β-CN milk and A2 β-CN milk in their second trial period, respectively. While a study by He et al. (2017) found that the ingestion of conventional (A1/A2 β-CN) milk after 1 h, 3 h, and 12 h resulted in significantly higher GIT symptom scores (bloating, abdominal pain, stool frequency, and stool consistency) in both study phases, in comparison with consuming A2 β-CN milk ($p < 0.0001$). As part of the same study, the authors reported that the symptom scores from the urine samples were consistently lower with A2 β-CN milk in both lactose absorbers (urinary galactose ≥ 0.27 mmol/L) and lactose malabsorbers (urinary galactose < 0.27 mmol/L). Pre-school children who completed the milk sequence in baseline containing conventional (A1/A2 β-CN) milk and A2 β-CN milk in phases one and two (including the washout period of 9 days) showed no change in daily stool frequency, however, this increased during the post-intervention period for both phases, but only for subjects who consumed A1 β-CN milk (Sheng et al., 2019). A study on the influence of A1 β-CN milk and A2 β-CN milk on chronic functional constipation (CFC) by Crowley et al. (2013) found CFC in 14 participants (out of 22; 64%) who consumed A1 β-CN milk, and in 16 participants (out of 25; 64%) who consumed A2 β-CN milk. The resolution of CFC (greater than 8 bowel motions per fortnight) was highest during the washout period in 18 participants (78%), proving that both types of milk had a beneficial effect when it comes to CFC based on BSS. Milan et al. (2020) made a comparison between three groups of participants (lactose intolerant, non-lactose dairy intolerant, and lactose tolerant) who consumed conventional milk (A1/A2 β-CN and lactose), A2 β-CN milk (A2 β-CN with lactose), or lactose-free conventional milk (A1/A2 β-CN without lactose). The study revealed that in all participants, lactose malabsorption and digestive comfort with lactose-containing milks was ameliorated with milk containing exclusively A2 β-CN ($p < 0.001$). However, lactose-free conventional milk (containing A1 β-CN and A2 β-CN) demonstrated the best results regarding GIT symptoms based on the number of participants.

In animals (mice and rats) treated with naloxone and saline in the jejunum, increased activity levels of colonic myeloperoxidase (MPO) and jejunal dipeptidyl peptidase IV (DPP-4) enzymes have been shown after consumption of bovine milk (especially those subjects fed with A1 β-CN milk) (Barnett et al., 2014). Namely, the activity of DPP-4 was 40% higher in the A1 β-CN saline group than in the A2 β-CN saline group (38.3 vs 27.3 pkat/μg protein; p = 0.002). MPO activity was 65% higher in the A1 β-CN saline group than in the A2 β-CN saline group (0.52 vs 0.32 units/3 min/mg protein, p = 0.04); and also 64% higher in the A1 β-CN saline group than in the A1 β-CN nalaxone group (0.52 vs 0.32 units/3 min/mg protein, p = 0.04) (Barnett et al., 2014). Comparably, Haq et al. (2014) showed that feeding mice with A1/A1, A1/A2, and A1/A2 of bovine β-CN milk increased MPO activity in the murine intestine ($p < 0.01$) by 179.06% and 31.68% as compared to A2/A2 β-CN. Guantario et al. (2020) suggested that milk had a positive role in partially counteracting the ageing effect on the gut health of mice (20 months old) across all three groups (control - CTRL/milk free diet, A2/A2 β-CN milk, and A1/A2 β-CN milk), particularly when the gastrointestinal system was exposed to A2 β-CN. The difference in DPP-4 activity among the three groups was not shown, however, the production of short-chain fatty acids in faecal samples presented a significant difference (CTRL = 60.1 ± 20.9; A1/A2 = 127 ± 79.2; A2/A2 = 131 ± 42.6), with short-chain fatty acids known to play a potential role in glucose homeostasis, lipid metabolism and body weight control. Moreover, the results suggested that the consumption of A2 β-CN milk could be a suitable strategy to obtain positive gut health outcomes in the ageing population.

3.4.2. Cardiovascular diseases (CVD)

Two human trials and one animal trial investigating the influence of different β-CN proteoforms on CVD are shown in Supplemental Table 3. Using a crossover design, Chin-Dusting et al. (2006) provided bovine milk (milk powder with β-CN = 10 g/d) A1 β-CN milk or A2 β-CN milk to 15 participants with a high risk of CVD. The authors did not find a significant difference on triglycerides (TGs), total cholesterol (TC) or blood pressure (BP: systolic blood pressure [SBP] and diastolic blood pressure [DBP]) between milk that contain A1 β-CN or A2 β-CN genetic variants at the end of the examination (week 12). Herein, the amount of TGs, TC, SBP and DBP in A1 β-CN group were 1.2 mmol/L, 5.6 mmol/L, 131 mmHg and 77 mmHg, respectively, followed by the A2 β-CN presented with 1.3 mmol/L (TGs), 5.7 mmol/L (TC), 131 mmHg (SBP) and 75 mmHg (DBP). Venn et al. (2006) investigated whether there was an effect of commercial milk and full-fat cheese containing either A1/A2 β-CN or A2 β-CN on TGs and TC (HDL and LDL) after 4.5 weeks of intervention (A1/A2 β-CN group: TGs = 1.33 mmol/L, TC = 5.60 mmol/L; and A2 β-CN group: TGs = 1.34 mmol/L and 5.63 mmol/L). Neither Chin-Dusting et al. (2006) nor Venn et al. (2006) RCT trials indicated that A1 β-CN had any detrimental impact on CVD in comparison to A2 β-CN. Furthermore, Kamiński et al. (2012) showed no correlation of A1/A1 β-CN diet with CVD in gilts (non-lactating sows) after a 6-week intake of A1 β-CN milk in comparison with A2 β-CN milk (TC: A1/A2 β-CN = 109 mg/dl and A2 β-CN = 106.67 mg/dl; and HDL: A1/A2 β-CN = 55.33 mg/dl and A2 β-CN = 54 mg/dl). Overall, the studies showed no indication that supplementation with A1 β-CN had any cardiovascular health implications in comparison to A2 β-CN milk.

3.4.3. Diabetes mellitus (DM)

The two crossover RCTs, one human *in vivo* (Chin-Dusting et al., 2006) and the other, animal *in vivo* (Chia et al., 2018), evaluated the influence of both A1 β-CN and A2 β-CN genetic variants on incidence of DM are depicted in Supplemental Table 4. Chin-Dusting et al. (2006) found that the mean plasma insulin concentration declined in both diet groups (A1 β-CN and A2 β-CN) without any significant difference (A1 β-CN [baseline = 11.8 mU/L; after 6 weeks = 10.1 mU/L; and after 12 weeks = 8.8 mU/L] and A2 β-CN [baseline = 11.8 mU/L; after 6 weeks = 7.8 mU/L; and after 12 weeks = 9 mU/L]). In the study of Chia et al. (2018), p. 5 non-obese diabetic (NOD) mice generations were exposed to a bovine milk powder diet containing 60.53/100 g β-CN of either A1 or A2 genetic variant. They observed no difference in DM incidence between the two diet groups from F0 to F2 generations (F1: A1 18.4% vs. A2 21.6%; F2: A1 18.2% vs. A2 13.2%). However, in generation F3, the incidence of DM doubled (A1 β-CN = 40%); A2 β-CN = 20.7%). Moreover, in generation F4, fasting blood glucose levels were notably higher in NOD mice fed with A1 β-CN (7.0 ± 0.4 mM), in comparison with A2 β-CN (5.5 ± 0.5 mM, $p < 0.05$) (Chia et al., 2018). This intervention

study presented the possibility of A1 β-CN milk to influence the glucose homeostasis and type 1 diabetes mellitus progression. Nevertheless, in order for this effect to be expressed, it takes a few generations; indicating that A2 β-CN milk did not possesses any diabetes protective effects.

3.4.4. Neurological disorders and mental health (ND and MH)

The influence of a bovine milk based diet on ND has been studied in two *in vivo* human RCTs (González-Domenech et al., 2020; Jianqin et al., 2015) (Supplemental Table 5). González-Domenech et al. (2020) reported differences in the concentrations of BCM7 in the urine of participants fed either a normal diet (ND), or a gluten-free and casein-free diet (GFCF) in an *in vivo* study. They found that BCM7 concentrations in urine were lower after consuming the GFCF (2.30 ± 3.0 ng/mL) diet in comparison with the normal diet (3.63 ± 4.4 ng/mL); however, that slight difference in the concentration of BCM7 in the urine did not establish a correlation between BCM7 and the behavioural symptoms of autism. Moreover, another study utilised the Subtle Cognitive Impairment Test (SGIT) in order to evaluate the speed and efficiency of information processing among participants (Jianqin et al., 2015). It was determined that participants who consumed milk containing A1/A2 β-CN genetic protein variant demonstrated slightly longer processing times and higher error levels on the SGIT comparable to participants who consumed only A2 β-CN milk genetic protein variant (Jianqin et al., 2015). With regards to mental health, small to no difference between A1 β-CN milk and A2 β-CN milk has been observed, even though the consumption of bovine milk and the activity of DPP-4 are potential factors in determining the pathogenesis of autism among children (Jarmołowska et al., 2019).

3.4.5. Health-related conditions

A summary of the outcomes describing the relationship between the specific β-CN proteoforms (bovine BCM7) and *in vivo* human health studies, apart from the aforementioned conditions (GIT, CVD, DM, ND), are presented in Supplemental Table 6. The RCT by Deth et al. (2015) found that BCM7 concentrations were not significantly greater in plasma samples after consumption of A1/A2 β-CN milk in contrast to A2 β-CN milk, with values of 0.87–0.98 ng/mL and 0.71–0.73 ng/mL, respectively. Moreover, the concentration of plasma glutathione was 1.99 ± 0.50 nmol/mL in A1/A2 β-CN, compared to 4.01 ± 0.61 nmol/mL in A2 β-CN. Namely, glutathione in conjunction with its associated enzymes, such as glutathione peroxidase and glutathione-S-transferase, build the glutathione redox mechanism which provides the ability to effectively prevent unnecessary oxidation reactions within the cells (Degroote et al., 2020). However, it has been stated that any modulations in glutathione and redox homoeostasis initiated by BCM7 can correspond to pathophysiological mechanisms contributing to the prevalence of neurodegenerative diseases (Deth et al., 2015). In one double-blinded study, athletes (team sport players) were randomly selected and divided into three groups consuming A1/A2 β-CN milk (first group), A2 β-CN milk (second group), or maltodextrin mixed with water (placebo – PLA: third group). After 48 h, sprint time recovered quicker in both A2 β-CN (3.3 ± 0.1 m), and A1/A2 β-CN (3.3 ± 0.3 m) consumers in contrast with the placebo group (3.6 ± 0.3 m) (Kirk et al., 2017). Furthermore, after 48 h the countermovement jump height recovered quicker in A2 β-CN and A1/A2 β-CN consumers in contrast with the PLA consumers, representing 33.4 ± 6.6 cm, 33.1 ± 7.1 cm, and 29.2 ± 3.6 cm, respectively. Significant differences between the effect of consumption of A1 β-CN milk and A2 β-CN milk on health conditions were not observed, however, A2 β-CN milk offered greater antioxidant capacity than A1 β-CN milk (Kirk et al., 2017). Moreover, an increased amount of interleukin-4 (IL-4) (16–54 pg/ml in all four included subjects) and IL-5 (12–24 pg/ml in all four included subjects) were found in the bronchoalveolar lavage and serum of the same male Balb/c on A1/A1 β-CN milk diet (Yadav et al., 2020). Pulmonary inflammation in immunodeficient male Balb/c mice (albino laboratory-bred strain that is more susceptible to airway allergy and inflammation) was examined over a period of 30 weeks and it was found that long-term feeding of A1/A1 β-CN milk induced significant Th2-driven allergic airway inflammation. The immunoglobulin E (IgE) and IgG levels in A1/A2 β-CN and A2/A2 β-CN milk along with infiltration of lymphocytes and eosinophils were considerably lower in comparison with that in the A1/A1 β-CN variant fed mice (Table 6) (Yadav et al., 2020).

4. Discussion

The controversy over the A1 β-CN milk and A2 β-CN milk hypothesis has been discussed publicly for at least two decades. Numerous arguments and counterclaims have been made as to how regular conventional milk, which depends on the allele frequencies in the dairy cow used for milk production aimed to manufacture milk or dairy products, normally comprising both A1 β-CN and A2 β-CN genetic variants, is related to a variety of health-condition issues, and whether consumption of A2 only milk would alleviate such problems (Haq, 2020; Summer et al., 2020; Truswell, 2006). The findings of the present systematic review show that the possible roles of A1 β-CN and A2 β-CN in health and chronic diseases still remain controversial and inconclusive, however, their advantages or disadvantages need to be studied further following the well-established criteria for food claim regulations. As a result of the tremendous interest in this field over the last decade, numerous *in vivo, in vitro,* and *ex vivo* studies have been performed in order to follow and define the bioavailability and the fate of BCM7 in the human gut (Haq et al., 2014; He et al., 2017; Ho et al., 2014; Jianqin et al., 2015; Yadav et al., 2020). While a few outcomes presented in the current study showed, to some extent, the disadvantages of A1 β-CN milk consumption compared to that of A2 β-CN milk, the variations between these two genetic variants were however not clinically admissible (stool frequency, stool consistency, computer-based tests). Therefore, the unconfirmed evidence from human clinical trials may easily be classified as emerging evidence.

The largest study (n = 600) included in this systematic review investigated consumption of conventional (A1/A2 β-CN) milk by Han Chinese adults in comparison to that of the A2 β-CN milk and the results showed that intake of A1/A2 β-CN milk may have inhibited lactase activity and enhanced/increased frequency of the adverse gastrointestinal symptoms (borborygmus, flatulence, bloating, abdominal pain, stool frequency, and stool consistency) (He et al., 2017). Nonetheless, the limitations in this study are that the participants self-reported with lactose intolerance and digestive discomfort. Additionally, if all participants were lactose intolerant it has to be taken into consideration the possibility of inducing the association between the beforehand mentioned symptoms and the absence of lactase, but not their relation to β-CN genetic variant, per se (He et al., 2017). However, the findings of this study did not specifically indicate the significant benefit of A2 β-CN milk consumption, and only a moderate change in gastrointestinal symptoms was achieved when participants had history of prior milk consumption in comparison to that of A1 β-CN milk. Several studies had substantial variations between A1 β-CN and A2 β-CN consumption when intermediate markers were tested (He et al., 2017; Ho et al., 2014; Jianqin et al., 2015). A study by Yadav (2020) observed immunosuppressed male Balb/c mice that were fed with A1/A1 β-CN, A1/A2 β-CN, or A2/A2 β-CN milk. The results showed that the included mice group, which consumed a diet consisting of A1/A1 β-CN milk expressed the highest inflammatory response (Yadav et al., 2020). This study can only be an indication that only those with immunocompromised health, such as human neonates and elderly may experience a pro-inflammatory effect of bovine milk containing A1 β-CN on the respiratory tract. During postnatal formation, the neonates' intestinal mucosa may become more permeable to large peptides compared to an adult intestinal mucosa, increasing the likelihood of possible migration of bovine BCM7 from the intestines to blood (Hohmann et al., 2021). Therefore, presently, several dairy companies have started moving towards offering infant formulae based on A2 β-CN milk. Nevertheless, conclusive evidence involving

human participants addressing the presence and fate of BCM7 in infant formulae is absent. Some studies, however, have demonstrated that upon simulated gastrointestinal digestion at a pH equal to an infant's stomach, A2 β-CN infant formulae released the lowest level of BCM7 in comparison to other commercially available infant forumale (Duarte-Vázquez, García-Ugalde, Villegas-Gutiérrez, García-Almendárez, & Rosado, 2017; Haq, 2020; Noni, 2008). Due to some inconclusive data, such as differences in stool consistency, stool frequency, and exercise-induced muscle injury, this systematic review raises one question - are these results decisive enough to demonstrate a cause - effect relationship of particular β-CN proteoform and human health; to raise awareness about public health?

The suggested differences between A1 β-CN and A2 β-CN are based on the mechanism of BCM7 and its physiological effects. This peptide is entrapped in its inactive state within the β-CN chain; however, it can be released after *in vivo*, *in vitro* or *ex vivo* digestion and consequently attached to μ-opioid receptors that are found throughout the central nervous system, gastrointestinal tract, and certain immune cells. The μ-opioid receptors, known as receptors that can recognise peptides that carry amino acid sequences containing Tyr at the N terminus, such as BCM7, are G-protein coupled receptors (GPCRs), are composed of α, β, and γ subunits. When the activation of the μ-opioid receptor is initiated by opioid peptides, including BCM7, both subunits of μ-opioid receptor Gα and Gβγ dissociate from one another and could possibly influence different intracellular effects or pathways, leading to biological and immunological changes that involve complications in cell populations and causing problems within their functionality (Kodukula & Zeng, 2018; Listos et al., 2019; Tyagi, Daliri, Kwami Ofosu, Yeon, & Oh, 2020). Thus, if BCM7 is liberated, not degraded and transferred through the epithelium, it may potentially lead to detrimental health effects (Bhushan Jawale, Kaluskar, & Sabnis, 2015). The biological mechanism of BCM7 at an intestinal level can be expressed only if it is not further degraded *in situ*; however, under normal gastrointestinal conditions, dipeptidyl peptidase IV (DPP-4) is an enzyme responsible for cleavage of BCM7 into its derived C terminally shortened fragments and myeloperoxidase (MPO) is an enzyme that acts as a marker for inflammation (Haq et al., 2014; Summer et al., 2020). Therefore, there is possibility that BCM7 has been hydrolysed by brush boundary enzymes, including the DPP-4, before accessing the chosen organ or being detected in the human plasma (Osborne et al., 2014). This mechanism of DPP-4 apparently degrades BCM7 into BCM5, which is particularly resistant to proteolytic degradation and more potent compared to BCM7. The BCM5 alters the intestinal motility because of its high degree of selectivity for μ-opioid receptors (Dalziel et al., 2014). On the contrary, Iwan et al. (2008) and Jarmołowska et al. (2019) suggested that the decreased amount or low enzymatic activity of DPP-4 may be an indication of increased levels of opioids in the intestines that can lead to translocation of BCM7 to a particular organ. Additionally, MPO is normally released from activated polymorphonuclear neutrophils (PMNs) that contain potent oxidant hypochloric acid; it indicates a microbicidal activity in addition to tissue damage, such as acute or chronic inflammation. Therefore, a published *in vivo* data showed that mice fed with BCM7 and BCM5 greatly improved the activity of MPO ($p < 0.001$) (Haq et al., 2014).

Even though most reported *in vitro* studies that were performed on animal cell lines, tissues or organs, particularly, examining peripheral blood mononuclear cells, blood serum and urine, have revealed possible negative consequences of BCM7, its protective effects on human epithelial cells, reduced inflammation and decreased oxidative stress have also been discussed in the literature (Zhang, Song, Liu, Liu, & Zhang, 2015; Zhang, Zhao, Ge, Wang, & Qi, 2019; Zhu, Li, Wu, & Li, 2018). This data was not included in this systematic review since these experiments have been performed using synthetic BCM7. While reported human trials could not establish the link between the β-CN genetic variants, BCM7 and μ-opioid pathways, the likelihood that this mechanism may initiate certain gastrointestinal symptoms appears plausible. As a consequence, research is expected to continue to evolve as new experiments are being developed and their findings released in the very near future.

5. Strength, limitations and future directions

The results described as part of this systematic review have shown repeatability across various independent clinical and research studies, and some negative effects of consumption of a particular β-CN milk (mainly A1), or BCM7. Nevertheless, there is insufficient evidence in the literature of the health related problems that may be caused by the consumption of A1 β-CN milk on the gut discomfort associated to delayed gastrointestinal transit time, and the reason behind that behaviour. It is possible that this deficit is due to a fundamental lack of knowledge in the area of CN micelle morphology that is also responsible, at least in part, for the differences in these two genetic protein variants of milk (Raynes, Day, Augustin, & Carver, 2015). The question of how CN micelle confirmation and its behaviour during processing, as a function of cow's genotype and consequently the genetic protein variants, may impact digestive patterns and release of BCM7 needs to be fully understood, in order to link this protein with its hypothesized health implications. In addition, inadequate clinical analysis, short intervention trials, insufficient number of participants and follow-up periods used in the included studies in this review sometimes hindered a detailed analysis of the findings. One of the difficulties in this systematic analysis was the evaluation of all various results and intermediate indicators (human or animal health markers) which typically demonstrated very short time-analysis experiments and trials. A few gastrointestinal RCTs described a strong correlation that β-CN (A1 or A1/A2) and BCM7 might induce inflammation, delaying the transit time, activation of DPP-4 (Barnett et al., 2014; Jianqin et al., 2015) and issues in humans with milk intolerance (Sheng et al., 2019). DPP-4 is active in immune response and non-specific inflammatory processes and its reduced activity is usually associated with compromised immune function (Erić-Nikolić et al., 2011). Based on the study done by Jarmołowska et al. (2019), the highest serum concentration of BCM7 among girls diagnosed with autism spectrum disorder was associated with the highest DPP-4 content. Since DPP-4 is the only enzyme capable of impairing the BCM7 structure, its activation only gives an indication of the presence of BCM7 in blood (Jarmołowska et al., 2019). The limited knowledge and contradictory statements regarding the mechanism of DPP-4 once attached to BCM7 require further research.

Milk proteins, especially CNs, are an important source of peptides with different biological actions and characteristics, such as antioxidative, antihypertensive, antimicrobial and immunomodulation (Sah, Vasiljevic, McKechnie, & Donkor, 2015). Nevertheless, in the included studies, it is difficult to characterise the digestion, absorption and identification of proteins and related peptides that occurred during these processes and their subsequent mechanism. As presented in the literature, the levels of absorbed proteins and peptides are very low and require very strong precision and responsiveness to correctly identify the related protein or peptide of concern, which remains a challenge (Hortin, 2006; Michalski, Cox, & Mann, 2011).

The evaluation of the potential risk between the consumption of A1 β-CN milk and elaboration of the fate of BCM7 during digestion has been presented in several review studies published over the last two decades (Chia et al., 2017; De Noni et al., 2009; Summer et al., 2020; Swinburn, 2004). Recently, two different systematic reviews presented results from both *in vivo* and *in vitro* trials and compared the differences of A1 β-CN and A2 β-CN in the gastrointestinal tract (Brooke-Taylor et al., 2017) and on mixed health-related outcomes (Küllenberg de Gaudry et al., 2019). In this systematic review, we examined the novelty and in-depth research on the presence and the effects of bovine milk components in humans and animals; most importantly, the utilisation of only naturally derived β-CN or BCM7 from A1 β-CN milk or A2 β-CN milk and only within *in vivo* studies. While an informed estimate may be taken on the

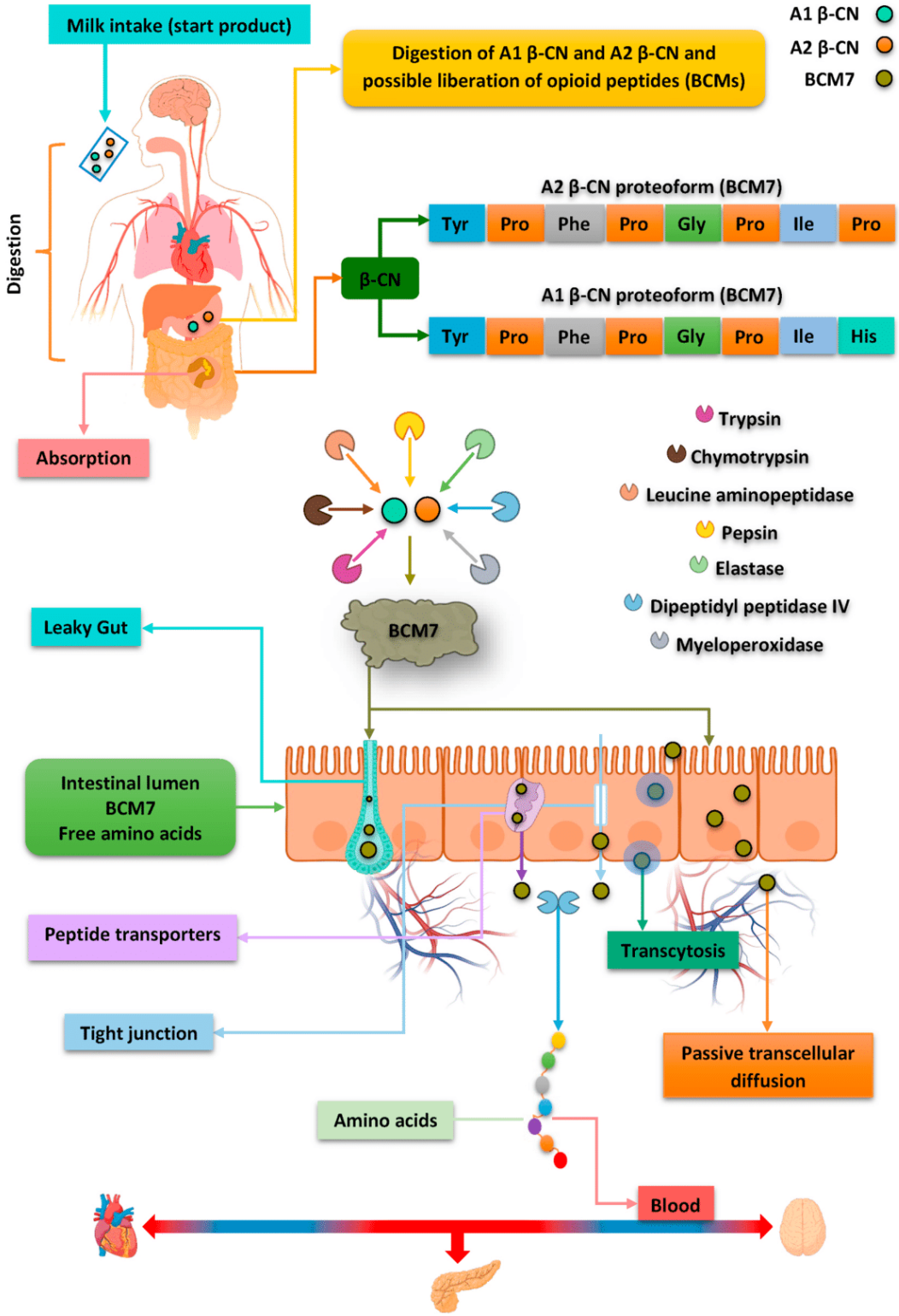

Fig. 5. Possible mechanism of *in vivo* digestion and absorption of β-CN and BCM7 (if liberated) from A1 β-CN and A2 β-CN genetic variants of bovine milk. Can an intact BCM7 cross the intestine/blood barrier and be transferred to other internal organs?

basis of *in vitro/ex vivo* studies performed by using trypsin, chymotrypsin, pancreatin, animal and human gastric and duodenal juices to successfully mimic the real *in vivo* conditions, the mechanism of how β-CN and the BCM peptides may be released after *in vivo* human digestion and their exact function/activity is still not completely understood. As presented in Fig. 5, a possible explanation could be that if BCM7 was about to be liberated from β-CN in the intestinal lumen after digestion, it may be transported across the epithelial cell monolayer into the bloodstream via one or more of the following routes: carrier-mediated permeation (peptide transporters), paracellular transport (tight junctions), transcytosis (vesicles), and passive transcellular diffusion; thus, it can possibly be tranfessred to the internal body organs; nevertheless, the

presence of intact BCM7 molecules in the blood after ingestion of bovine milk has not been shown *in vivo* (Hohmann et al., 2021; Xu, Hong, Wu, & Yan, 2019) (Fig. 5).

6. Concluding remarks

The debate between A1 β-CN milk and A2 β-CN milk and which one is most favourable to health will continue to be a captivating topic in human nutrition. We hypothesized that high consumption of A1 β-CN results in an increased occurrence of chronic diseases, but the current evidence does not confirm such a relationship. The results from the clinical trials included in this systematic review demonstrated that A2 β-CN can have some beneficial effects on the gastrointestinal system, but it does not completely support that A1 β-CN has negative effects on human health. Notably, those causes for the observed effects on the gastrointestinal tract are still poorly understood, their assessment is a matter of debate, and their clinical significance is not clearly established; they may be initiated from the origin and nature of other underlying conditions (increased gut permeability), but their involvement cannot be ruled out. In light of the concern, larger peptides in addition to individual amino acids, dipeptides and tripeptides can easily penetrate during the absorption by the epithelial cells in the intestines of elderly patients, infants or those suffering from symptoms of increased gut permeability (Shani-Levi et al., 2017).

The most appropriate solution going forward to offer an explanation of the health impact of A1 β-CN and A2 β-CN should come from long-standing clinical trials and research with more participants from different geographical regions, gender, age (infants, adolescence and older people), and physiological states. These studies should document the important role of particular β-CN variants and BCM forms and their alleged role in the development of non-communicable diseases. There is still ample opportunity for research across the fundamental-applied spectrum on this interesting subject dealing with the 'β-CN (A1 or A2)/BCM7 controversy'. Thus, given the aforementioned role of A1 β-CN milk and A2 β-CN milk, specific genetic variants of β-CN, and BCM7, further functional research is also necessary to unravel the mechanisms of action of these identified peptides and gene variants responsible for the observed health associations.

Declaration of competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tifs.2021.02.073.

Author contributions

D.D. conceived the study and research question, and D.D., T.V. and N.M.D. designed the review. D.D. wrote the original draft, conceptualised, reviewed, edited the manuscript, and designed the tables and figures. T.V. and N.A.M. provided critical feedback and analysis, secured funding, reviewed and edited the manuscript and supervised the study. N.M.D., T.O.C. and S.M.P. provided critical feedback and analysis, reviewed and edited the manuscript. All authors have contributed to the manuscript and reviewed the final version.

Data availability statement

The data used, analysed and elaborated in this systematic review have been stated in figures and tables/supplemental tables within the manuscript.

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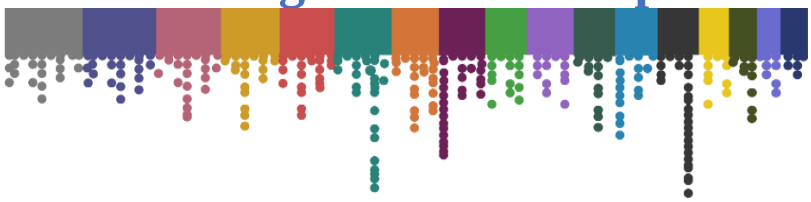
Main findings: Part II. Chapter 10.



Impact of β-casein phenotype on the physical properties of skim milk powders and their subsequent digestion characteristics

- Greater rehydration properties were observed in A1/A2 skim milk powder
- Random coils were mainly present in A2/A2 skim milk powder
- Heat stability was lower in A2/A2 milk at pH 7.4
- A2/A2 digesta had more stable protein structure under gastric conditions
- Milks with β-casein A1 showed faster gastric digestion

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This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

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I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

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Davor Daniloski	81	Designed and wrote the original draft, conceptualised, reviewed, edited, revised the manuscript, designed the experiments, the tables and the figures, and data analysis	[redacted]	17/03/2024
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André Brodkorb	3	Concept development, manuscript editing and revision		17/03/2024
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Impact of β-casein phenotype on the physical properties of skim milk powders and their subsequent digestion characteristics

Davor Daniloski [a,b], Yonas Hailu [b], André Brodkorb [b], Todor Vasiljevic [a], Noel A. McCarthy [b,*]

[a] Victoria University, Advanced Food Systems Research Unit, Institute for Sustainable Industries and Liveable Cities and College of Health and Biomedicine, Melbourne, Victoria, 8001, Australia

[b] Teagasc Food Research Centre, Food Chemistry and Technology Department, Moorepark, Fermoy, P61 C996, Cork, Ireland

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ABSTRACT

This study investigated the physical properties of skim milk powders containing β-casein A1/A1, A1/A2 or A2/A2 phenotypes and determined their digestion properties. Rehydrated skim milk powders all had a type A heat coagulation profile (HCT); although A2/A2 milk had a different local maximum and minimum HCT, compared to A1/A1 and A1/A2 milks. Differences in the rehydration properties of milk powders appeared to be related to the presence of β-casein A1 in reconstituted A1/A1 and A1/A2 milks since they were characterised by smaller casein micelles and greater levels of κ-casein, compared to A2/A2 milk. All reconstituted samples displayed protein aggregation and coagulum formation within the first 5 min of gastric digestion, at which time the pH ranged from 6.0 to 5.5. During digestion of reconstituted A2/A2 milk, casein breakdown was slower, compared to that of A1/A1 or A1/A2 milks. The final gastric clot obtained from the A2/A2 sample possessed a tight protein network, containing a greater level of calcium and aggregated β-sheets. In this regard, the dry weight of the separated clot after the final gastric phase was significantly higher by 29 and 68 % in A2/A2 digesta compared to that in A1/A1 or A1/A2 digests, respectively. Overall, this study shows that for rehydrated skim milk powders there was a significant difference in the level of gastric protein-breakdown between milks containing β-casein A1 and milk containing only β-casein A2. This may have significant implications during *in vivo* gastric digestion and emptying.

1. Introduction

Bovine milk is a nutritionally dense, but highly perishable food source, comprising of a variety of macro - and micro - components that play an essential role in human nutrition. Converting bovine milk into dried powder format by partial or almost complete removal of water, increases its shelf-life and enables it to be transported and stored for extended periods without substantial loss of quality, at either cool or ambient temperatures (McSweeney & Fox, 2013; Singh & Creamer, 1991). Two main commercial milk powders are skim milk (SMPs) and whole milk (WMPs) powders, generally classified as either regular (non-instant) or instant (Kelly & Fox, 2016; Sharma, Jana, & Chavan, 2012). Due to its high protein (~35%, w/w) and calcium content, and concomitant low fat level (≤1 %, w/w), SMPs are used in various applications, such as in tea and coffee whiteners, condensed milks, nutritional formulations but also in recombined milk for yoghurt and cheese manufacture (Lin, Kelly, O'Mahony, & Guinee, 2018). Skim milk powders possess several physical, functional, and rehydration properties that depend on interrelated factors (Zhang, Pandiselvam, & Liu, 2022), such as protein and mineral profile, heat treatment, and storage conditions to name a few (Harper, Holsinger, Fox, & Pallansch, 1963; Hill, Boland, Harris, & Paterson, 2000; Kelly & Fox, 2016).

Bovine milk is comprised of two major protein groups, classified as caseins and whey proteins. The casein fraction accounts for about 80 % of the total protein content and consists of αs_1-, αs_2-, β- and κ-caseins, assembled in spherical structures known as casein micelles (Huppertz, Fox, & Kelly, 2018). β-Casein is an intrinsically disordered protein with a strong amphipathic nature and comprises ~40 % of the total casein, and is derived in milk predominately in two proteoforms, A1 and A2 (Aschaffenburg, 1963). Although nearly identical proteins, a difference of one amino acid at position 67, within the structure of β-casein, with histidine for β-casein A1 and proline for β-casein A2 opened a scientific debate and initiated the market in some countries into promoting A2/A2 milk and dairy products (Daniloski, McCarthy, Huppertz, & Vasiljevic, 2022a).

A number of previous studies (McLean, Graham, Ponzoni, &

* Corresponding author.
E-mail address: noel.mccarthy@teagasc.ie (N.A. McCarthy).

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McKenzie, 1984, 1987; Daniloski et al., 2022a; Robitaille, 1995) have shown that milk rennetability, heat stability, emulsification and foaming characteristics are influenced in part by the genetic polymorphisms of the milk proteins. In this regard, a study by Hill et al. (2000) showed that the selection of β-lactoglobulin B/B and κ-casein A/A improved the production of milk powders for recombining into ultra-high temperature treated milks, they also had an effect on the milk's heat stability compared to reconstituted milks carrying other phenotypes of both milk proteins. Further, addition of SMP carrying κ-casein B/B in bovine milk aimed for cheese production, improved the renneting characteristics of the milk achieving higher cheese yields (Fitzgerald et al., 1999); nevertheless, the impact of β-casein A1 and A2 proteoforms on the properties of milk powders has not been reported in the literature. Contrarily, over the last several years, substantial research has been performed on the technological properties of A1/A1, A1/A2, and A2/A2 milks, gels, emulsions, and some ingredients (Daniloski et al., 2022a; Daniloski, McCarthy, Auldist, & Vasiljevic, 2022e; Darewicz & Dziuba, 2007; Hemar, Banjar, Otter, & Yang, 2021; Poulsen et al., 2013). The studies have found that milk comprised of β-casein A2 has usually been associated with poorer acid gelation (Daniloski, McCarthy, Gazi, & Vasiljevic, 2022d) and rennet coagulation properties (Poulsen et al., 2013), but with better emulsification (Daniloski et al., 2022e) and foam formation capabilities (Nguyen, Schwendel, Harland, & Day, 2018). While milk with β-casein A2 appears to be less suitable for cheese or yoghurt making, the weak gel it produces could potentially be responsible for its proposed greater digestibility (Milan et al., 2020), which might be advantageous for certain applications. These differences in techno-functional properties between milks with different β-casein phenotypes may have consequences for digestibility.

Therefore, there has been increased interest in characterising the digestibility of milks with different protein variants. Results from previous clinical trials demonstrated that milk with β-casein A2 can have some beneficial effects on the gastrointestinal system, such as reduced abdominal pain and bloating (Ho, Woodford, Kukuljan, & Pal, 2014; Jianqin et al., 2016; Milan et al., 2020). Very recently, Ramakrishnan, Zhou, Dydak, and Savaiano (2023) showed that gastric emptying was faster in reduced fat A1/A2 milk, as opposed to that of the A2/A2 milk. The authors hypothesised that the faster gastric emptying was due to differences in both clot formation and the structure of the gastric digesta. For almost a century, the physiological importance of curd firmness formed through the gastric coagulation of caseins has been focused on the properties of the so called 'soft' and 'hard' curd milks (Brennemann, 1911). Softer milk gels empty faster from the stomach and have less adverse abdominal effects, with improved protein digestibility (Ye, 2021). It is well established that the rate of gastric digestion and stomach clearance is related to the hardness of the gastric clot (Huppertz & Chia, 2021). Thus, this study aimed to investigate the structural and functional properties of A1/A1, A1/A2, and A2/A2 SMPs and determine their subsequent gastric digestibility behaviour.

2. Materials and methods

2.1. Powder analysis

2.1.1. Pilot-scale production of A1/A1, A1/A2, and A2/A2 skim milk powders and preparation of reconstituted skim milk

Morning milk from 28 individual Irish Holstein Friesian cows was collected from the Animal and Grassland Research and Innovation Centre at Teagasc, Moorepark, Fermoy, Co. Cork, Ireland. Cows were identified and selected based on their β-casein phenotype using the Irish Cattle Breeding Federation database (www.icbf.com).. To further clarify the protein genotype, a Reversed Phase - High Performance Liquid Chromatography (RP-HPLC) was performed (see below: section 2.2.1). All cows chosen had the same genetic polymorphism for β-lactoglobulin, α_s- and κ-casein, but differed only in the β-casein phenotypes (A1/A1, A1/A2, and A2/A2). All cows included in the trial (regardless of the protein genotype) were in late-lactation and were all grass-fed (95–97 % of their diet consisting of ryegrass (*Lolium perenne* L.), through rotational grazing, at 95 % of annual dry matter intake, with concentrates consisting of the remaining 5 % of annual dry matter intake, between March and October) (Timlin et al., 2023). The milks (~250 L per β-casein phenotype) from the individual cows (A1/A1 cows = 8; A1/A2 cows = 10; A2/A2 cows = 10) were transported from the Moorepark Dairy Farm to the Bio-functional Food Engineering pilot plant at Teagasc and pooled according to their β-casein phenotype before treatment.

The raw milk was preheated to 50 °C in an APV plate heat exchanger (SPX Flow Technology, Crawley, West Sussex, UK), followed by separation in a Westfalia centrifugal disk separator (GEA Westfalia, Oelde, Germany). Subsequently, the milk samples were heat-treated at 80 °C for 30 s using a pilot-scale UHT/HTST system (MicroThermics, Raleigh, NC, USA). The skim milk powders were produced using an Anhydro single-stage spray dryer (SPX Flow Technology, Denmark), equipped with a two-fluid nozzle atomisation system and configured in a counter-current flow mode, while the air inlet and outlet temperatures were set at 180 °C and 85 °C, respectively (Hailu, Maidannyk, Murphy, & McCarthy, 2023; Magan et al., 2019; McSweeney, Aydogdu, Hailu, O'Mahony, & McCarthy, 2022). After spray drying, powders were stored in aluminium foil bags at 8 °C for the duration of the study. Fig. 1 outlines the experimental design of the study.

2.1.2. Compositional properties of skim milk powders

The free moisture and ash contents of the SMPs were determined using a TGA701 thermogravimetric analyser (LECO Corporation, St Joseph, Michigan, USA) at 102 and 550 °C, respectively. The nitrogen content of SMPs were determined by the accredited Technical Services Laboratory at Teagasc, using the Kjeldahl method. Protein content was determined using a nitrogen to protein conversion factor of 6.38 (ISO, 2014, pp. 1–18). Fat content was determined by Nuclear Magnetic Resonance (NMR) (Rapid Trac analysis system, Oracle, CEM Corp., Charlotte, NC, USA). The lactose content was calculated by difference. Total and soluble mineral levels of both, SMPs and their dispersions were assessed using inductively coupled plasma emission spectroscopy.

2.1.3. Physicochemical traits of skim milk powders

2.1.3.1. Particle and bulk density. The particle density of the powders was measured using a Micromeritics Accupyc II 1340 gas pycnometer as described in GEA analytical methods No. 11a (GEA Niro, 2006a). The bulk and tapped density (500 taps) of the SMPs were measured as per GEA method No. A2a (GEA Niro, 2006b) jolting volumeter STAV II (Funke Gerber, Berlin, Germany). The volumes of interstitial and occluded air of the samples were then calculated using the method described by McSweeney et al. (2022).

2.1.3.2. Flowability and compressibility. The flowability of powders was analysed using a powder flow tester (Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA). The powder samples were filled into the aluminium trough (volume of 230 cm^3 and 15.2 cm internal diameter) until a dome - shaped volume of powder was obtained using a curved blade. A standard flow function test was carried out over five normal stresses (1.0, 1.9, 2.9, 3.9, and 4.8 kPa) and three over-consolidation stresses at each normal stress. The flow function graph was obtained by plotting the major principal consolidating stress as a function of unconfined failure strength. The index of flow was calculated from the inverse of the slope of the flow function curve. The index corresponds to the strength that has to be overcome for a powder to flow when consolidated (Fitzpatrick, Barry, Delaney, & Keogh, 2005). The compressibility, expressed as compressibility index, was calculated as the percentage increase from loose bulk density to tapped bulk density following the equation already described in the study of McSweeney, Maidannyk, Montgomery, O'Mahony, and McCarthy (2020).

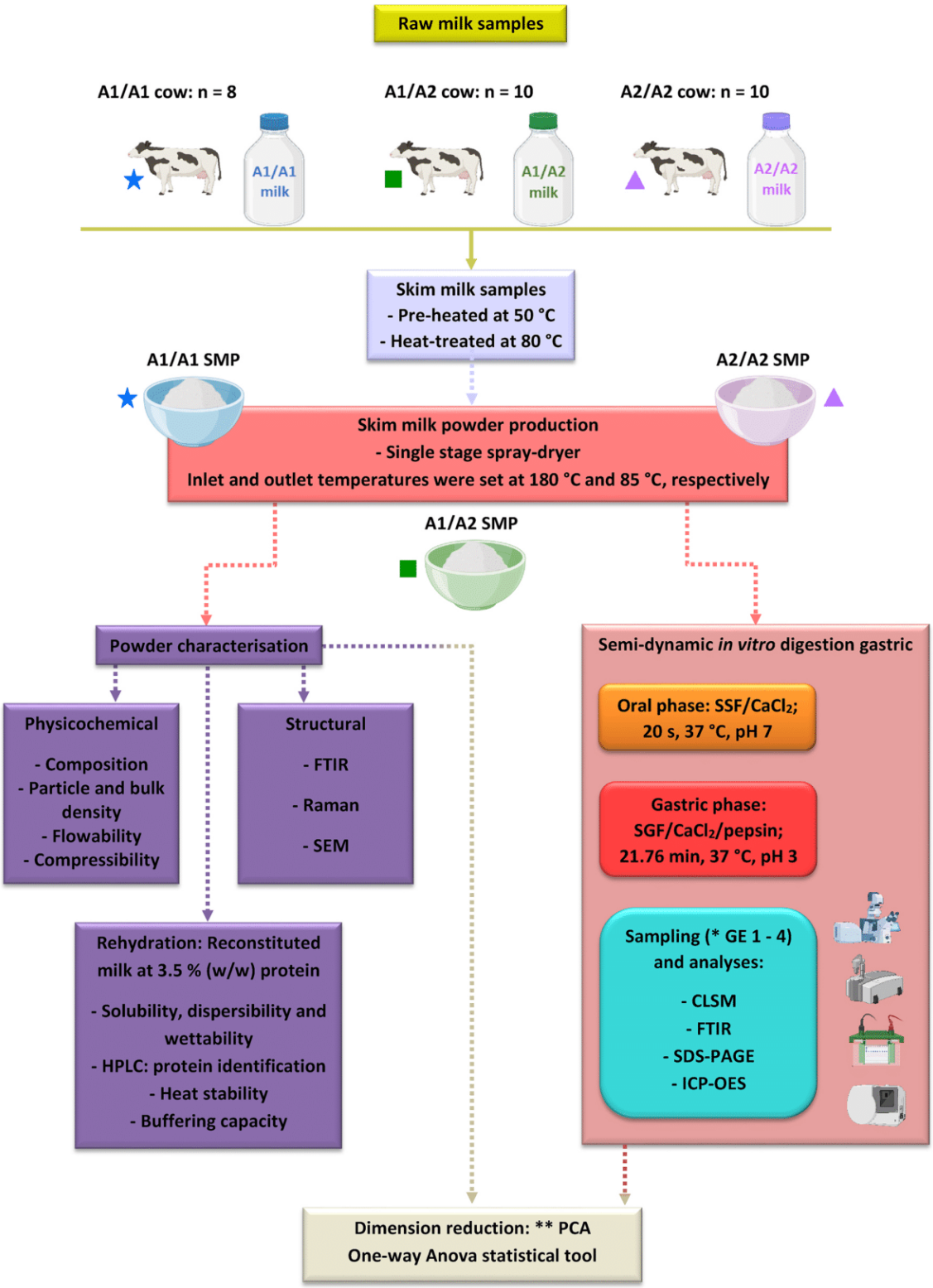

Fig. 1. Schematic showing the general approach of sample selection and analysis; * GE 1, 2, 3, and 4 representing the gastric emptying points at 5.30, 10.80, 16.00, and 21.76 min, respectively. ** PCA (Principal Component Analysis).

2.1.3.3. Colour determination. Colour was evaluated using a spectrophotometer (Konica Minolta Sensing Europe B·V., Nieuwegein, the Netherlands) equipped with a specialised granular attachment for powders as described by Hailu et al. (2023). The obtained results were expressed as L* (lightness), a* (green/red colour) and b* (blue/yellow colour) values. The particle size of the SMPs was determined using a Malvern Mastersizer (Mastersizer 3000; Malvern Instruments Ltd, Malvern, Worcestershire, UK) equipped with an Aero S dry dispersion unit. The refractive index was set at 1.37 (Jenness, 1962). The air pressure was set at 2 bar for all samples, and the feed rate ranged between 25 and 100 %. Size measurements were recorded as the volume-weighted mean particle diameter ($D_{[4,3]}$), median diameter (D_{50}) and cumulative

diameters (D_{90} and D_{10}) representing powder particles smaller than the size indicated (McSweeney, Maidannyk, O'Mahony, & McCarthy, 2021b).

2.1.3.4. Solubility, dispersibility, and wettability of skim milk powders. The solubility of SMPs was measured by the total solids content of the supernatant obtained following centrifugation at 3000*g* for 10 min, expressed as a percentage of the total solids content of the initial dispersion (McSweeney et al., 2021b). Following this, methods described previously by Hazlett, Schmidmeier, and O'Mahony (2021) and Fitzpatrick et al. (2016) were used to evaluate the dispersibility and wettability of the dispersions, respectively. Briefly, for dispersibility, 10 g of SMP was added to the surface of 250 mL Milli-Q water (purified by a Milli-Q® apparatus, Millipore Corp., Bedford, MA, USA) in an 80 mm diameter beaker. The dispersion was stirred for 30 s using a metal spatula, allowing one motion across the diameter of the beaker per s, after which, the sample was left to stand for an additional 30 s. The dispersions were then passed through a 150 μm analytical sieve into a receiver beaker. The sieved solution (10 mL) was pipetted into pre-weighed moisture dishes, dried at 103 °C for 2 h, cooled in a desiccator and weighed to calculate total solids of the sieved material. For wettability, 10 g of SMP was placed onto the surface of 250 mL of water (25 °C) in a 600 mL volume glass beaker. After 20 min, the remaining surface powder was carefully removed using a spatula. This powder was dried in an oven (102 °C) and its original water content was determined.

2.1.4. Conformational and morphological properties of milk powders

2.1.4.1. Secondary structure of milk proteins in skim milk powders. The Fourier Transform Infrared (FTIR) spectra of the SMPs were recorded on a temperature controlled Attenuated Total Reflectance (ATR)-FTIR (Bruker, INVENIO 100453, Billerica, MA, USA) at 37 °C, over the wavenumber range from 4000–600 cm^{-1}. The FTIR, was equipped with a Haake K20/DC30 external water bath (Thermo Haake, Karlsruhe, Germany). Using Bruker Opus 5.5 software, the spectra were obtained in an absorbance mode from 100 scans of each sample with an instrument resolution of 4 cm^{-1}. In addition, the background (air) was collected before every sample and was measured with a blank Diamond ATR cell, utilising the same instrumental conditions as for the sample spectra acquisition. The FTIR spectra were derived upon baseline corrections (Daniloski, McCarthy, O'Callaghan, & Vasiljevic, 2022c). Additionally, the SMPs were analysed using an Alpha300 R confocal Raman spectroscopy (WITec, GmbH, Ulm, Germany) equipped with a 532 nm laser and a 50× microscope objective (0.55 numerical aperture) in the Raman shift range between 4000 and 600 cm^{-1}. The laser power was set at 20 mW, the integration time at 0.5 s, and the number of accumulations at 10. At least 5 spectra, collected at different random locations, were considered for each type of SMP (Panthi, Shibu, Ochalski, & O'Mahony, 2023).

2.1.4.2. Microscopy analysis of skim milk powders. Scanning electron microscopy images of the SMPs (500× magnification) were captured using a Zeiss Supra 40 P field emission scanning electron microscopy (SEM) (Carl Zeiss SMT Ltd., Cambridge, UK) at 2.00 kV. Samples were prepared using double sided adhesive microscope stubs coated with chromium (K550*X*, Emitech, Ashford, UK) (Hailu et al., 2023).

2.2. Rehydration properties of skim milk powders with differing β-casein phenotypes

Skim milk powders were rehydrated in Milli-Q water (purified by a Milli-Q® apparatus, Millipore Corp., Bedford, MA, USA) to the same protein content (3.5 %, w/w). To ensure complete solubilisation, the rehydrated milk dispersions were gently mixed at ambient temperature for 1 h using a magnetic stirrer bar and left to hydrate overnight at 4 °C prior to further analysis and subsequent oral and gastric digestion.

2.2.1. Physicochemical properties of reconstituted skim milk

2.2.1.1. Protein analysis. To estimate the total and individual casein (α -, β -, and κ - caseins) and whey protein (β - lactoglobulin and α - lactalbumin) fractions, RP-HPLC was performed using a Poroshell 300SB-C_{18} (2.1 mm diameter, 75 mm length, 5 μm; Agilent Technologies, Ireland) column, equipped with a Zorbax poroshell guard column (1.0 mm diameter, 17 mm length, 5 μm; Agilent Technologies). From the rehydrated SMPs (see section 2.1.1.), 200 μL was diluted in 7 M urea buffer containing 20 mM bis-Tris propane and 3 % dithiothreitol (DTT) (pH 7.5, all three purchased from Sigma-Aldrich, St. Louis, MO, USA) at a 1:20 ratio (v/v). Diluted samples were left for 1 h at ambient temperature prior to filtration using a 0.2 mm syringe filter (Agilent Technologies, Econofltr, PES 25 mm) (Hailu, O'Mahony, Fenelon, & McCarthy, 2022). The sample injection volume was set at 20 μL, with a total run time of 40 min per sample (including column re-equilibration).

2.2.1.2. Particle size and zeta potential (ζ). Particle size and ζ potential of the milks at 4 °C were analysed and processed by a light scattering technique (Zetasizer-Nano ZS, Malvern Instruments, Malvern, Worcestershire, UK) coupled with dispersion technology software (Malvern Instruments, Version 5). Samples were prepared by 100 - fold dilution with simulated milk ultrafiltrate (SMUF). The salts used for the SMUF preparation were purchased from Sigma-Aldrich, St. Louis, MO, USA. A refractive index of 1.37 for skim milk and 1.33 for dispersant (SMUF) were used (Jenness, 1962).

2.2.2. Heat stability and buffering capacity of reconstituted skim milk

The SMP dispersions were adjusted to different pH values in the range 6.2–7.4 (at 0.1 pH unit increments) at ambient temperature using 0.1 M HCL or 0.1 M NaOH (Sigma-Aldrich, Arklow, Co. Wicklow, Ireland). Glass tubes (100 mm long, 13 mm inner diameter) containing 3 mL of sample were immersed in a silicone oil bath. The heat coagulation time at 140 °C was recorded as the time between immersing the sample in the oil bath and visible clots appearing within the test tubes, as described by O'Connell and Fox (2000).

Buffering capacity was measured with a Titrando 842 Autotitrator with a TIAMO v.2.2 software package (Meterohm, Ireland Ltd., Carlow, Ireland). Under continuous stirring at 700 rpm at 25 °C, 50 mL per sample were titrated from their initial pH to pH 2.0, through the controlled addition of 0.5 M HCL (20 mL increments with 30 s equilibration after each addition, Sigma-Aldrich, Arklow, Co. Wicklow, Ireland), followed by alkalisation to pH 9 through the addition of 0.5 M NaOH (Sigma-Aldrich, Arklow, Co. Wicklow, Ireland) (Aydogdu, O'Mahony, & McCarthy, 2022). The buffering index value (dB/dpH) of the skim milk dispersions was determined using the Van Slyke equation (Van Slyke, 1922).

2.3. In vitro semi-dynamic gastric digestion of reconstituted skim milk

Reconstituted skim A1/A1, A1/A2, and A2/A2 milk samples were digested *in vitro* using the standardised simulated semi-dynamic gastrointestinal digestion (GID) method (Mulet-Cabero, Egger, et al., 2020). Each gastric digestion was simulated at 37 °C for 21.76 min based on the caloric value of the milk sample, and it was carried out in a jacketed glass vessel (ref. 6.1418.250, Metrohm, Ireland). An overhead stirrer

(OHS 200 Digital, VELP R Scientifica, Italy or CAT R 100 CT, Ingenieurbüro CAT M. Zipperer GmbH, Germany) fitted with a 3D - printed stirrer head was used to mix the digesta with a low speed between 10 and 20 rpm. Pepsin (porcine pepsin, P-6887, Sigma-Aldrich, Arklow, Co. Wicklow, Ireland) activity was tested according to Brodkorb et al. (2019) (4017.12 U/mL of digesta). Gastric lipase was omitted due to the extremely low level of lipids present in all three reconstituted milk samples.

The electrolyte simulated fluids, namely, eSSF (Simulated Salivary Fluid) and eSGF (Simulated Gastric Fluid) were prepared as described in the INFOGEST standardised static *in vitro* digestion (Brodkorb et al., 2019). The only difference is that the eSGF was adjusted to pH 7 instead of pH 3 because the pH decrease during gastric phase was obtained by a gradual addition of HCL. For all milk samples, 45 mL of milk (3.5 %, w/w protein) was supplemented with 6.27 mL eSSF. Milk samples and eSSF were mixed with a plastic spatula at pH 7.0 for 20 s to reproduce the salivary phase of digestion. The gastric phase was performed by adding 34.64 mL eSGF, porcine pepsin (4000 U/mL digest, Sigma-Aldrich, Arklow, Co. Wicklow, Ireland) along with 0.13 M HCL (Sigma-Aldrich, Arklow, Co. Wicklow, Ireland) to reach pH 3.0. Gastric emptying was simulated by taking four samples, referred to as GE 1–4 in the text at 5.30, 10.80, 16.00, and 21.76 min, respectively. Aliquots of the samples (500 μL - 1 mL) were kept in ice for a very short time and used for structural characterisation of the digesta (see section 2.3.1.). Furthermore, the other aliquots of the samples needed for protein composition of initial and digested samples were snap frozen with liquid nitrogen, stored at - 80 °C overnight, freeze-dried and mixed thoroughly.

2.3.1. Characterisation of digested samples

The pH of the digesta emptied from the gastric vessel at ~ 5-min intervals was measured using a CyberScan pH meter 510 (Eutech Instruments, Singapore). Samples were taken manually, at certain time points (5.30, 10.80, 16.00, and 21.76 min), from the bottom of the vessel using a serological pipette with a tip internal diameter between 2.07 and 2.20 mm. This diameter was used as it is approximately the upper particle size limit that has been seen to pass through the pyloric opening into the duodenum (Mulet-Cabero, Mackie, Wilde, Fenelon, & Brodkorb, 2019). The structure of the digesta from each time point was observed using confocal laser scanning microscopy (CLSM) images (proteins were displayed in green) (Mulet-Cabero et al., 2019).

The protein contents of the digested milks in all four gastric phases were determined in triplicate using a LECO FP628 nitrogen analyser (LECO Corporation, UK) and by multiplying the nitrogen concentration by a nitrogen-to-protein conversion factor of 6.38 (Mulet-Cabero, Torcello-Gómez, et al., 2020). To understand the process of gastric emptying during digestion, digesta samples were analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and were used according to the manufacturer's instructions and following the method of Laemmli (1970), with some modifications. Specifically, the freeze-dried samples obtained from all four gastric phases/emptying points (section 2.3) were examined by SDS-PAGE. Furthermore, FTIR (see section 2.1.4.) operating at 37 °C was used to evaluate the structural properties of all four gastric emptying freeze-dried digested samples, taking into consideration the background (air) that was collected before every sample measurement (Liao et al., 2021).

Despite all the analyses done on the final gastric phase (see above), after ~ 22 min of digestion, the remaining curd after the final gastric phase (if any) was collected and filtered through a cheese cloth with a 1-mm pore size to separate the curd from the aqueous phase (note: additional triplicate digestion procedures were performed on all milk samples for this phase of the study). Removal of the pepsin from the surface of the curd was achieved by washing off with SGF, followed by weighing of the curd immediately. The curd was then heated at 90 °C for 3 min to inactivate the pepsin and placed in the oven at 105 °C for 24 h to determine its dry weight (Li et al., 2022). Also, mineral content and SEM (see sections 2.1.1. and 2.1.4., respectively) of the dried final gastric phase curds (GE 4) were studied to depict possible differences in the digesta among the milk samples.

2.4. Data analysis and processing

Three independent batches of SMPs and digesta were analysed for each sample with either β-casein A1/A1, A1/A2, or A2/A2, and the results were expressed as the mean of three average values (n = 3 ± the standard deviation). Mean values were then compared by one-way analysis of variance (ANOVA) using Minitab statistical software (Version 20; Minitab, Pennsylvania, USA) with a significance of 95 %. Tukey's test was used to detect pairwise significant differences. Atmospheric compensation for absorbance of CO_2 and H_2O as vapour and solute was performed on the raw FTIR data. Data processing of the chosen spectral measurements in FTIR and Raman included mean centering which was carried out by Spectragryph software (version 1.2.7, Oberstdorf, Germany). Subsequently, second order derivative of each individual spectrum derived from the SMPs (FTIR and Raman) and their counterpart digesta samples from all gastric phases (FTIR) were evaluated using Origin software. The multivariate analysis was conducted with 95 % confidence. At least 5 spectra were considered for each type of SMP and digested sample (Byler, Farrell, & Susi, 1988; Daniloski, Markoska, McCarthy, & Vasiljevic, 2023; Daniloski et al., 2022b).

3. Results and discussion

3.1. Powder properties

3.1.1. Gross composition and physical properties of skim milk powders with different β-casein phenotypes

The general composition of the SMPs is shown in Table 1. As

Table 1
Composition and physicochemical properties of skim milk powders.

Parameter	Skim milk powders		
	A1/A1	A1/A2	A2/A2
Protein (%)	39.48 ± 2.26 ^{b}	40.64 ± 0.11 ^{ab}	41.71 ± 0.16 ^{a}
Lactose (%)	46.90 ± 0.02 ^{a}	45.53 ± 0.34 ^{ab}	44.24 ± 0.21^{b}
Fat (%)	0.58 ± 0.06 ^{c}	0.67 ± 0.01 ^{b}	0.79 ± 0.02 ^{a}
Ash (%)	8.42 ± 0.05 ^{a}	8.29 ± 0.02 ^{a}	8.30 ± 0.01 ^{a}
Moisture (%)	4.53 ± 0.20 ^{b}	4.93 ± 0.05 ^{a}	4.96 ± 0.00 ^{a}
Colour - L	91.32 ± 2.02 ^{a}	91.67 ± 2.24 ^{a}	91.64 ± 2.33 ^{a}
Colour - a*	−2.41 ± 0.26 ^{b}	−2.62 ± 0.15 ^{b}	−3.06 ± 0.17 ^{a}
Colour - b*	10.00 ± 0.63 ^{c}	11.02 ± 0.59 ^{b}	12.26 ± 0.55 ^{a}
Calcium (mM)	36.03 ± 0.29 ^{ab}	35.32 ± 0.59 ^{b}	36.86 ± 0.46 ^{a}
Phosphorus (mM)	32.02 ± 0.15 ^{a}	32.19 ± 0.29 ^{a}	32.47 ± 0.55 ^{a}
Magnesium (mM)	5.32 ± 0.02 ^{a}	5.56 ± 0.07 ^{a}	5.23 ± 0.02 ^{a}
Potassium (mM)	37.39 ± 0.09 ^{a}	36.17 ± 1.20 ^{ab}	35.80 ± 0.72 ^{b}
Sodium (mM)	21.09 ± 0.44 ^{a}	20.57 ± 1.70 ^{ab}	19.21 ± 0.75 ^{c}
Insoluble calcium (mM) in rehydrated milk at 4 °C	21.89 ± 1.19 ^{ab}	21.25 ± 0.25 ^{b}	22.28 ± 0.83 ^{a}
Particle size of rehydrated milk at 4 °C	168.37 ± 2.00 ^{b}	168.60 ± 5.60 ^{b}	211.07 ± 13.09 ^{a}
Zeta potential of rehydrated milk at 4 °C	−16.63 ± 1.03 ^{a}	−14.03 ± 0.83 ^{b}	−13.97 ± 1.46 ^{bc}

Mean values (± standard deviation, n = 3 measurements) within a row that do not share a common superscript letter are significantly different ($p \leq 0.05$).

expected, all samples had similar levels of protein, lactose, minerals, and moisture ($p > 0.05$). Slight ($p > 0.05$) differences were observed for the L* values (whiteness) of the SMPs (Table 1). Due to their higher fat content (Table 1), both SMPs carrying β-casein A2 possessed greater b* values ($p < 0.05$), thus appeared more yellow compared to that of the A1/A1 sample. It is worth mentioning that the differences in colour between SMPs may be due to the interactions of many variables, but in this case it is mainly related to the higher fat content in the A2/A2 SMP. This is simply a result of differences in cream separation efficiency at pilot-scale.

Average particle size and particle distribution of SMP powders are shown in Table 2 and Fig. 2 C, respectively. There was a shift in profile towards a broader particle size distribution in A1/A2 SMP (Fig. 2 C). Particularly, $D_{[4,3]}$ and the D_{90} size values were significantly higher in A1/A2 SMP (46.9 and 103 μm) compared to both homozygous systems, for which $D_{[4,3]}$ and D_{90} size values accounted for more than 36 and 74

Table 2
Functional properties of skim milk powders.

Parameter	Skim milk powders		
	A1/A1	A1/A2	A2/A2
Particle density (g/cm^3)	1.10 ± 0.00 ^{a}	1.11 ± 0.00 ^{a}	1.13 ± 0.00 ^{a}
Loose bulk density (g/cm^3)	0.48 ± 0.00 ^{ab}	0.50 ± 0.00 ^{a}	0.51 ± 0.01 ^{a}
Tapped bulk density (g/cm^3)	0.67 ± 0.02 ^{b}	0.72 ± 0.00 ^{a}	0.72 ± 0.00 ^{a}
Flow index	3.50 ± 0.05 ^{b}	4.05 ± 0.23 ^{a}	3.78 ± 0.02 ^{b}
Jenike classification	Cohesive	Easy flowing	Cohesive
Compressibility index (%)	23.40 ± 0.66 ^{a}	22.20 ± 0.07 ^{b}	23.28 ± 1.69 ^{a}
Wettability (%)	30.99 ± 4.11 ^{b}	39.19 ± 2.78 ^{a}	28.74 ± 2.69 ^{c}
Solubility (%)	95.46 ± 0.67 ^{b}	96.76 ± 0.47 ^{a}	95.22 ± 1.25 ^{b}
Dispersibility (%)	93.16 ± 0.01 ^{a}	93.65 ± 0.01 ^{a}	93.04 ± 0.04 ^{a}
Percentage value (D_{50}, *μm)*	24.41 ± 0.47 ^{b}	32.68 ± 0.06 ^{a}	26.09 ± 0.10 ^{b}
Percentage value (D_{90}, *μm)*	76.61 ± 1.61 ^{b}	103.02 ± 2.52 ^{a}	74.80 ± 1.15 ^{b}
Mean particle diameter ($D_{[4,3]}$ μm)	36.10 ± 1.02 ^{b}	46.92 ± 1.04 ^{a}	37.00 ± 0.57 ^{b}

Mean values (± standard deviation, n = 3 measurements) within a row that do not share a common superscript letter are significantly different ($p \leq 0.05$).

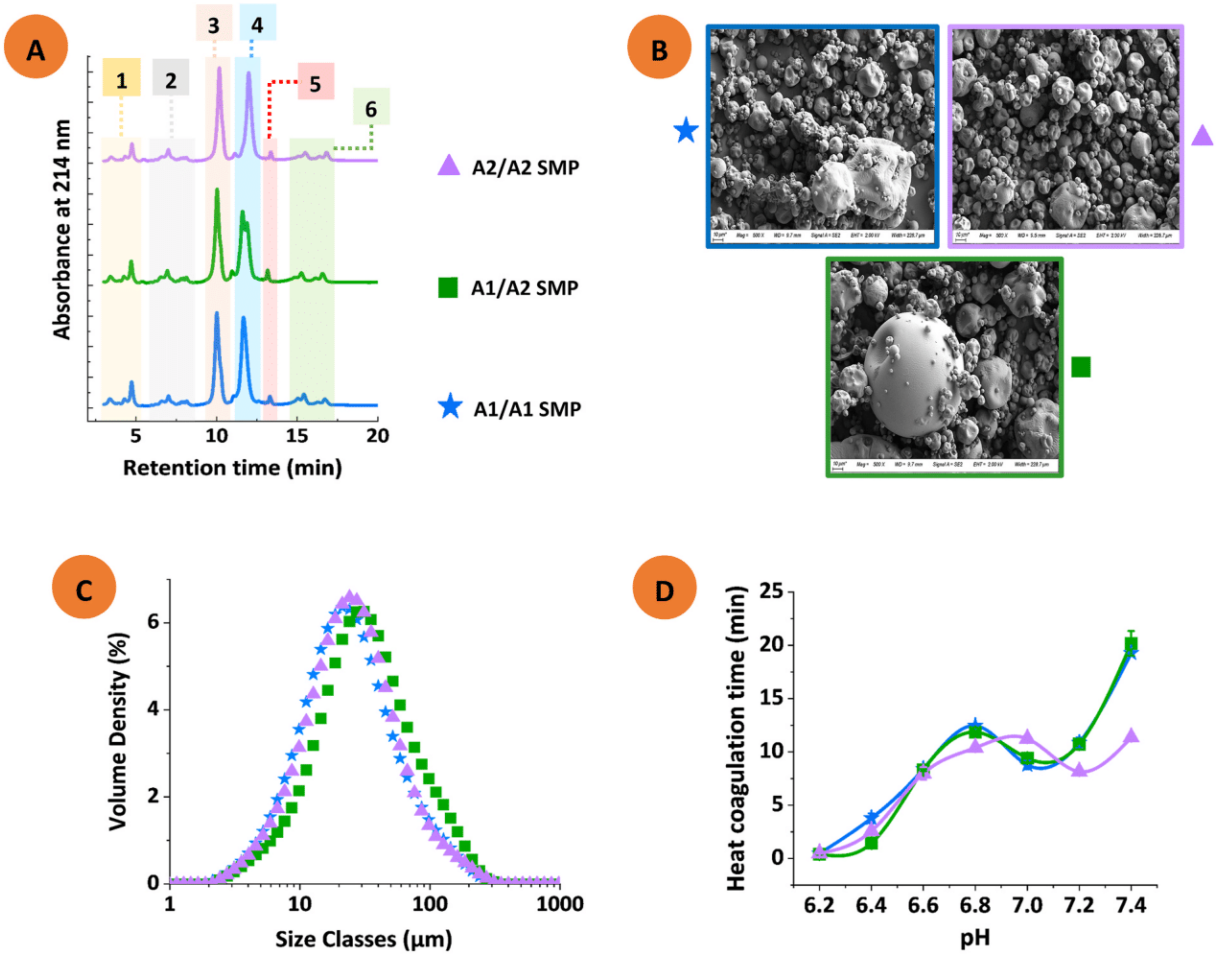

Fig. 2. A) RP-HPLC chromatographic profiles used for identification of different milk samples (1 = κ-casein A/A; 2 = αs_2-casein A/A; 3 = αs_1-casein B/B; 4 = β-casein [genetic variant indicated in the figure]; 5 = α-lactalbumin; 6 = β-lactoglobulin A/B). **B)** Representative SEM micrographs of SMPs, scale bars represent 10 μm. **C)** Particle size distribution of SMPs. **D)** pH-heat coagulation time profiles at 140 °C for the SMPs reconstituted to 3.5 % (w/w) protein. Data shown are average values of data from three collections. Error bars represent standard deviation.

μm, respectively ($p < 0.05$). The particle size values found in this study are comparable to those reported by Bista, Murphy, O'Donnell, and O'Shea (2022) for a number of un-heated and heat-treated SMPs. Both, A1/A1 and A2/A2 samples had a cohesive flow behaviour with flow indexes lower than 4 (Table 2). The flowability index of A1/A2 SMP was higher ($p < 0.05$, 4.05) than that of other powders ($p < 0.05$), thus it was classified as an easy-flowing powder (Fitzpatrick et al., 2005). The higher flow index for A1/A2 SMP is related to a larger powder particle size, as opposed to the other powder samples, and thus reduced contact area among powder particles during flow (Fitzpatrick, Barringer, & Iqbal, 2004). With a decrease in particle size, the surface area per unit mass of powder increases and more surface area is available for cohesive forces to resist powder flow, resulting in reduced flowability (Bista et al., 2022), as in A1/A1 and A2/A2 SMPs. Also, reduced flowability of powders may be affected by a higher fat content (Nijdam and Langrish (2006), particularly noticed in A2/A2 SMP, and might have occurred due to reduced inter-particle mobility caused by weak particle bridging.

The impact of β-casein phenotype on powder particle density and powder bulk density is presented in Table 2. Particle density and bulk density values were similar for all SMPs, ranging between 1.10 and 1.13 g/cm^3, and 0.67 and 0.72 g/cm^3, respectively. Commercial SMPs with a bulk density of around 0.70 g/cm^3 were reported previously (Fitzpatrick et al. (2005); similar to the results in the present study. In general, powder with large particle size and particle porosity is indicative of a degree of agglomeration that allows for better reconstitution properties, and, especially, greater dispersibility (Bell, Hanrahan, & Webb, 1963; Sharma et al., 2012). While the particle size for A1/A2 SMP was greater compared to the other systems, all three SMPs were comparable, with good dispersibility ($p > 0.05$, Table 2); therefore, in this case the influence of β-casein phenotype appears to be limited ($p > 0.05$).

3.1.2. *Physicochemical and rehydration properties of skim milk powders with different β-casein phenotypes*

Protein profile and concentration data are shown in Table 3. Although all three SMP dispersions were reconstituted to the same protein content (section 2.1.1.) and had comparable levels of whey protein and αs- and β-casein ($p > 0.05$), the amount of total κ-casein was greater in the samples carrying β-casein A1 ($p < 0.05$). These differences in total κ-casein levels may impact casein micelle size and its electrostatic potential (Day, Williams, Otter, & Augustin, 2015). Bijl, de Vries, van Valenberg, Huppertz, and van Hooijdonk (2014) and Daniloski et al. (2022b) generally agreed that higher κ-casein content was negatively correlated with casein micelle size. This is in line with the present study, since the particle size of reconstituted skim A2/A2 milk was greater (211 nm, Table 1) than those with β-casein A1 ($p < 0.05$, ~ 170 nm). Additionally, since κ-casein maintains the net negative charge of the casein micelles and it sterically prevents calcium-induced aggregation (McSweeney & Fox, 2013), in both, A1/A1 and A1/A2 SMP dispersions, the zeta potential (ζ) became more negative, which actually reflects the greater levels of κ-casein in these dispersions, compared to the other system (Table 3, 5.48 mg/mL in A1/A1 or 6.25 mg/mL in A1/A2 vs 4.77 mg/mL in A2/A2 samples).

Despite the similar protein content and the poor wetting behaviour that all three SMPs showed, the wettability of A1/A2 SMP was greater at 39.19 %, than that of A2/A2 SMP ($p < 0.05$, ~ 28 %; Table 2) upon 20 min of analysis. Poor wetting behaviour has previously been attributed to a greater fat in the powder (Fitzpatrick et al., 2016), which would explain the present results for A2/A2 SMP. Solubility of all SMPs generally followed the same trend as those observed for wettability and particle size distribution data ($p < 0.05$, Table 2). The A1/A2 SMP displayed solubility of approximately 97 %, compared with 95 % for A1/A1 and A2/A2 SMPs ($p < 0.05$). The slower solubilisation in both homozygous SMPs is most likely due to strong attraction among the milk proteins, in particular the casein micelles (Fitzpatrick et al., 2016). Namely, these strong interactions inhibit water's ability to protrude and disperse the powder particles, shown in the micrographs of both, A1/A1 and A2/A2 SMPs (section 3.1.3).

3.1.3. *Morphological and conformational properties of skim milk powders with different β-casein phenotypes*

The morphology of SMP particles are displayed in Fig. 2 B and show a dimpled structure with distinct shrivelled or collapsed particles, and resemble the wrinkled SMP particles reported by Mistry, Hassan, and Robison (1992). This possibly arises from the localised compaction of casein micelles, thus leading to shrinkage of the protein material during drying (McSweeney et al., 2020; Mimouni, Deeth, Whittaker, Gidley, & Bhandari, 2010). Felfoul et al. (2022) previously stated that the presence of pores at the particle surface might facilitate water diffusion into the powder, thus enhancing its rehydration ability.

Conformational differences among the SMPs were observed in slightly higher amounts of α-helical structures in A1/A1 and A1/A2 SMPs ($p < 0.05$), followed by a high and comparable levels of β-turns in

Table 3
Protein composition of reconstituted A1/A1, A1/A2, and A2/A2 skim milk as determined by Reversed Phase - High Performance Liquid Chromatography (RP-HPLC).

Protein content (mg/mL)	Sample (reconstituted dispersions)		
	A1/A1 SMP	A1/A2 SMP	A2/A2 SMP
κ-casein A/A	5.48 ± 0.09 ^{b}	6.25 ± 0.10 ^{a}	4.77 ± 0.06 ^{c}
αs_2-casein	2.83 ± 0.03 ^{a}	2.82 ± 0.11 ^{a}	2.76 ± 0.14 ^{a}
αs_1-casein	12.04 ± 0.05 ^{a}	11.86 ± 0.03 ^{b}	12.12 ± 0.01 ^{a}
β-casein A1	11.00 ± 0.09 ^{a}	n/a	n/a
β-casein A1/A2	n/a	11.07 ± 0.02 ^{a}	n/a
β-casein A2	n/a	n/a	11.26 ± 0.05 ^{a}
β-lactoglobulin A/B	2.95 ± 0.02 ^{a}	2.89 ± 0.03 ^{a}	2.76 ± 0.03 ^{b}
α-lactalbumin	0.29 ± 0.01 ^{a}	0.28 ± 0.01 ^{a}	0.25 ± 0.03 ^{a}
Estimated protein content after reconstitution (RP-HPLC)	34.59 ± 0.02 ^{a}	35.17 ± 0.18 ^{a}	33.92 ± 0.05 ^{b}
Reconstituted SMP with initial protein content (Kjeldahl)		35.00 ± 0.04	

Mean values (± standard deviation, n = 3 measurements) within a row that do not share a common superscript letter are significantly different ($p \leq 0.05$); n/a = not applicable.

all three samples ($p > 0.05$, Fig. 3 and Table 4), which in the past was attributed to bonding between κ-casein and β-lactoglobulin (Dalgleish & Corredig, 2012). Very recently, Daniloski, McCarthy, and Vasiljevic (2022f) showed that dissociation of micellar κ-casein and its aggregation with β-lactoglobulin due to heating of milk was related to appearance of β-turn structures in the samples. On the other hand, lower presence of α-helices (1650 cm^{-1}) in A2/A2 SMP compared to the other systems might be related to the presence of the additional proline[67] in the polypeptide chain of β-casein A2. This amino acid was considered to break α-helices, while on the contrary, in many instances, hisitidine[67] may promote α-helical conformation in both A1/A1 and A1/A2 SMPs (Daniloski et al., 2023). By using molecular modelling, very recently, Markoska, Daniloski, Vasiljevic, and Huppertz (Markoska et al., unpublished data) fingerprinted the structure of two peptides with 11 amino acids from both, β-casein A1 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser-Leu) and β-casein A2 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile--Pro-Asn-Ser-Leu). The authors correlated the lower presence of α-helix with the low intensity of the hydrogen-alpha (Hα) chemical shift of proline in the spectra of β-casein's A2 peptide, also observed in another study (Daniloski et al., 2023). Additionally, the peptide from β-casein A2 was more packed into the tertiary conformation compared to the other peptide's structure that was more spacious (Markoska et al., unpublished data). In that case, the high rigidity imposed by the proline cyclic structure prevented rotations about the *N*–C^{a} bond, hence, led to tighter packing within the molecule (Raynes, Day, Augustin, & Carver, 2015) and greater probability for attenuation of the helical structures (Huang & Nau, 2003). It is worth mentioning that while these conformational changes might occur within the peptide, the milk as a system is comprised of various milk proteins and their phenotypes, which can individually affect the structural modulations within the molecule.

In the past, Kher, Udabage, McKinnon, McNaughton, and Augustin (2007) postulated that α-helices were not correlated to the solubility of milk protein concentrate powders. However, A1/A1 caseinate powder has been shown to possess lower solubility, compared to that of the A2/A2 caseinate due to its high amount of α-helices (Daniloski et al., 2022e). These motifs display a tight structure with no cavities and may be detrimental to the specific conformational change that is required for the solubility of the sodium caseinate powders (Daniloski et al., 2022e), which might be the case in A1/A1 SMP in the current study.

Another difference among A1/A1, A1/A2, and A2/A2 SMPs was in the intramolecular and aggregated β-sheets. The β-sheets were approximately 8 % higher in A1/A1 SMP compared to that in A1/A2 and A2/A2 samples. The β-sheets in milk are predominately found in β-lactoglobulin (50 % of its motifs) (Uhrínová et al., 1998). Since A1/A1 sample possessed greater levels of β-lactoglobulin that tends to appear in β-sheet motif, it would be expected that the appearance of β-sheets was likely governed by the structural orientation of this globular protein ($p < 0.05$, Table 3). Interestingly, observing the properties of milk protein concentrate powders (Kher et al., 2007) and micellar casein powder (Nasser et al., 2018), it was found that a higher presence of β-sheets in the samples affected and decreased their solubility. Hence, it was established that β-sheets probably form a supporting hydrophobic core, thus leading to an exposure of the hydrophobic regions of the milk proteins (Farrell et al., 2002), followed by altered rehydration

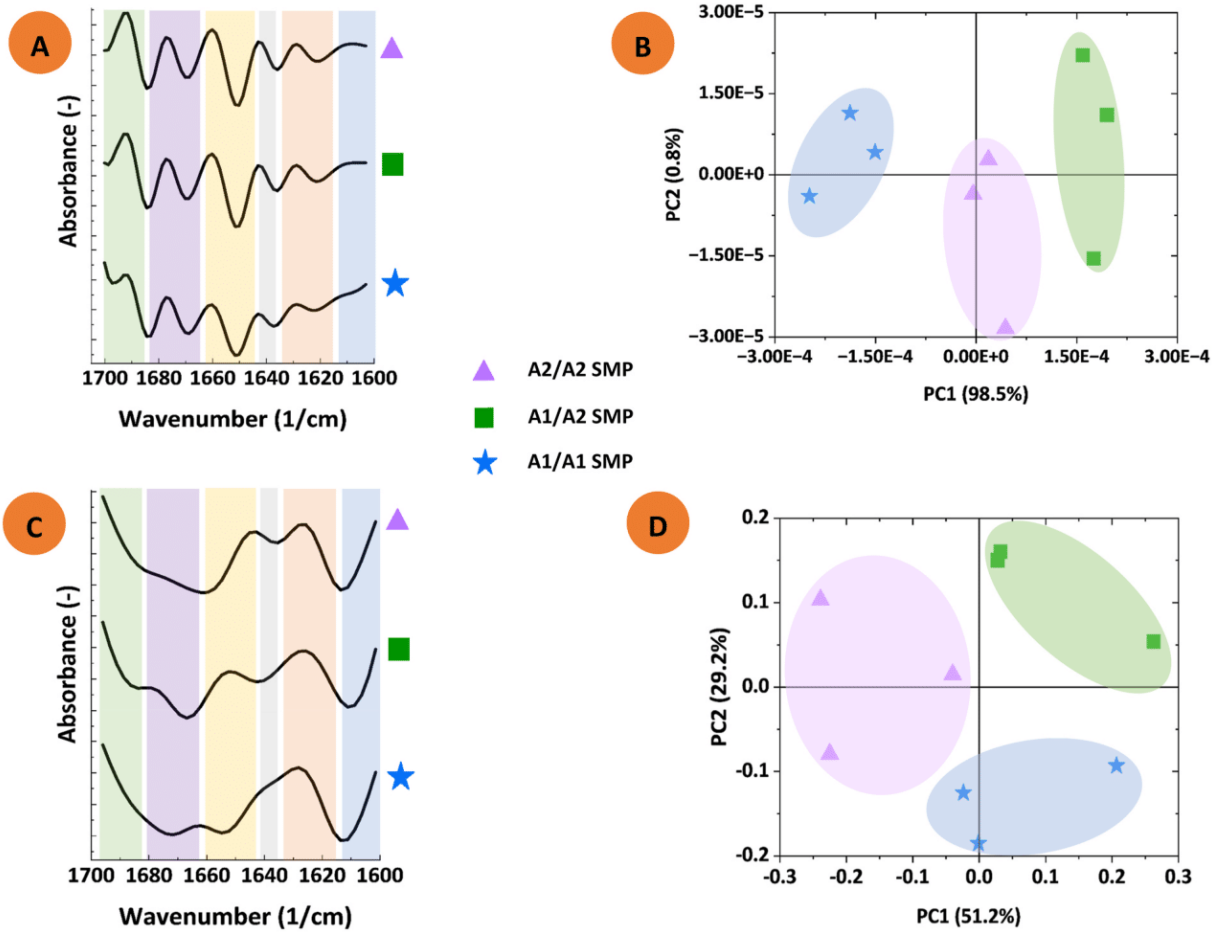

Fig. 3. **A)** Average of three second derivative spectra of Amide I region of SMPs depicted by FTIR. **B)** Scatter plot of the PCA scores of the second derivative FTIR spectra of SMPs. **C)** Average of three second derivative spectra of Amide I region of SMPs depicted by Raman **D)** Scatter plot of the PCA scores of second derivative Raman spectra of SMPs.

Table 4
Total percentage areas of different secondary structures in Amide I of A1/A1, A1/A2, and A2/A2 skim milk powders depicted with FTIR and Raman spectroscopies.

Band Assessment	Band frequency (cm^{-1})	Peak area (%)					
		A1/A1	A1/A2	A2/A2	A1/A1	A1/A2	A2/A2
		FTIR			Raman		
Side chain	1614–1601	2.41 ± 0.79 ^{a}	1.23 ± 0.15 ^{a}	1.66 ± 0.28 ^{a}	0.53 ± 0.05 ^{d}	5.42 ± 0.32 ^{a}	5.95 ± 0.06 ^{c}
Intramolecular β-sheet	1637–1615	15.06 ± 0.42 ^{c}	12.7 ± 1.72 ^{ab}	12.38 ± 0.67 ^{a}	16.83 ± 0.76 ^{b}	23.26 ± 3.10 ^{a}	16.70 ± 3.05 ^{b}
Random coil	1645–1638	6.76 ± 1.60 ^{a}	9.06 ± 0.44 ^{ab}	11.1,6 ± 0.49 ^{ab}	10.52 ± 0.18 ^{c}	11.03 ± 0.23 ^{b}	18.48 ± 0.94 ^{a}
α-helix	1664–1646	33.85 ± 3.50 ^{a}	30.57 ± 1.09 ^{ab}	29.58 ± 0.67 ^{b}	34.34 ± 1.89 ^{a}	25.86 ± 5.33 ^{b}	24.53 ± 0.64 ^{b}
β-turn	1681–1665	16.11 ± 2.94 ^{b}	20.03 ± 0.38 ^{a}	20.17 ± 0.31 ^{a}	17.74 ± 0.26 ^{c}	15.55 ± 0.43 ^{d}	17.27 ± 8.78 ^{b}
Aggregated β-sheet	1700–1682	25.81 ± 1.20 ^{a}	25.88 ± 0.82^{a}	25.04 ± 0.36 ^{a}	19.04 ± 0.60 ^{bc}	18.87 ± 5.03 ^{c}	17.08 ± 0.95 ^{c}

Mean values (± standard deviation, n = 3 measurements) within a row that do not share a common superscript letter are significantly different ($p \leq 0.05$).

characteristics of the powders, as in the present A1/A1 SMP.

The aggregated β-sheets predominantly tend to be more stable, but it may undergo a conformational change during processing of milk into SMP (Ye, Zhou, Shi, Chen, & Du, 2017). Therefore, fewer areas of aggregated β-sheet strands in the A2/A2 SMP, likely mean unfolding and loss of its secondary structure (Fig. 3 and Table 4, $p < 0.05$) (Nishinari, Zhang, & Ikeda, 2000). This translates into the conversion of β-sheet structures into random coils (Farrell, Qi, Wickham, & Unruh, 2002), particularly in A2/A2 SMP. The number of random coils was greater in A2/A2 SMP by a maximum of 54 % compared to that in A1/A1 and A1/A2 samples. Very recently these structures were specifically found in un-treated, heat-treated, and acidified A2/A2 milks (Daniloski et al., 2022d; Daniloski et al., 2022f; Daniloski et al., 2022b). Notably, random coils have been assigned to β-casein, with higher levels in the A2/A2 sample (Table 3, $p < 0.05$), accounting for 70 % of the secondary structure (Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015). Namely, unfolding of the secondary structure depicted by greater levels of random coils of a protein leads to alteration of its functional properties, including solubility (Haque et al., 2010) as in the case of A2/A2 SMP (Tables 2 and 3).

Principal Component Analysis (PCA) was performed separately for FTIR and Raman spectroscopies assessing the secondary structure of proteins (Fig. 3). In FTIR, two principal components (PCs) were able to explain 99.3 % of the total variability. The first PC (PC1), representing 98.5 %, while PC2 accounted for 0.8 % of the total variance. Within Raman and the SMP samples, both PCs explained 80.4 % of the variance. Generally, PC1 was positively linked to A1/A2 SMP regardless of the used spectroscopy, while A1/A1 and A2/A2 SMPs were found either in the negative or the positive side. Presumably, PC1 has separated A1/A2 SMP based on its slightly different properties compared to both

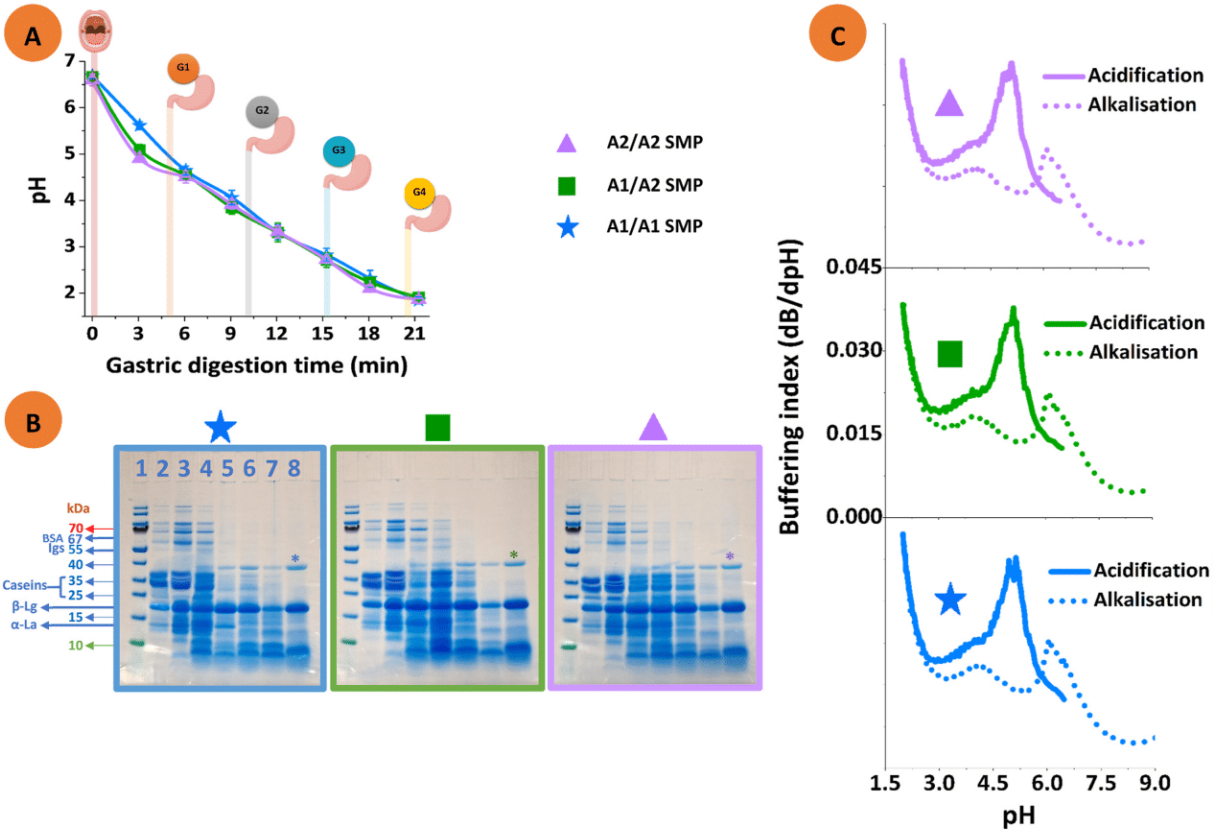

Fig. 4. A) Changes in pH of reconstituted skim milk samples during gastric digestion. **B)** SDS-PAGE patterns under reducing conditions of oral and GE 1–4 phases obtained during gastric digestion at different times. **Line 1**: Molecular marker; **Lane 2**: Skim milk; **Lane 3**: Oral phase; **Lanes 4–7**: GE 1 - GE 4; **Lane 8**: Dried clot after GE 4; BSA (Bovine Serum Albumin); Igs (Immunoglobulins); β-Lg (β-lactoglobulin), α-lactalbumin (α-La), * Pepsin. **C)** Acid-base buffering curves of undigested reconstituted milk samples.

homozygous systems (see section 3.1., Fig. 3B and D). In contrast, all three SMPs were assigned to both positive and negative axes of PC2, which might indicate the possibilities of many conformational similarities with the samples possessing β-caseins A1/A1, A1/A2 or A2/A2 (Fig. 3B and D).

3.1.4. Acid-base titration and heat coagulation time of reconstituted skim milk

Changes in the acid-base buffering curves were observed for A1/A1, A1/A2, and A2/A2 SMP dispersions (Fig. 4 C). When all three reconstituted milks were acidified with HCL, buffering peaks appeared at approximately pH 5.0 for all milk samples. This peak is due to the solubilisation of colloidal calcium phosphate (Lucey, Hauth, Gorry, & Fox, 1993). During back-titration with base, the peak at pH 5.0 was absent, and the buffering peak at pH 6.0 appeared due to the formation of insoluble calcium phosphate (Lucey et al., 1993).

The heat coagulation time (HCT) as a function of pH in the range between 6.2 and 7.4 for reconstituted A1/A1, A1/A2, and A2/A2 milks is shown in Fig. 2 D. While all three reconstituted milk types predicted a typical heat stability for type - A profile (Rose, 1961), reconstituted A1/A1 and A1/A2 milks had similar maximum and minimum HCTs at comparable pH values ($p < 0.05$), as opposed to the A2/A2 sample. Namely, their minimum HCTs were at ~ 9 min (pH 7.0) and their maximum HCTs were < 12 min (pH 6.8). In contrast, reconstituted A2/A2 milk had its minimum and maximum HCTs at pH 7.2 (8.2 min) and at pH 7.0 (11.33 min), respectively. The HCT for both samples carrying β-casein A1 at pH 7.4 was around 20 min. It seems that in A2/A2 sample the HCT curve shifted to the right, however, its HCT at pH 7.4 was significantly lower (11.2 min), thus leading to lower heat stability compared to those of A1/A1 and A1/A2 samples ($p < 0.05$, Fig. 2 D) in this pH region. Similarly, Gai, Uniacke-Lowe, O'Regan, Goulding, & Kelly (2023) found that A2/A2 milk was less heat stable compared to both milks with β-casein A1. The authors suggested that a possible reason for these difference in heat stability might be due to differences in the phenotypes of either κ-casein or β-lactoglobulin among the samples (Gai, Uniacke-Lowe, O'Regan, Goulding, & Kelly, 2023), which is not the case in the present study since all three milks possessed the same genetic variants for the above-mentioned proteins.

The local shift particularly in the minimum heat stability for reconstituted A2/A2 milk can be driven by a change in aggregation kinetics, particularly evident in milks with larger casein micelles ($p < 0.05$, Table 1) and that have lower κ-casein content (Horne & Muir, 1990; Singh & Creamer, 1991). In this regard, even though there is no general consensus on the relation between κ-casein levels and heat stability of milk, selection of small casein micelles with high levels of κ-casein (Tessier & Rose, 1964) and increased electrostatic protein charge tend to give milks with greater heat stability (Singh, 2004), as in the case with A1/A1 and A1/A2 casein micelles and their counterpart milks in the present research. Another reason for the difference in heat stability among the reconstituted milks might be due to heat-induced formation of the β-lactoglobulin/κ-casein complex in the serum (Fox & Morrissey, 1977; Lucey & Horne, 2022), taking into account that the total κ-casein amount was lower in A2/A2 SMP dispersion ($p < 0.05$, Table 3). There is usually more β-lactoglobulin in type - A milks with its local minimum in heat stability associated with the formation of heat-induced whey protein-κ-casein complexes (Dumpler, Huppertz, & Kulozik, 2020). However, this type of interaction was not analysed in the present study.

3.2. Behaviour of reconstituted skim milk samples during gastric digestion

3.2.1. pH profiles and gastric emptying

Milk and milk proteins have been shown to be highly digestible (Dupont & Tomé, 2020). However, small changes in the structure can have a significant impact on their digestive behaviour in the stomach in terms of mechanism and kinetics. In particular, the gastric phase with its relatively slow change in pH and concurrent addition of digestive enzymes can give rise to substantial gastric restructuring of the protein matrix (Mulet-Cabero, Mackie, Brodkorb, & Wilde, 2020). The pH profiles of reconstituted A1/A1, A1/A2, and A2/A2 milks during the gastric digestion are shown in Fig. 4 A. Whilst, there was no significant difference among the milks in the change in pH with digestion time ($p > 0.05$), where pH decreased from pH 6.7 to pH 1.9 after ~ 22 min, both reconstituted milks with β-casein A2 experienced a steep decrease in pH between pH 5.7 and 4.9, which was not as noticeable in A1/A1 milk. The main difference observed at pH below 5.7 is believed to be associated with the solubilisation of colloidal calcium phosphate (Lucey et al., 1993), and which can strongly influence the casein micelle digestion behaviour in the stomach (Mulet-Cabero, Mackie, et al., 2020). It is known that H^+ ions are accepted by polar and charged amino acids, including histidine, which is the only amino acid difference between β-casein A1 and A2 (Daniloski et al., 2022d). Subsequently, the formation of ionic and hydrogen-bonds between H^+ ions and histidine may take place (Scheiner, Kar, & Pattanayak, 2002), and thus greater coagulation properties. Generally, faster protein coagulation normally results in faster pH reduction (Li et al., 2022).

Caseins are insoluble at pH 4.6 (isoelectric point), whereas native whey proteins remain soluble but can aggregate if they are denatured or covalently linked to κ-casein (Huppertz & Chia, 2021; Huppertz et al., 2018). In the present study, protein aggregation commenced after ~2 min of gastric digestion at a pH value of about 5.5 for all three samples, which is a significantly higher pH than the isoelectric point of caseins. The *in vitro* gastric digestion is based on *in vivo* gastric emptying data and is strongly correlated with the caloric value of a meal (Brodkorb et al., 2019). As skim has no fat, it is relatively low in calories, hence the pH drops relatively quickly and reaches ~ pH 2.0 after only 21.76 min. The formation of clots or aggregates is normal for untreated milk, which is due to the acid coagulation process unaffected by the conditions of the enzymes (Lucey and Singh, 1997). Furthermore, the initial coagulation could be driven by the action of pepsin that has been reported to favour the hydrolysis of κ-casein into para-κ-casein, which reduces the steric repulsion between casein micelles and destabilises its overall structure (Dalgleish & Holt, 1988; Miranda & Pelissier, 1983). At this stage the serum phase became clear, indicating the casein micelles were incorporated into the clot formed after 5 min. Similar results were found in the study of Ye, Cui, Dalgleish, and Singh (2016a) when observing the gastric digestion of skim milk. However, their initial milk sample size was 200 g compared to 45 g of milk carrying various β-caseins in the present study. Additionally, the milk digestion time was 220 min (10 times longer than in the present analysis) due to the dynamic *in vitro* digestion method used that was different to the semi-dynamic method used in the current study (Ye et al., 2016a).

3.2.2. Microstructure of the gastric digesta

The microstructure of the clots formed from reconstituted A1/A1, A1/A2, and A2/A2 milks during the four gastric phases are shown in Fig. 5 A. After 5.30 min of digestion at ~ pH 4.6, large aggregated structures were observed in all three reconstituted milk types. These structures possessed a dense, closely knit network, with a more porous network depicted for the A2/A2 sample. Previously, Daniloski et al. (2022d) showed the formation of a "soft" gel at pH 4.6 from A2/A2 milk, with a low storage modulus and discontinuous branching network. After 10–15 min of digestion (GE 2 and GE 3), a network structure with larger voids was observed in reconstituted A1/A1 and A1/A2 milks, characterised by a highly porous matrix. As the digestion time progressed, the structure of A2/A2 digesta became denser and the pores in the matrix were smaller compared to both samples with β-casein A1 (Fig. 5 A). Although, the network of A2/A2 digesta appeared to fuse into a smoother clot, the porosity of the network structure split into numerous clots of aggregated protein, evident in the last GE point for all three samples. This was due to the combined action of pepsin, mechanical force in the gastric vessel (reflecting the human stomach), and the gradual decrease in pH driven by HCL addition (Mulet-Cabero, Mackie,

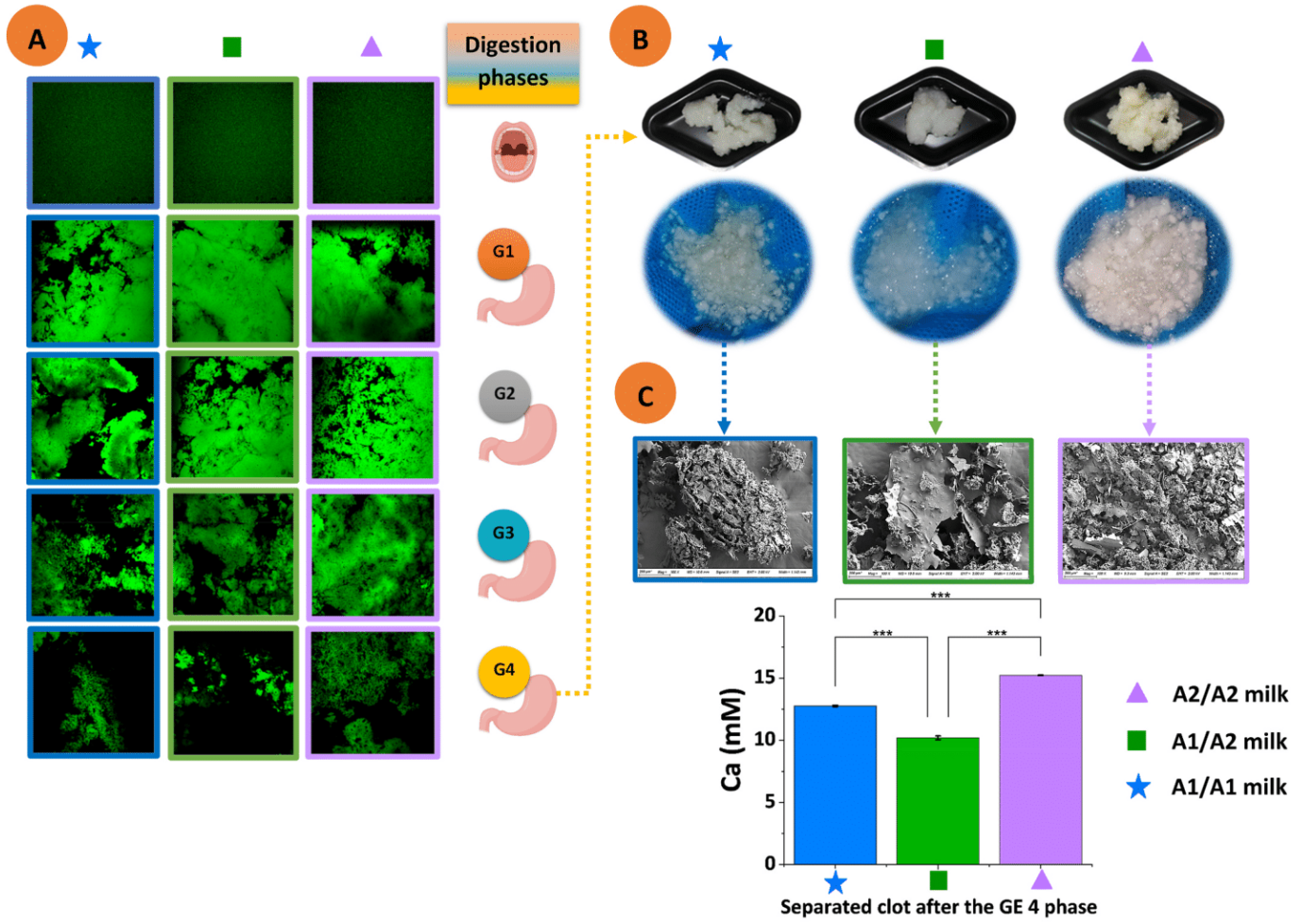

Fig. 5. A) CLMS micrographs of the clots obtained during the gastric digestion of reconstituted milks at different times, scale bars represent 25 μm. **B)** Images of wet clots formed during the gastric digestion of 45 g of reconstituted samples obtained at GE 4 (final gastric phase). **C)** Representative SEM micrographs of the separated clot after the GE 4 phase (scale bars represent 200 μm), including their calcium content. *** Statistical significance $p < 0.001$.

et al., 2020). As a result, these factors mentioned above led to aggregation of caseins at lower pH, separating them to some extent from β-lactoglobulin, which is resistant to the action of pepsin in its native form (Kitabatake & Kinekawa, 1998; Mulet-Cabero, Egger, et al., 2020). The disappearance of the protein network upon gastric digestion has been observed in numerous previous studies during *in vitro* digestion of milk (Mulet-Cabero et al., 2019, 2020a; Ye et al., 2016b). In particular, as the aggregated protein curds became smaller in size as digestion progressed, the clots in A2/A2 milk remained consistently larger than in A1/A1 and A1/A2 milks (Fig. 5 A).

In this regard, the final weight (wet and dried weights) of the separated clot after the GE 4 phase was significantly higher in the reconstituted A2/A2 milk (3.23 g ± 0.03 and 0.51 g ± 0.01) as opposed to reconstituted A1/A1 (2.50 g ± 0.05 and 0.38 g ± 0.02) or A1/A2 (2.05 g ± 0.01 and 0.25 g ± 0.01) milks ($p < 0.05$, Fig. 5 B). Comparisons of the wet and dry weights of the clots, but also their microstructure (Fig. 5 C), showed that there was a considerable difference in the remaining protein curd at the end of digestion in the reconstituted A2/A2 milk, indicating their resistance to gastric digestion (Zhang & Vardhanabhuti, 2014). Notably, the dried clots of A1/A1 and A1/A2 samples possessed significantly lower calcium content as opposed to the A2/A2 clot ($p < 0.05$, Fig. 5 C). Namely, higher calcium amount in milk is related to an improved acid gelation and rennet coagulation properties, smaller casein micelle size, and firmer gel (Poulsen et al., 2013), which may be the case for the knit network of A2/A2 digested clot (Fig. 5 C). The reconstituted A1/A1 and A1/A2 milks appear to possess faster gastric protein digestion in terms of curd formation and breakdown. Very recently, during human *in vivo* digestion with lactose-maldigesting subjects ($n = 10$) using reduced fat A1/A2 (75/25%) and A2/A2 milks, it was found that A2/A2 milk had a slower gastric transit (Ramakrishnan et al., 2023). Also, the authors found that consumption of A2/A2 milk resulted in a gastric volume that was approximately 2.5 times the volume after 120 min as compared to the consumption of A1/A2 milk (Ramakrishnan et al., 2023).

3.2.3. Protein hydrolysis and conformational features of digesta

Using SDS-PAGE, protein composition and the extent of hydrolysis was shown to have a similar profile in all three reconstituted milks; the bands corresponding to the samples before digestion did not differ (Fig. 4 B). There was an increase of the protein breakdown at first two GE points, after which it levelled off, showing no increase at GE 3 point (Fig. 4 B). Following this, the level of protein breakdown significantly increased at GE 4 for all samples, yet was still lower for the reconstituted A2/A2 milk. Hence, its corresponding GE 4 band was more intense, signifying more undigested protein in this sample, compared to A1/A1 and A1/A2 milks (Fig. 4 B and 5 A). As previously shown, the protein network in A2/A2 milk was characterised by a continuous branching network and bigger clots compared to the other systems (Fig. 5 A and 5

B). Supposedly, when a dense clot is formed, the diffusion of pepsin into the clot is slow (Ye et al., 2016a). However, the small protein clots of A1/A1 and A1/A2 digesta may allow for increased pepsin-protein interactions in the clot, hence promoting increased hydrolysis.

The caseins in all samples were detectable in the first emptying point, also followed by the GE 2 (pH between 4 and 5) where these proteins could also be observed together with some liberated peptides (Fig. 4 B). The low protein hydrolysis in all samples presumably occurred because of the casein gelation at these pH conditions, which resulted in the pepsin susceptible bonds being buried in the aggregated casein (Guo, Fox, Flynn, & Kindstedt, 1995). After 15 min of digestion, most of the caseins were hydrolysed because of the higher activity of pepsin at low pH. Therefore, many small molecular weight peptides were present during digestion and could be seen from GE 2 onwards. This rapid degradation of caseins by the action of pepsin is also in accordance with other results reported after static *in vitro* digestion on reconstituted skim milks from SMPs (Sánchez-Rivera, Ménard, Recio, & Dupont, 2015) and in whole bovine milks (Mulet-Cabero et al., 2019).

The initial rate of hydrolysis by pepsin at pH below 6.0 is faster for κ-casein than for other caseins *in vitro* (Mulvihill & Fox, 1979), which is also depicted in reconstituted A1/A1, A1/A2, and A2/A2 milks. Particularly, in all samples, the κ-casein band started fading after 10 min of digestion (at the second GE point). This indicated that pepsin hydrolysed κ-casein to para κ-casein early on in the digestion process, which initiated the aggregation of casein, also seen in the studies of Ye et al. (2016a) and Mulet-Cabero et al. (2019). Similarly, the band referring to αs_2-casein expectedly diminished during digestion and showed that this protein (as the other caseins) in A2/A2 digesta was resistant to breakdown until the GE 3, before it was completely hydrolysed in the last gastric phase (Fig. 4 B). The αs_2-casein is the most hydrophilic of all caseins, due to the three clusters of anionic groups in the amino acid sequence, composed of phosphoseryl and glutamyl residues (Farrell et al., 2004). The overall hydrophilic nature of αs_2-casein could make it more accessible to digestive enzymes.

Contrarily, between 10 and 15 min of digestion, the β-casein band was more intense than the αs_1-casein band in all three milks (Fig. 4 B), indicating a slower rate of β-casein hydrolysis compared to that of αs_1-casein. Breakdown of β-casein was reported to be less extensive than that of αs_1-casein using chymosin, bovine pepsin, or porcine pepsin (Fox, 1989), as well as during *in vitro* digestion of camel milk (Li, Ayyash, Ye, & Singh, 2023). The greater hydrophobic nature of β-casein results from most of its hydrophobic and aromatic amino acid side-chains being well buried in its hydrophobic core, lowering the specificity and affinity of pepsin towards β-casein, thus preventing proteolysis (Dalgalarrondo, Dufour, Chobert, Bertrand-Harb, & Haertlé, 1995). Fox (1969) also found that at higher temperatures, β-casein was less suspectable to proteolysis compared to αs_1-casein. This was attributed to the hydrophobic nature of β-casein partially folded into poly-L-proline- and α-helical conformations resulting in greater resistance to proteolysis (Christensen, 1955; Fox, 1969). Interestingly, the level of random coil motifs in all three samples increased during digestion, but especially in A1/A1 and A1/A2 milks ($p < 0.05$, Table 5). Hence, if the secondary structures are totally diminished, proteins start to behave as random coils, leading to soft gel formation (Nishinari et al., 2000), as expected during digestion. As previously mentioned, random coil conformations have been assigned to β-casein (Fox et al., 2015). Since β-casein levels were higher compared to αs- and κ-casein in the GE points of all three samples, it is expected that this protein is the driver for the formation of random coils in its counterpart digesta (Table 5).

Reduction of α-lactalbumin may have resulted from both, dilution, and pepsin hydrolysis, especially at pH values lower than 4 (Fig. 4 B). This has been depicted previously; pepsin was shown to be active towards α-lactalbumin during *in vitro* digestion of milk, including infant formula, but only at pH < 4 (Li et al., 2022; Roy, Ye, Moughan, & Singh, 2021). With increasing digestion time, only β-lactoglobulin observed in the digesta was not hydrolysed by pepsin in its native form (Miranda & Pelissier, 1983). Miranda and Pelissier (1983) reported that β-lactoglobulin and α-lactalbumin were still present in considerable amounts in the stomach contents of rats after 30 min of digestion of skim milk. Additionally, Mahé et al. (1996) reported that casein empties from the stomach mainly as peptides, whereas β-lactoglobulin exits the stomach mainly as intact protein (Mahé et al., 1996), similar to the results in the current study. Notably, in the last GE point, A2/A2 digesta had greater amount of β-sheet structures compared to the other samples ($p < 0.05$). This was rather unexpected since β-sheets refer to a stable secondary structure and acid gel with improved gelation properties (Daniloski et al., 2022d); its levels were higher compared to the other motifs in all GE points for all samples. This could be due to presence of β-lactoglobulin and its affinity to appear in β-sheet conformational motifs (Uhrínová et al., 1998).

Second derivative of the spectra in the Amide I region possessed similar grouping of samples according to the digestion stages, though not as precise as that for the PCA analysis. Thus, PCA of the Amide I region possessed a data variation ranging between 80 and 99 % being

Table 5
Total percentage areas of different secondary structures in Amide I of A1/A1, A1/A2, and A2/A2 digesta depicted with FTIR spectroscopy.

Sample	Peak area (%) - Oral phase					
	Side chain	Intramolecular β-sheet	Random coil	α-helix	β-turn	Aggregated β-sheet
A1/A1	4.98 ± 0.24 ^{ab}	14.76 ± 0.88 ^{b}	10.12 ± 0.05 ^{c}	20.26 ± 0.13 ^{a}	13.93 ± 1.12 ^{b}	35.95 ± 0.09 ^{b}
A1/A2	3.86 ± 1.69 ^{b}	16.41 ± 0.86 ^{a}	12.02 ± 1.36 ^{b}	16.69 ± 0.46 ^{b}	19.02 ± 2.44 ^{a}	32.00 ± 3.43 ^{c}
A2/A2	5.34 ± 1.90 ^{a}	12.31 ± 3.14 ^{c}	13.47 ± 1.06 ^{a}	9.68 ± 0.27 ^{c}	19.35 ± 2.15 ^{a}	39.84 ± 5.85 ^{a}
	Peak area (%) - GE 1 phase					
A1/A1	3.41 ± 0.05 ^{a}	13.85 ± 0.44 ^{c}	10.46 ± 0.39 ^{c}	19.22 ± 0.92 ^{a}	19.05 ± 0.13 ^{a}	34.02 ± 1.15 ^{a}
A1/A2	2.55 ± 0.07 ^{b}	14.86 ± 1.67 ^{b}	14.63 ± 0.99 ^{a}	16.02 ± 1.96 ^{c}	18.88 ± 0.82 ^{b}	33.06 ± 3.73 ^{b}
A2/A2	2.47 ± 1.43 ^{b}	15.70 ± 1.04 ^{a}	12.57 ± 2.43 ^{b}	18.64 ± 1.60 ^{b}	19.41 ± 4.20 ^{a}	31.21 ± 0.91 ^{c}
	Peak area (%) - GE 2 phase					
A1/A1	8.20 ± 2.39 ^{a}	12.84 ± 0.02 ^{b}	14.29 ± 1.17 ^{b}	15.72 ± 0.97 ^{b}	12.49 ± 1.97 ^{c}	36.46 ± 0.64 ^{a}
A1/A2	3.15 ± 0.26 ^{b}	11.57 ± 2.91 ^{c}	13.29 ± 1.41 ^{c}	16.62 ± 1.70 ^{c}	20.32 ± 1.60 ^{a}	35.06 ± 1.34 ^{b}
A2/A2	2.34 ± 0.63 ^{c}	13.83 ± 1.03 ^{a}	15.29 ± 0.68 ^{a}	20.48 ± 3.78 ^{a}	19.37 ± 1.15 ^{b}	28.69 ± 0.29 ^{c}
	Peak area (%) - GE 3 phase					
A1/A1	3.33 ± 0.78 ^{a}	10.70 ± 0.47 ^{c}	21.56 ± 0.95 ^{b}	20.72 ± 2.30 ^{a}	16.04 ± 0.16 ^{c}	27.64 ± 1.50 ^{b}
A1/A2	2.39 ± 0.27 ^{b}	11.71 ± 0.13 ^{b}	28.68 ± 1.45 ^{a}	16.45 ± 0.53 ^{b}	20.02 ± 0.93 ^{b}	20.75 ± 0.14 ^{c}
A2/A2	2.71 ± 0.01 ^{b}	12.85 ± 0.23 ^{a}	19.12 ± 1.15 ^{c}	14.33 ± 1.56 ^{c}	22.33 ± 1.14 ^{a}	28.66 ± 0.95 ^{a}
	Peak area (%) - GE 4 phase					
A1/A1	3.34 ± 0.87 ^{a}	10.58 ± 0.51 ^{b}	37.64 ± 2.64 ^{a}	11.68 ± 0.47 ^{b}	18.84 ± 2.68 ^{b}	17.93 ± 1.80 ^{b}
A1/A2	3.09 ± 0.26 ^{a}	11.61 ± 0.31 ^{a}	37.08 ± 1.05 ^{a}	14.86 ± 1.12 ^{a}	21.75 ± 1.12 ^{a}	11.60 ± 1.00 ^{c}
A2/A2	3.29 ± 1.09 ^{a}	11.66 ± 0.87 ^{a}	29.74 ± 1.05 ^{b}	12.00 ± 0.24 ^{b}	17.03 ± 0.72 ^{c}	26.28 ± 2.05 ^{a}
Wavenumber (cm^{-1})	1614–1601	1637–1615	1645–1638	1664–1646	1681–1665	1700–1682

Mean values (± standard deviation, n = 3 measurements) within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$).

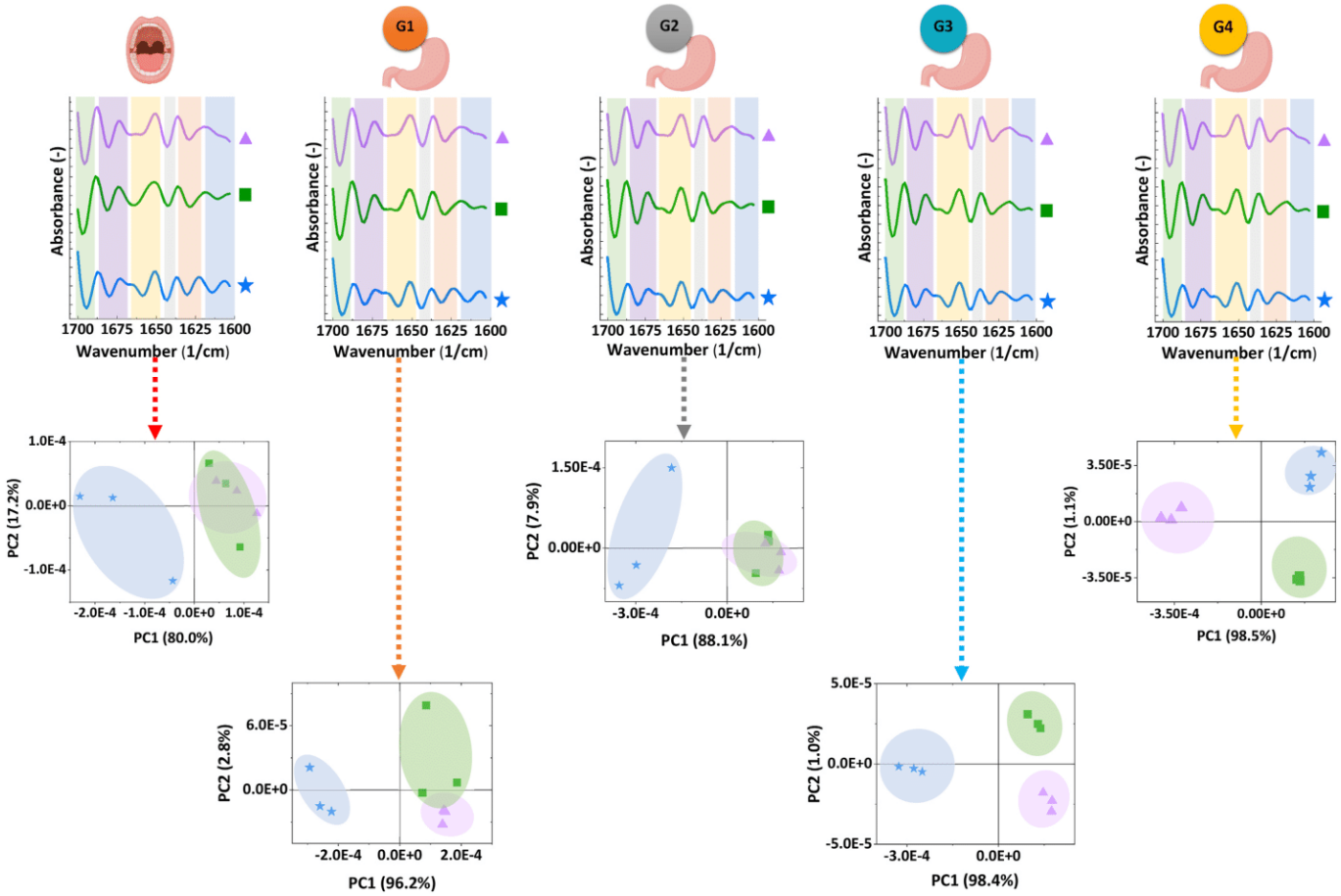

Fig. 6. Second derivative spectra and scatter plot of the PCA scores of Amide I region of oral and GE 1–4 phases of the reconstituted milk samples depicted by FTIR.

explained in PC 1 and between 1 and 17 % in PC 2 (Fig. 6). Although, A1/A1 digesta between oral and GE 1 - GE 2 phases differ significantly compared to the other samples ($p < 0.05$), the major difference between the Amide I band data and those for the other spectral regions was the clear differentiation of A2/A2 GE 4 digesta which remained isolated from the rest of the samples (also shown in the micrographs and protein hydrolysis results, Fig. 4 B and 5 A). It should be noted that the main differences in FTIR spectra of GE 4 samples was in the region around ~ 1644 cm^{-1} (in A1/A1 and A1/A2 samples the spectra shifted to the left). This shift actually shows the OH bond and the possibility for bond cleavage during digestion (Verma et al., 2022), and may corroborate with the greater extent of protein hydrolysis observed in these two samples compared to A2/A2 milk in the last gastric phase.

4. Conclusion

This study showed how genetic variants of β-casein influenced the physical properties of skim milk powders and effected their subsequent digestion behaviour. Overall, β-casein phenotype had only minor effects on the physical properties of skim milk powders, with A1/A1 and A1/A2 samples having better rehydration properties, than A2/A2 SMP. This may be related to increased levels of random motifs in A2/A2 milk, due to the presence of proline at position 67 in the polypeptide chain of β-casein. However, this study has shown that dairy processors may also need to be cognisant of altered heat stability, as there was differences over the HCT profile between A1 and A2 milks, although at a pH of 6.6 there was no significant difference observed. During gastric digestion, the faster rate of protein breakdown in A1 milks, compared to A2 milks was definitive. This could be useful for the production of specific nutritional formulations with tailored digestion properties. Overall, the dairy industry should be cognisant that the physical properties of skim milk powder may change, depending on β-casein phenotype, but it is more likely that from a human nutrition perspective, gastric digestion will be significantly affected.

CRediT authorship contribution statement

Davor Daniloski: Data curation, Investigation, Writing – original draft, Writing – review & editing. **Yonas Hailu:** Data curation, Formal analysis, Methodology. **André Brodkorb:** Data curation, Methodology, Writing – review & editing. **Todor Vasiljevic:** Investigation, Supervision, Writing – review & editing. **Noel A. McCarthy:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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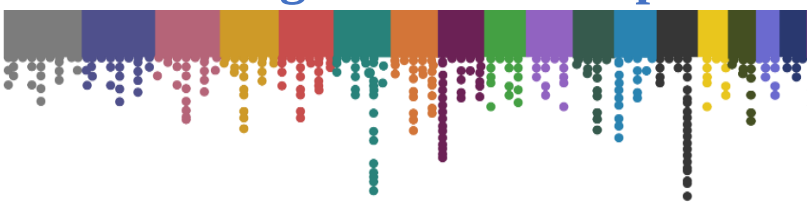

Physicochemical and simulated gastric digestion properties of A1/A1, A1/A2 and A2/A2 yoghurt

- Physical properties of the yoghurts were driven by their matrices
- A2/A2 yoghurt possessed lower water retention and greater syneresis
- Yoghurts carrying β-casein A1 had more aggerated β-sheets
- A2/A2 yoghurt matrix led to slower protein breakdown during gastric digestion
- Digesta from A1/A1 and A1/A2 yoghurts showed a porous microstructure

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Davor Daniloski	80	Designed and wrote the original draft, conceptualised, reviewed, edited, revised the manuscript, designed the experiments, the tables and the figures, and data analysis	[redacted]	17/03/2024
Todor Vasiljevic	4	Concept development, manuscript editing, revision, and supervision		17/03/2024
Daniela Freitas	3	Concept development, manuscript editing and revision		17/03/2024
Talita A. Comunian	3	Concept development, manuscript editing and revision		17/03/2024
Andre Brodkorb	4	Concept development, experimental design, manuscript editing and revision		17/03/2024
Noel A. McCarthy	6	Concept development, experimental design, manuscript editing, revision, submission, and supervision		17/03/2024

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Abstract

This study aimed to determine the physicochemical properties of yoghurts from skim milk containing either β-casein A1/A1, A1/A2 or A2/A2 and establish their behaviour during simulated gastric digestion. Yoghurts with β-casein A1 had significantly higher storage modulus, water holding capacity, and lower syneresis compared to A2/A2 yoghurt. Microscopy images also showed a more porous microstructure and greater pore size in A2/A2 yoghurts. The main conformational variability included approximately 35 % lower amounts of aggregated β-sheets in A2/A2 yoghurt as opposed to the other yoghurt systems. The A2/A2 sample was also characterised by higher levels of β-lactoglobulin in the yoghurt serum phase, larger casein micelles and lower levels of κ-casein. During gastric digestion, coagulum formation in all three yoghurts occurred within the first 5 min when the pH was ranging from 3.8 to 4.3. However, the protein breakdown of A1/A1 and A1/A2 yoghurts was faster with the final gastric clot characterised with a loose protein network and between 20 and 50 % greater levels of random coils, as opposed to that of A2/A2 digesta. Overall, the use of A2/A2 milk in yoghurt production results in prolonged gelation times, with altered digestibility compared to yoghurts with β-casein A1.

Keywords:

Yoghurt; gelation properties; β-caseins A1/A1, A1/A2, and A2/A2; structure; gastric digestion.

1. Introduction

Bovine milk and derivatives thereof are comprised of several components, yet its milk proteins in particular, are subject of an ongoing debate regarding their composition, nature, technological role, and biological significance to human health (McSweeney & Fox, 2013). Two main categories of proteins are found in milk. Caseins, flexible and unordered proteins, predominantly present in the form of casein micelles, classified as αs-, β- and κ-caseins; and whey proteins, including α-lactalbumin, β-lactoglobulin and other minor proteins, globular in nature and heat labile (Huppertz & Gazi, 2022; Walstra, Walstra, Wouters, & Geurts, 2005). These two families of dairy proteins hold paramount importance in the technological properties of milk, profoundly influencing gel formation during the manufacture of fermented dairy-based products, such as in set-yoghurt production (Lucey, 2017; Lucey, Tamehana, Singh, & Munro, 1998).

During acid gelation, the *hairy* κ-casein layer curls, the casein particles aggregate, simultaneously micellar calcium phosphate solubilises, as the pH decreases from ~ pH 6.70 to pH 4.60 (Lucey & Singh, 1997). Prior to yoghurt production, milk is usually heated at a high temperature in order to denature and aggregate whey proteins, particularly β-lactoglobulin (Anema, 2021). Denatured whey proteins can interact with κ-casein on the surface of casein micelles during heat treatment but is pH dependent (Ozcan, Horne, & Lucey, 2015). Factors including, protein, fat and mineral concentration and profile (i.e., amino acid and fatty acid profile), heat treatment (time-temperature combination), starter culture used, influencing manufacturing properties of acid and yoghurt gels have been investigated extensively for many decades (Lucey et al., 1998; Robinson, Lucey, & Tamime, 2006; Walstra et al., 2005). However, a number of studies (Aschaffenburg, 1961; Aschaffenburg & Drewry, 1955; Jakob & Puhan, 1992; Ketto et al., 2018; Poulsen & Larsen, 2021) have also shown that the genetic polymorphism of milk proteins appears to affect the properties of acid gels, including yoghurt. From these studies, there is a general agreement that A/A phenotypes of both κ-casein and β-lactoglobulin are associated with improved acid gelation properties (Hallén, Allmere, Lundén, & Andrén, 2009). It was also established that protein from β-casein A2/A2 cows is a contributing factor in poorly coagulating milks (Poulsen & Larsen, 2021). Furthermore, A2/A2 milk had a detrimental effect on the properties of acidified milk gels and yoghurts when compared to A1/A1 and A1/A2 milks across New Zealand and Australia (Daniloski, McCarthy, Gazi, & Vasiljevic, 2022a; Nguyen, Schwendel, Harland, & Day, 2018a), and most recently,

in milk obtained from Irish Holstein cows (Gai, Uniacke-Lowe, O'Regan, Goulding, & Kelly, 2023).

While there have been differences found in the physicochemical properties of acid gels and yoghurts based on protein genotype (Poulsen & Larsen, 2021), very few studies have investigated the effect of the yoghurt matrix on its gastric digestibility. Yoghurt digestion is affected by milk composition, particularly the protein structure and mineral levels, which can differ with breed, stage of lactation, milk processing, and casein genotype (Dupont & Tomé, 2020; Mulet-Cabero, Mackie, Brodkorb, & Wilde, 2020; Ye, 2021). For example, Sheng, Nielsen, Poulsen, and Larsen (2021) reported significantly lower *in vitro* gastric digestion of milk with B/B κ-casein compared with that of A/A and A/B milks, which was linked to its casein micelle size. In contrast, the effect of β-casein phenotypes on intestinal digestion properties have been previously reported (De Noni & Cattaneo, 2010; Nguyen, Busetti, Johnson, & Solah, 2018); however, the literature is scare on how any casein phenotypes alters yoghurt gastric digestion. Therefore, this study aimed to investigate if β-casein phenotype influences yoghurt properties, specifically gel strength and microstructure, and if these differences subsequently affect digestibility of yoghurts containing the two well-defined β-casein proteoforms, A1 and A2.

2. Materials and methods

2.1. Skim milk powder production

Approximately 250 L of milk was collected for each β-casein phenotype (i.e., A1/A1, A1/A2 and A2/A2) from 28 individual Irish Holstein Friesian breed (A1/A1 cows = 8; A1/A2 cows = 10, and A2/A2 cows = 10) from the Moorepark Dairy Farm in the Teagasc Animal and Grassland Research and Innovation Centre (Teagasc, Moorepark, Fermoy, Co. Cork, Ireland). Upon receipt, the raw whole milk from individual cows was pooled based on β-casein phenotype, subsequently the cream was separated at 60 °C using a Westfalia centrifugal disk separator, GEA Westfalia, Oelde, Germany), pasteurised at 80 °C for 30 s (pilot-scale UHT/HTST system, MicroThermics, Raleigh, NC, USA), and spray dried (Anhydro single-stage spray dryer, SPX Flow Technology, Soeborg, Denmark) (McSweeney, Maidannyk,

Montgomery, O'Mahony, & McCarthy, 2020). The spray dried milk powders were stored in double sealed plastic bags at 8 °C and away from direct sunlight.

Skim milk powders were rehydrated in Milli-Q water (purified by a Milli-Q apparatus, Millipore Corp., Bedford, MA, USA) by adding around 11 g of milk powder to 89 mL of water resulting in a similar protein content, approximately 3.50 % (w/w). The rehydrated skim milk dispersions were then stirred for 1 h at room temperature and left to hydrate overnight at 4 °C prior to further analyses and yoghurt production. All chemicals used in the study were of analytical grade and were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA) unless otherwise specified.

2.2. Yoghurt preparation

The yoghurts were prepared using rehydrated raw skim milk dispersions by initially heating at 85 °C for 10 min, followed by cooling to 43 °C. As per the manufacturer's guidelines a yoghurt starter culture was added at 2 % w/w (YC-380: *Lactobacillus delbrueckii subsp. Bulgaricus* and *Streptococcus thermophilus*, CHR Hansen, Hørsholm, Denmark, 500U · $2500L^{-1}$). The inoculated milk was then distributed into containers and incubated at 43 °C until a pH of 4.6 was reached. During fermentation, pH of the milk was measured using a CyberScan pH meter 510 (Eutech Instruments, Singapore). Yoghurt samples were stored at 4 °C overnight for further analyses, including physicochemical, structure-functionality, and digestion properties (Nguyen, Afsar, & Day, 2018b). The samples were named as A1/A1, A1/A2, or A2/A2 yoghurts depending on the β-casein phenotypes present in the samples.

2.3. Chemical composition analysis on milk, milk serum, and yoghurt serum

The total protein content of milk and supernatant samples obtained from milk and yoghurt was established by the Kjeldahl method (ISO, 2014). To obtain supernatants for distribution of caseins and whey proteins in rehydrated skim milks (pre-warmed at 43 °C) and yoghurts, the samples were centrifuged at 100,000 · g for 1 h at 43 °C in a Beckman Coulter Ultra L - 70 centrifuge (Beckman Instruments, Indianapolis, IN, USA). The clear supernatants were carefully removed and used for protein profiling and mineral determination. The distribution of individual caseins between the milk and milk serum, but also the yoghurt serum was determined by Reversed Phase-High Performance Liquid Chromatography (RP-HPLC). An

Agilent 1200s HPLC equipped with a quaternary pump, heated column compartment, temperature-controlled autosampler and multiwavelength detector was used with the data being processed using the ChemStation software (Agilent Technologies Ireland Ltd, Little Island, Cork, Ireland). Separation and identification of each single milk protein was achieved using Poroshell 300SB-C_{18} column (2.1 mm diameter, 75 mm length, 5 µm; Agilent Technologies, Ireland), equipped with a Zorbax poroshell guard column (1.0 mm diameter, 17 mm length, 5 µm; Agilent Technologies, Ireland). Elution was attained with acetonitrile:water:TFA mixture (100:899:1,v/v/v; mobile phase A), and acetonitrile:water:TFA mixture (900:99.1:0.9, v/v/v, mobile phase B) (Bobe, Beitz, Freeman, & Lindberg, 1998). The total and soluble mineral content in all three, rehydrated skim milk, milk serum (before yoghurt manufacture), and yoghurt serum obtained as described above, were determined using an Inductively Coupled Plasma - Atomic Emission Spectrometer (ICP - AES Multitype, Shimadzu Corporation, Kyoto, Japan). The samples were ashed and dissolved in 1 M nitric acid before analysing the mineral content (Daniloski et al., 2022a). The particle size and zeta-potential of unheated and heated milk samples diluted four-fold in Milli-Q water were measured at 43 °C using Malvern Zetasizer Nano ZS (Zetasizer-Nano ZS, Malvern Instruments, Malvern, Worcestershire UK) (Jenness, 1962).

2.4. Small and large deformation studies of yoghurt samples

The viscoelastic properties of the milk samples were established using a Discovery HR-1 hybrid rheometer (TA Instruments, New Castle, DE, USA), equipped with a concentric cylinder maintained at 43 °C, with bob diameter (28 mm), bob length (42 mm), cup diameter (30 µm), operating gap (5,920 µm) and loading gap (9,000 µm). An aliquot (19 mL) of the freshly inoculated milk was immediately transferred into the concentric cylinder. The storage (G') moduli of the samples were determined at constant 1 % strain and a frequency of 1 Hz until pH 4.6 was reached. This was monitored by measuring the pH of simultaneously incubated samples of inoculated milk, which were maintained at the same temperature as the sample in the rheometer for the same duration. This was followed immediately by a frequency sweep from 1 to 63.1 Hz at a constant strain of 1 %. Gelation was defined as the point at which the *G'* of the gel was ≥ 1 Pa (Lucey et al., 1998).

Following overnight refrigerated storage, the yoghurt samples (100 mL, per sample) were analysed using a 35-mm flat-disk backward extrusion rig on a Texture Expert Exceed system

(Stable Microsystems, Godalming, UK) at 4 °C. Probe force was calibrated using a 2 kg weight mounted on a 5 kg load cell. Trigger force was set at 2 g. The probe penetrated the sample to a depth of 25 mm and returned to the starting point. Pre-test, test, and post-test probe speeds were set at 1 mm · s^{-1}. The sample firmness, consistency, cohesiveness, and index of viscosity were recorded (Bourne, 1978).

For the water retention, 30 mL of yoghurt was produced (Section 2.2.) in a 50 mL Falcon tube (Falcon, Blue Max; Becton Dickinson and Co., Franklin Lakes, NJ, USA). Subsequently, the tubes were centrifuged for 20 min at 5000 x g and 20 °C in a centrifuge (Sorvall Lynx 6000) using a Fiberlite F21-8x50y rotor (Thermo Fisher Scientific, Waltham, MA, USA). The supernatant was carefully decanted, collected, and weighed. The water holding capacity (WHC) of the yoghurts was expressed as a percentage, considering the weight of the yoghurt gel (pellet) after supernatant was expelled, relative to the weight of the total sample (Daniloski et al., 2022a). The level of spontaneous whey separation from undisturbed yoghurts was measured according to the siphon method as explained by Sah, Vasiljevic, McKechnie, and Donkor (2016).

2.5. Conformational features of yoghurt samples

The yoghurt samples were prepared (Section 2.2.) in 50 mL Falcon tubes and rapidly immersed into liquid nitrogen at - 196 °C for 15 s. The frozen samples were immediately stored at - 80 °C overnight to prevent ice re-crystallisation including any changes in their microstructure. After that, the samples were lyophilised in a pilot scale freeze dryer (Edwards Pirani 501 freeze dryer; Edwards Ltd, Crawley, UK) for 48 h to obtain absolute water-free samples. The prepared powders were used for conformational characteristics of these yoghurts.

Infrared spectra of the freeze-dried yoghurt samples were recorded using an Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectrometer (Bruker, BioATR II cell, INVENIO 100453, Billerica, MA, USA) controlled by a Haake K20/DC30 external water bath (Thermo Haake, Karlsruhe, Germany) at 37 °C. Before measuring the samples, the background spectrum was scanned with a blank diamond ATR cell using the same instrumental conditions as for the sample spectra acquisition. The yoghurt samples were scanned in the range of 4,000 to 900 cm^{-1}, a resolution of 4 cm^{-1}, and by averaging 100 scans of each spectrum, followed by atmospheric compensation for the absorbance of CO_2 and H_2O as vapour and solute (OPUS software version 7.5).

The freeze-dried yoghurts were also analysed using a WITec Alpha300 R Confocal Raman Spectroscopy (Ulm, Germany) featuring a 532 nm Laser and a 50 x Microscope Object (0.55 numerical aperture). Namely, the samples were placed on microscopy glass slides and equilibrated at 37 °C before analysis using a Linkam PE94 heating/cooling microscopy stage (Linkam Scientific Instruments Ltd., Tadworth, UK). The Raman shift range was from 4,000 to 600 cm^{-1} with a laser power of 20 mW, an integration time of 0.5 s and number of accumulations of 10. Raman measurements were performed in a similar manner to those described by Pax and Sheehan (2020) with slight modifications.

Scanning electron microscopy images of the freeze-dried yoghurts were captured using a Zeiss Supra 40P field emission scanning electron microscopy (SEM) (Carl Zeiss SMT Ltd., Cambridge, UK) at 2.00 kV with a magnification of 4,000. Samples were prepared using double sided adhesive microscope stubs coated with chromium (K550X, Emitech, Ashford, UK) (Sah et al., 2016). Confocal laser scanning microscopy (CLSM) was used to visualise the microstructure of the fresh yoghurt samples. A small amount (~ 0.1 g) of each yoghurt was transferred to a glass slide using a spatula. Stock solutions of Fast green FCF (0.1 % w/v in water) was added to the sample (50 µL) followed by a 15 min incubation. After blotting excess stain with tissues, the sample was covered with a 0.17 mm thick glass coverslip and visualised with a 63x oil-immersion objective on an inverted CLSM (Leica SP8, Leica Microsystems Baden-Württemberg, Germany). The excitation/emission wavelengths for Fast Green FCF were 633/660 - 750 nm, respectively (set to appear green). With these configurations, the protein, appears greenish blue in CLSM images (Mulet-Cabero, Mackie, Wilde, Fenelon, & Brodkorb, 2019)

2.6. Semi-dynamic *in vitro* gastric digestion

2.6.1. In vitro semi-dynamic gastric digestion of yoghurt

To assess variations in the simulated dynamic gastric digestion of the yoghurt samples, the standardised simulated *in vitro* model operating at 37 °C for a period of 21.76 min was applied (Mulet-Cabero et al., 2020a). The duration of gastric digestion was based on the caloric value of the food, treated as a meal. The digestion was carried out in a jacketed glass vessel (ref. 6.1418.250, Metrohm, Dublin, Ireland) and for mixing the digesta (low speed between 10 - 20

rpm) an overhead stirrer (OHS 200 Digital, VELP R Scientifica, Usmate Velate, Italy or CAT R 100 CT, Ingenieurbüro CAT M. Zipperer GmbH, Staufen, Germany) fitted with a 3D - printed stirrer head. No amylase was added during the oral phase as no starch was incorporated in the yoghurts. Pepsin activity was tested according to Brodkorb et al. (2019) (4,017.12 U · mL^{-1} of digesta, P-6887), but gastric lipase was omitted due to the absence of lipids in the food.

The simulated electrolyte fluids, namely, Simulated Salivary Fluid (eSSF) and Simulated Gastric Fluid (eSGF at pH 7) were prepared as described in the INFOGEST standardised static *in vitro* digestion (Brodkorb et al., 2019). For all yoghurt samples, 45 mL of prepared refrigerated yoghurt (section 2.2.) was first mixed with 6.27 mL eSSF at 37 °C. Milk samples and eSSF were stirred with a plastic spatula at pH 7 for 20 sec to reproduce the salivary phase of digestion. The gastric phase was performed by gradually adding 34.64 mL eSGF, porcine pepsin along with a slow addition of 0.13 M HCL to gradually reduce pH down to 2.0. Gastric emptying was simulated by taking four samples, named as gastric emptying points (GE) 1 - 4 in the text.

2.6.2. Properties of digested samples

The digesta were taken from the bottom of the vessel using a serological pipette with a tip internal diameter between 2.07 and 2.20 mm mimicking the upper limit of particle size that pass through the pyloric valve into the duodenum (Mulet-Cabero et al., 2019). The GE samples were collected from the digests' supernatants at 5.30 (GE 1), 10.80 (GE 2), 16.00 (GE 3) and 21.76 min (GE 4). The pH of the digesta emptied from the gastric vessel at an interval of 5 min was measured by a CyberScan pH meter 510 (Eutech Instruments, Singapore). Each sample (8 mL) was collected after halting stirring for 30 s to allow particles to sediment. Aliquots of the samples (1 mL) were placed on ice for a short period and immediately used for structural characterisation of the digesta. The other aliquots of the samples, needed for protein composition and profiling, were snap frozen with liquid nitrogen and stored at - 80 °C until further analysis.

The structure of the digesta from each time point was observed using CLSM images and the samples were kept on ice for a short time (Mulet-Cabero et al., 2019). Furthermore, FTIR (see section 2.5.) operating at 37 °C evaluated the structural properties of the digested samples, taking into consideration the background (sSGF and pepsin) that was collected before every sample's measurement.

The protein contents of the digested yoghurts in all four gastric phases were determined using LECO FP628 nitrogen analyser (LECO Corporation, UK). The yoghurt samples obtained before and during simulated gastric digestion were analysed by Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4 - 12 % Tris-glycine polyacrylamide gels (Invitrogen, CA, USA) for protein profiling. Samples were first reconstituted 1:60 in 0.1 M NaOH. The reconstituted samples (5.1 µL) were then added to fresh Eppendorf tubes to which sample buffer (7.5 µL), reducing agent (3 µL) and deionised water (14.4 µL), equating to a final volume of 30 µL. The samples were heated for 10 min at 80 °C and allowed to cool to room temperature. For each sample, 10 µL were loaded in the gel and the electrophoresis was performed with a Xcell SureLock NovexMini Cell apparatus (Invitrogen, Loughborough, UK) at 200 V for 45 min, in NuPAGE® MOPS running buffer with NuPAGE® Antioxidant (Invitrogen, Loughborough, UK). Gels were stained overnight with InstantBlue™ and destained in an ultra-pure water prior to analysis, according to the supplier instruction (Invitrogen, Loughborough, UK) (Arranz et al., 2023)

2.7. Statistical analysis and spectra processing

Three independent batches of yoghurt and digesta were produced for each sample with either β-casein A1/A1, A1/A2, or A2/A2, and the results were expressed as mean of the three average values (n = 3 ± standard deviation). Mean values were then compared by one-way analysis of variance (ANOVA) Minitab statistical software (Version 20; Minitab, Pennsylvania, USA) with a significance of 95 %. Tukey's test was used to detect pairwise significant differences. The second derivative spectra of both, FTIR and Raman on the powdered yoghurts were analysed using Origin software (Origin Pro 2023, v. 95E, OriginLab Corporation, Northampton, MA, USA) (Daniloski, McCarthy, O'Callaghan, & Vasiljevic, 2022b). The multivariate analysis was conducted with 95 % confidence. At least 5 spectra (5 repetitions) were considered for each type of yoghurt and digested sample.

3. Results

3.1. Composition and physicochemical properties of A1/A1, A1/A2, and A2/A2 milks and their corresponding yoghurts

The protein and mineral composition of rehydrated skim milks, milk serums, and supernatants from the yoghurts are shown in Figure 1 A, Tables 1 and 2. Not significant differences were observed among the milks based on protein level ($p > 0.05$), since all milks were rehydrated to the same protein content (section 2.1.). While the levels of αs-and β-caseins, but also the level of calcium (Ca), were slightly higher in both homozygous milks ($p < 0.05$), the amount of κ-casein, phosphorus (P), potassium (K), and sodium (Na) were higher in A1/A1 and A1/A2 milks compared to that of A2/A2 milk ($p < 0.05$). The average particle sizes of A1/A1 and A1/A2 milks (~ 154 nm) were smaller compared to those of A2/A2 milk (167 nm, $p < 0.05$, Table 3). As expected, due to the lower content of κ-casein, the ζ potential of A2/A2 milk was between 3 and 9 % lower than that in both milks with β-casein A1 (ζ potential was less negative, $p < 0.05$, Table 3). No significant differences were observed in the ratio of caseins to whey proteins among all three milk groups (Table 1, $p > 0.05$). However, β-lactoglobulin was present in significantly higher levels in A2/A2 milk for almost 8 and 17 % than in A1/A2 and A1/A1 milks, respectively.

A greater amount of non-sedimentable αs- and β-caseins and higher level of sedimentable κ-casein were present in A1/A1 yoghurt relative to both yoghurts carrying β-casein A2 ($p < 0.05$). The A2/A2 yoghurt possessed the highest content of β-casein in its structure. Although only 1 - 5 % of the whey proteins were solubilised in the serum, the soluble β-lactoglobulin was significantly higher in the serum of A2/A2 yoghurt compared to its amount in the other yoghurt systems (Table 1, $p < 0.05$). Calcium and phosphorus are nearly entirely in the supernatant phase of the yoghurts due to pH drop (solubilisation of colloidal calcium phosphate from the micelle into the serum).

3.2. Rheological, physical, and textural properties of yoghurt gels

The storage moduli of skim milk yoghurts are shown in Figure 1 C, measured as a function of time. Yoghurts produced from A1/A1 and A1/A2 milks had a similar trend in G' evolution (Figures 1 C and D, and Table 4), where the G' value was greater than 1 Pa · s after ~ 112 and 114 min, respectively. This is significantly faster than that shown for A2/A2 milk where the G' value > 1 Pa · s occurred after ~ 145 min (Figure 1 C), with the final G' value almost 20 % less than that of the other yoghurts with β-casein A1 (Table 4). The viscoelastic behaviour (frequency sweep from 1 up to 63.1 Hz) of the yoghurt samples are displayed in Figure 1 D and show that the highest average G' value was observed in A1/A1 yoghurt, followed by A1/A2

and A2/A2 yoghurts, indicating the formation of a stronger, more cohesive gel matrix, which were more resistant to deformation in both yoghurts with β-casein A1 ($p < 0.05$). However, above a frequency of 63.1 Hz there was a deviation away from linear behaviour in the A1/A1 yoghurt, indicating gel breakage and increased brittleness in comparison to the other yoghurts (Figure 1 D).

Average texture profile data for each yoghurt is shown in Table 5. The highest gel firmness (1.91 N) and consistency (34.57 N · s) were exhibited by A1/A1 yoghurt. The A1/A2 yoghurt yielded 9 % higher firmness value than did the A2/A2 sample ($p < 0.05$). Maximum gel cohesiveness (- 0.47 N) and index of viscosity (~ - 7 N · s) were exhibited by both yoghurts with β-casein A1. Lower values were observed in A2/A2 sample for all texture profile components, indicating that A2/A2 milk led to softer yoghurt ($p < 0.05$, Table 5). Notably, the ability of the yoghurts to retain water was also distinguishable based on β-casein phenotype ($p < 0.05$, Table 5). In particular, after maintaining the samples at 4 °C for 24 h, the WHC of A1/A1 and A1/A2 yoghurts was between 1 and 4 % greater, but their syneresis was between 7 and 33 % lower compared to both, WHC and syneresis, in A2/A2 yoghurt, thus reflecting their relative compactness and firmness.

3.3. Conformational properties of the yoghurts

FTIR and Raman spectra of the yoghurts are shown in Figure 2 and Table 6. Integration of the second derivative spectra yields relative amounts of secondary structural features. Both yoghurts containing β-casein A1 had a similar level of random coils, where their presence was lower by almost 25 % depicted by FTIR and approximately 80 % as per Raman results compared to their level in A2/A2 yoghurt (Table 6, $p < 0.05$). Interestingly, A1/A1 yoghurt possessed the highest number of α-helixes, also presented with a peak shoulder at 1660 cm^{-1}, but the lowest level of β-turns in comparison to both yoghurts carrying β-casein A2 ($p < 0.05$). Although the level of structural aggregated β-sheets seemed to be high in all three yoghurt types, possibly due to the higher protein aggregation, in particular the aggregation of whey proteins, and new molecular rearrangements during fermentation stages ($p < 0.05$), the amount of these motifs was between 10 and 35 % lower in A2/A2 yoghurt as opposed to A1/A1 and A1/A2 yoghurts.

The microstructure properties of the yoghurts examined using both CLSM and SEM are shown in Figure 1 B. The CLSM microstructure of all three gels consisted of a protein network

(appearing as green) and the serum pores (appearing as black). The A2/A2 yoghurt displayed a more open porous microstructure and least dense protein network, whereas A1/A1 and especially A1/A2 yoghurts contained a more homogenous and compact structure (Figure 1 B). The microstructure observed using SEM confirmed the most porous structure in A2/A2 yoghurt and the densest protein network in both yoghurts with β-casein A1. Namely, the SEM images also show that the protein aggregation was different among the yoghurt networks (Figure 1 B).

3.4. Behaviour of the yoghurts during simulated gastric digestion

3.4.1. Gastric pH profiles and protein breakdown

The pH profiles of the yoghurts under simulated gastric conditions are presented in Figure 3 A. Although similar rapid decrease of the pH was observed during the first 3 min of digestion for all samples (pH from 4.6 to 4), the pH of both homozygous yoghurts changed only slightly up to 6 min; they then decreased gradually, reaching a pH of around 2 after ~ 22 min. In contrast, the decrease of pH in A1/A2 yoghurt underwent two plateau periods, including the one between 7 and 9 min, and the other from 13 to 15 min. These alternations in the pH curve were different among the samples probably due to differences in their buffering capacity, most likely induced by structural or kinetic differences in the curd formation caused by the milk fermentation, even though the yoghurts had the same protein content.

The protein profiles of yoghurts were analysed by SDS-PAGE under reducing conditions (Figure 4). As expected, the yoghurts before digestion (0 min) had a typical milk-like protein profile. The caseins in all samples were detectable at the first emptying point, followed by the GE 2 (pH 3.5) where these proteins could also be observed together with some liberated peptides (Figure 4). In all samples, the κ-casein band started fading following 10 min of digestion; right after the GE 2 point. Furthermore, after 15 min of digestion (GE 3), pepsin almost hydrolysed both β- and αs-caseins, nevertheless the β-casein band was more intense than the αs-casein band in all three yoghurts (Figure 4, Bands 14 - 16 within the caseins section of the SDS-PAGE gel), indicating a slower rate of β-casein hydrolysis compared to that of αs-casein. Towards the end of the digestion time (between GE 3 [15 min] and GE 4 [~ 22 min]), the A1/A2 sample appeared to contain little intact caseins and consisted mainly of peptides (Figure 4). This degradation of caseins after 22 min of digestion was also observed in A1/A1

yoghurt, whereas the protein breakdown was lowest in A2/A2 yoghurt (Figure 4). The intensity of the whey protein bands was almost constant during the whole duration of gastric phase (underwent a slight decrease from GE 1 to GE 3), nevertheless, the presence of β-lactoglobulin even in GE 4 was still visible in A1/A1, but mainly in A2/A2 yoghurt (Figure 4). In this regard, since the GE 4 band of A2/A2 yoghurt was more intense, it specified more undigested proteins in this sample, compared to A1/A1 and A1/A2 yoghurts.

3.4.2. Conformational features and microstructure of the gastric digesta

During the early stage of digestion (between oral and GE 1 phases), a close-knit network of protein was observed for A1/A1 and A1/A2 yoghurts, whereas a more open fragmented network was observed in A2/A2 yoghurt (Figure 3 B). Also, the digesta matrices of both phases (oral and GE 1) were comprised of more α-helixes, and intramolecular β-sheets within all three samples, but particularly the yoghurts with β-casein A1 ($p < 0.05$, Table 7). The large protein aggregates observed in GE 1 became smaller in GE 2, but interestingly greater in A2/A2 digesta as opposed to the other digesta samples (Figure 3 B). Herein, the A1/A1 digesta between GE 1 and GE 2 possessed the lowest level of α-helixes ($p < 0.05$, 114 % lower compared to A1/A2 and 94 % than in A2/A2 GE 2 digesta, Table 7). At the final digestion times (from 15 to 22 min), the protein matrix appeared to decrease, and the structure of the curds was much more open, with blocks of aggregated protein. These protein aggregates in GE 4 phase were visibly larger in A2/A2 digesta and they seemed to remain associated with the protein matrix. It is worth mentioning that the level of β-turns, between GE 3 and GE 4 in all three digesta samples marginally increased ($p < 0.05$), nevertheless, the random coils significantly increased (almost 18 %) in A1/A1 and A1/A2 digesta ($p < 0.05$). Thus, the protein breakdown shown in Figure 4, along with the opening of the protein matrix (Figure 3 B) and high level of random coil motifs (Table 7), appeared to be greater in both yoghurts with β-casein A1, presumably because of the greater protein breakdown by pepsin.

4. Discussion

The transformation of milk into a fine-stranded protein network through acid-induced gelation is a fundamental process in yoghurt production. Casein and highly aggregated whey protein (whey protein - whey protein and whey protein - casein aggregates) provide the main structural

network for yoghurt but a number of other factors play a key role in determining the gel strength of set-style yoghurts, such as protein, fat and mineral concentration and profile, level of heat treatment and starter culture (Lucey, 2002; Lucey, 2017; Lucey et al., 1998). During fermentation, the production of lactic acid and the concomitant decrease in pH, results in the solubilisation of colloidal calcium phosphate (Table 2) and dissociation of micellar casein (Jin et al., 2016). This process will lead to their destabilisation and adulteration of the caseins' secondary conformational state (Markoska, Huppertz, & Vasiljevic, 2021). Previously Lucey, Johnson, and Horne (2003) stated that much of the secondary structure of casein is random coil, present in an open, highly hydrated state. The level of random coils in A1/A1 and A1/A2 yoghurts was lower compared to their amount in A2/A2 yoghurt ($p < 0.05$). In this regard, Daniloski et al. (2022a) and Nishinari, Zhang, and Ikeda (2000) showed that the lower amount of random coils and greater levels of β-sheets, specifically aggregated β-sheets, were responsible for the induced stable protein structure, and therefore firmer gels, as in the present study's yoghurts with β-casein A1 ($p < 0.05$, Tables 4 - 6). Hence, β-sheet formation in the yoghurts seems to be involved in protein aggregates, gel network formation, and improvement of the yoghurt firmness ($p < 0.05$). Previously, during acid gelation of these three milks, a similar trend was observed, and it was found that mineral content and distribution, casein micelle size, level and phenotypes of β-lactoglobulin, but specifically β-caseins A1 and A2 and κ-casein, all affected the rate of gelation and final gel strength (Daniloski et al., 2022a; Hallén et al., 2009; Poulsen & Larsen, 2021). As known in the literature, the fundamental difference between the two β-casein proteoforms is an amino acid residue in position 67, with histidine for β-casein A1 and proline for β-casein A2 (Aschaffenburg, 1961). During acid gelation, hydrogen ions are released and are especially accepted by polar and charged amino acids, including histidine, leading to the formation of ionic and hydrogen-bonds and thus likely fine and stable structure (Scheiner, Kar, & Pattanayak, 2002), but also improved acid gel formation (Daniloski et al., 2022a). Additionally, the study of Bautista, Dahiya, and Speck (1966) showed that by fermenting a milk with *Streptococcus thermophiles* (used also within this study, see section 2.2.), a yoghurt starter culture that possessed the affinity towards histidine, resulted in increased acid production and gel firmness, that might be the case in the present A1/A1 and A1/A2 yoghurts, both carrying histidine within their protein structure.

During the formation of yoghurt gels, the system relies on re-arrangement of the bonds and interactions among individual caseins making up the original casein micelles. Therefore, an improved gel firmness may be associated with a greater number of such bonds and increased

interaction owing to the rearrangements of the casein particles into a more compact structure, hence leading to an increased number of bonds and firmer gel (Lucey, Wilbanks, & Horne, 2022). If it is assumed that all κ-casein is located on the surface of the casein micelle (Huppertz & Gazi, 2022), the reason behind this phenomenon can be related to the difference in κ-casein levels in all three yoghurts. Accordingly, greater κ-casein content indeed leads to more interactions, at least at the surface of the casein micelles during gelation (Lucey & Horne, 2018). The A2/A2 milk in the current study had the lowest amount of κ-casein as opposed to the other milks. Additionally, since the isoelectric point of κ-casein is fairly high at pH 6.70, the average casein isoelectric point would also be high in a sample rich in κ-casein (Walstra et al., 2005). This means that a sample with a lower amount of κ-casein would coagulate slower and become softer in the fermentation process; namely, it coagulates at a lower pH on account of the lower isoelectric point (De Kruif, 1997). Again, that appears to be the case for A2/A2 yoghurt in the current study that also possessed a smaller *tan δ*, which implies a longer relaxation time (Lucey, 2017). Additionally, a lower level of κ-casein led to creation of greater casein micelle size ($p < 0.05$, Tables 1 and 2); a key driver for milk with impaired gelation properties (Poulsen & Larsen, 2021), which might be the case for A2/A2 milk in the current study.

Although, the amount of κ-casein seems to play a key role in governing the yoghurt properties, the effect of heat-induced whey protein complexes with κ-casein has not yet been fully evaluated (Anema, 2021). However, many scholars explained that whey protein complexes interact with κ-casein on the surface of the casein micelles upon heat treatment, which consequently creates a firmer gel (Donato, Alexander, & Dalgleish, 2007; Lucey et al., 1998; Lucey et al., 2022). This was a case in the current study, since the amount of non-denatured β-lactoglobulin was lower in both yoghurts with β-casein A1 ($p < 0.05$, Table 1) which translates to higher whey proteins-casein micelle connections; A2/A2 yoghurt showed impaired gelation properties ($p < 0.05$, Figures 1 C and D, Table 4). Additionally, following the published data, various secondary orientations of β-lactoglobulin and κ-casein during heating were found to be responsible for creation of β-sheets detected in the samples (Grewal, Chandrapala, Donkor, Apostolopoulos, & Vasiljevic, 2017; Grewal, Huppertz, & Vasiljevic, 2018; Markoska, Huppertz, Grewal, & Vasiljevic, 2019) which corresponds to the present study's results. This might be the reason for the higher number of β-sheets in both yoghurts with β-casein A1, since they contained greater concentration of κ-casein and higher whey protein denaturation compared to their amount in A2/A2 yoghurt (Tables 1 and 6). Additionally, the presence of

these complexes and the possible κ-casein-whey protein aggregates on the surface of the casein micelles might affected the enzymatic coagulation of the yoghurts during the gastric digestion (Huppertz & Gazi, 2022; Ye, 2021). Interestingly, whilst A1/A1 and A1/A2 compared to A2/A2 yoghurts, exhibited greater β-sheet motifs, firmer gels with greater onset of gelation and shorter time of fermentation, upon simulated gastric digestion they possessed smaller protein aggregates and underwent faster protein breakdown (Figures, 3 B and 4, $p < 0.05$), presumably creating soft curds.

A number of previous studies showed that the properties and the size of gastric curd particles formed during yoghurt digestion are strongly influenced by the conformational state of the milk proteins present in the starting milk (Buchheim, 1984; Doan, 1938; Gaudichon et al., 1994; Mulet-Cabero et al., 2020; Scanff et al., 1990; Ye, 2021). However, less is known about the matrix structure formed during simulated gastric digestion of yoghurt. Yoghurt is a unique matrix in that the pH is already under acidic conditions, where solubilisation of calcium phosphate and dissociation of the casein micelle have already taken place with aggregation due to low electrostatic repulsion at the isoelectric point (Mulet-Cabero et al., 2020). The enzyme induced coagulation, initiated by the pepsin during digestion, can specifically catalyse the hydrolysis of κ-casein (Figure 4) (Creamer & Plowman, 1995), but κ-casein-cleaving sites by pepsin are still unknown (Fox & Walley, 1971; Ye, 2021). During gastric digestion of dairy products, the interaction of β-lactoglobulin and κ-casein does not normally hinder the enzymatic hydrolysis of κ-casein (Huppertz & Chia, 2021). Nevertheless, the enzymatic coagulation in the stomach might be amplified if heat treatment of yoghurt or dairy product has resulted in high levels of whey protein denaturation (Huppertz & Chia, 2021). The process normally results in an "opening" of the globular structure and rendering the whey proteins more sensitive to the action of pepsin, as demonstrated for β-lactoglobulin *in vivo* (Barbé et al., 2013) and α-lactalbumin *in vitro* (Inglingstad et al., 2010). This could be attributed to the weakly cross-linked protein structure and altered secondary protein conformation with greater levels of random coils observed in A1/A1 and A1/A2 digesta in both GE 3 and GE 4 phases (Table 7) (Daniloski et al., 2022a). Additionally, in the present study, the significantly higher level of β-lactoglobulin present in the supernatant phase of A2/A2 yoghurt, indicates a lower level of heat-induced aggregation ($p < 0.05$). This may be a cause of the lower gel strength observed in Figures 1 C and D and the slower gastric digestion found in A2/A2 yoghurt (Figures 3B and 4). Considering that the gastric digestion and protein breakdown of yoghurt and milk is to some extent similar (Dupont & Tomé, 2020; Gaudichon et al., 1994), very recently, during human *in*

vivo digestion of reduced fat A1/A2 and A2/A2 milks, it was found that A1/A2 milk had a faster gastric transit (Ramakrishnan, Zhou, Dydak, & Savaiano, 2023), which corroborates the present results.

5. Conclusion

This present study revealed how genetic variants of β-casein influenced yoghurt properties, with A2/A2 milk having significantly lower gel strength and greater yoghurt gel porosity, compared to those of A1/A1 and A1/A2 samples. Overall, the production of yoghurt using A2/A2 milk will have significant consequences for processors with ended times required for gelation and a slight reduction in final gel strength, compared to milks containing β-casein A1. The differences found in both, physical and microstructural properties of yoghurts from different β-casein phenotypes continued during simulated gastric digestion. The associated findings of the yoghurts with β-casein A1 may be related to the increased content of aggregated β-sheets and greater levels of κ-casein with increased levels of denatured β-lactoglobulin in A1/A1 and A1/A2 yoghurts after heat treatment. The creation of random coils, greater protein breakdown, and denaturation of whey proteins could contribute to the faster gastric digestibility of A1/A1 and A1/A2 yoghurts and should be examined further.

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Author Contributions

Davor Daniloski conceived the study and research question; designed and wrote the original draft, conceptualised, reviewed, edited the manuscript, designed the tables and the figures. **Davor Daniloski** prepared the methodology, formal analysis and investigation. **Daniela Freitas, Talita A. Comunian,** and **André Brodkorb** gave critical feedback and analysis, reviewed and edited the manuscript. **Todor Vasiljevic** and **Noel A. McCarthy** provided critical feedback and analysis, secured funding, reviewed and edited the manuscript and supervised the study. All authors have contributed to the manuscript and reviewed the final version.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare no conflict of interest.

List of Figures

Figure 1. A) RP-HPLC chromatographic profiles used for identification of different milk samples (1 = κ-casein A/A; 2 = αs_2-casein A/A; 3 = αs_1-casein B/B; 4 = β-casein [genetic variant indicated in the figure, A1/A1 = blue star, A1/A2 = green square, and A2/A2 = purple triangle]; 5 = α-lactalbumin; 6 = β-lactoglobulin A/B). **B)** Representative CLSM and SEM micrographs of yoghurts, scale bars represent 25 µm and 20 µm, respectively. **C)** Storage modulus (*G'*) as a function of time during fermentation with yoghurt starter culture at 42 °C of A1/A1 (blue star), A1/A2 (green square), and A2/A2 (purple triangle) milks. **D)** Storage modulus (*G')* of A1/A1 (blue star), A1/A2 (green square), and A2/A2 (purple triangle) milks obtained from small strain oscillation frequency sweep of the yoghurts. Data shown are average values of data from three collections. Error bars represent standard deviation.

Figure 2. A) Averaged of three second derivative spectra of Amide I region of yoghurts depicted by FTIR. **B)** Averaged of three second derivative spectra of Amide I region of yoghurts depicted by Raman. A1/A1 (blue star), A1/A2 (green square), and A2/A2 (purple triangle).

Figure 3. A) Change in pH of the yoghurts during gastric digestion **B)** CLMS micrographs of the clots obtained during the gastric digestion of yoghurts at different times, scale bars represent 25 μm. A1/A1 (blue star), A1/A2 (green square), and A2/A2 (purple triangle). Data shown are average values of data from three collections. Error bars represent standard deviation.

Figure 4. SDS-PAGE patterns under reducing conditions of oral and GE 1 - 4 phases obtained during the gastric digestion at different times. BSA (Bovine Serum Albumin); Igs (Immunoglobulins); β-Lg (β-lactoglobulin), α-lactalbumin (α-La). A1/A1 (blue star), A1/A2 (green square), and A2/A2 (purple triangle).

Table 1. Milk protein composition in skim milk, milk serum and yoghurt serum.

Protein content (mg/mL)						
Sample	κ-casein	αs_2-casein	αs_1-casein	β-casein	β-Lactoglobulin	α-Lactalbumin
A1/A1 milk	6.10 ± 0.05 ^{a}	2.11 ± 0.33 ^{b}	11.84 ± 0.43 ^{ab}	10.72 ± 0.16 ^{a}	3.35 ± 0.21 ^{a}	1.49 ± 0.01 ^{a}
A1/A2 milk	5.90 ± 0.22 ^{ab}	2.68 ± 0.03 ^{a}	11.01 ± 0.39 ^{b}	10.44 ± 0.30 ^{a}	3.65 ± 0.04 ^{a}	1.54 ± 0.01 ^{a}
A2/A2 milk	5.26 ± 0.18 ^{b}	2.51 ± 0.16 ^{ab}	12.43 ± 0.05 ^{a}	10.74 ± 0.16 ^{a}	3.94 ± 0.02 ^{a}	1.50 ± 0.03 ^{a}
A1/A1 serum (milk)	1.10 ± 0.25 ^{c}	0.35 ± 0.04 ^{c}	0.82 ± 0.29 ^{c}	2.16 ± 1.22 ^{bc}	3.24 ± 0.04 ^{a}	0.65 ± 0.01 ^{bc}
A1/A2 serum (milk)	1.34 ± 0.17 ^{c}	0.25 ± 0.01 ^{c}	0.87 ± 0.37 ^{c}	2.70 ± 0.97 ^{b}	3.41 ± 0.09 ^{a}	0.68 ± 0.03 ^{b}
A2/A2 serum (milk)	1.00 ± 0.01 ^{c}	0.19 ± 0.02 ^{c}	0.58 ± 0.01 ^{c}	2.77 ± 0.02 ^{b}	3.70 ± 0.35 ^{a}	0.64 ± 0.06 ^{b}
A1/A1 serum (yoghurt)	1.08 ± 0.10 ^{c}	0.06 ± 0.00 ^{c}	0.28 ± 0.01 ^{c}	0.99 ± 0.08 ^{c}	0.18 ± 0.07 ^{b}	0.39 ± 0.02 ^{cd}
A1/A2 serum (yoghurt)	0.99 ± 0.23 ^{c}	0.05 ± 0.01 ^{c}	0.22 ± 0.11 ^{c}	0.72 ± 0.46 ^{c}	0.38 ± 0.06 ^{c}	0.26 ± 0.05 ^{d}
A2/A2 serum (yoghurt)	0.90 ± 0.35 ^{c}	0.02 ± 0.00 ^{c}	0.21 ± 0.05 ^{c}	0.59 ± 0.21 ^{c}	1.75 ± 0.38 ^{bc}	0.29 ± 0.08 ^{d}

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$). The analysis of all three sample types [milk, serum (milk), and serum (yoghurt)] was performed separately.

Table 2. The average total and soluble mineral concentrations of milk, milk serum, and yoghurt serum with different β-casein phenotypes.

Sample	Ca (mM)	K (mM)	Mg (mM)	Na (mM)	P (mM)
A1/A1 milk	31.18 ± 0.52 ^{ab}	33.05 ± 0.04 ^{ab}	4.55 ± 0.01 ^{ab}	18.67 ± 0.38 ^{ab}	28.75 ± 0.09 ^{a}
A1/A2 milk	30.70 ± 0.01 ^{ab}	33.98 ± 0.61 ^{a}	4.69 ± 0.04 ^{a}	20.79 ± 1.15 ^{a}	28.23 ± 0.29 ^{a}
A2/A2 milk	31.47 ± 0.28 ^{a}	32.81 ± 0.73 ^{ab}	4.56 ± 0.04 ^{ab}	17.63 ± 0.26 ^{ab}	27.02 ± 0.30 ^{a}
A1/A1 serum (milk)	9.29 ± 0.97 ^{c}	32.68 ± 2.20 ^{abc}	2.90 ± 0.07 ^{c}	17.98 ± 1.74 ^{ab}	11.64 ± 0.75 ^{c}
A1/A2 serum (milk)	9.45 ± 1.00 ^{c}	29.33 ± 0.21 ^{c}	3.05 ± 0.09 ^{c}	16.67 ± 0.13 ^{bc}	12.24 ± 0.87 ^{c}
A2/A2 serum (milk)	9.19 ± 0.24 ^{c}	29.48 ± 0.56 ^{bc}	2.95 ± 0.01 ^{c}	15.77 ± 1.84 ^{bc}	10.55 ± 0.10 ^{c}
A1/A1 serum (yoghurt)	30.76 ± 0.15 ^{ab}	32.12 ± 0.20 ^{abc}	4.47 ± 0.03 ^{b}	17.48 ± 0.26 ^{ab}	21.85 ± 0.01 ^{b}
A1/A2 serum (yoghurt)	29.28 ± 0.25 ^{ab}	31.21 ± 1.19 ^{abc}	4.71 ± 0.02 ^{a}	18.98 ± 0.48 ^{ab}	21.73 ± 0.45 ^{b}
A2/A2 serum (yoghurt)	29.90 ± 0.33 ^{ab}	29.31 ± 0.23 ^{c}	4.59 ± 0.02 ^{ab}	13.65 ± 0.11 ^{c}	20.26 ± 0.61 ^{b}

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$).

Table 3. The average casein micelle size, zeta potential of the milks and initial, final and next day pH of yoghurts with different β-casein phenotypes.

Sample	A1/A1 milk	A1/A2 milk	A2/A2 milk
Particle size (nm)	153.27 ± 1.91 [b]	155.40 ± 1.15 [b]	167.10 ± 9.54 [a]
Zeta potential (mV)	- 14.43 ± 0.46 [a]	- 13.63 ± 0.75 [a]	- 13.23 ± 0.12 [a]
Initial pH of milk	6.63 ± 0.01 [a]	6.62 ± 0.01 [a]	6.55 ± 0.01 [b]
Final pH of yoghurt	4.59 ± 0.00 [a]	4.61 ± 0.03 [a]	4.60 ± 0.01 [a]
Next day pH of yoghurt	4.56 ± 0.01 [ab]	4.57 ± 0.01 [a]	4.54 ± 0.01 [b]
Fermentation time (min)	146.48 ± 5.66 [b]	148.04 ± 7.07 [b]	176.58 ± 3.54 [a]

Mean values within a row that do not share a common superscript letter are significantly different ($p \leq 0.05$).

Table 4. Rheological parameters measured during yoghurt production.

Sample	Gelation point time, *G'* = 1 Pa (min)	*G'* (Pa)			Tan δ		
		Time (min)					
		120	140	End	120	140	End
A1/A1 milk	112.50 ± 9.86 [b]	11.86 ± 0.70 [a]	32.15 ± 0.45 [a]	39.44 ± 5.73 [a]	0.512 ± 0.02 [a]	0.567 ± 0.05 [a]	0.574 ± 0.07 [ab]
A1/A2 milk	114.01 ± 3.94 [b]	5.33 ± 0.49 [b]	28.92 ± 4.56 [b]	37.76 ± 1.61 [ab]	0.483 ± 0.02 [a]	0.562 ± 0.04 [a]	0.583 ± 0.03 [a]
A2/A2 milk	145.01 ± 7.55 [a]	0.02 ± 0.00 [c]	0.09 ± 0.02 [c]	31.63 ± 3.40 [b]	0.056 ± 0.03 [c]	0.512 ± 0.07 [b]	0.566 ± 0.01 [b]

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$).

Table 5. Textural and chemical parameters of the yoghurts with different of β-casein phenotypes.

	Sample		
Treatment	A1/A1 yoghurt	A1/A2 yoghurt	A2/A2 yoghurt
Consistency (N · sec)	34.57 ± 1.97 [a]	31.39 ± 0.25 [ab]	29.73 ± 2.46 [b]
Index of viscosity (N · sec)	- 7.21 ± 0.94 [a]	- 6.81 ± 0.67 [a]	- 5.70 ± 1.85 [b]
Firmness (N)	1.91 ± 0.03 [a]	1.78 ± 0.12 [a]	1.63 ± 0.01 [a]
Cohesiveness (N)	- 0.47 ± 0.04 [a]	- 0.47 ± 0.10 [a]	- 0.35 ± 0.08 [a]
Water holding capacity (%)	50.75 ± 0.16 [a]	48.73 ± 0.77 [b]	48.28 ± 0.83 [b]
Syneresis (%)	16.50 ± 1.37 [b]	21.44 ± 1.34 [a]	23.21 ± 0.69 [a]

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$).

Table 6. Total percentage areas of second derivative Amide I band in A1/A1, A1/A2, and A2/A2 yoghurts depicted with FTIR and Raman spectroscopies.

Band Assessment	Band frequency (cm^{-1})	Peak area (%)					
		A1/A1	A1/A2	A2/A2	A1/A1	A1/A2	A2/A2
		FTIR			Raman		
Side chain	1,614 - 1,601	1.54 ± 0.48 ^{a}	0.85 ± 0.16 ^{a}	1.01 ± 0.49 ^{a}	2.27 ± 0.66 ^{b}	4.79 ± 0.92 ^{a}	2.59 ± 0.08 ^{b}
Intramolecular β-sheet	1,637 - 1,615	8.69 ± 0.22 ^{abc}	9.21 ± 0.25 ^{ab}	10.02 ± 0.27 ^{ab}	17.60 ± 1.16 ^{b}	15.74 ± 0.12 ^{bc}	12.19 ± 2.73 ^{c}
Random coil	1,645 - 1,638	8.91 ± 0.20 ^{b}	9.30 ± 0.80 ^{b}	11.91 ± 1.26 ^{ab}	3.00 ± 2.21 ^{d}	5.85 ± 0.08 ^{d}	11.04 ± 1.16 ^{c}
α-helix	1,664 - 1,646	29.36 ± 0.19 ^{b}	29.10 ± 0.46 ^{b}	28.87 ± 0.57 ^{b}	22.80 ± 6.64 ^{b}	16.57 ± 2.74 ^{d}	18.87 ± 0.53 ^{c}
β-turn	1,681 - 1,665	17.58 ± 0.60 ^{b}	17.59 ± 0.08 ^{b}	18.17 ± 2.41 ^{b}	18.18 ± 1.54 ^{bc}	25.09 ± 5.06 ^{ab}	29.85 ± 4.33 ^{a}
Aggregated β-sheet	1,700 - 1,682	33.92 ± 0.58 ^{a}	33.94 ± 0.52 ^{a}	30.01 ± 3.85 ^{ab}	36.15 ± 8.55 ^{a}	31.96 ± 0.97 ^{ab}	25.46 ± 0.21 ^{b}

Mean values within a row that do not share a common superscript letter are significantly different ($p \leq 0.05$).

Table 7. Total percentage areas of second derivative Amide I band in A1/A1, A1/A2, and A2/A2 digesta depicted with FTIR spectroscopy.

Sample	Peak area (%) - Oral phase					
	Side chain	Intramolecular β-sheet	Random coil	α-helix	β-turn	Aggregated β-sheet
A1/A1	4.06 ± 0.80 ^{c}	22.23 ± 3.74 ^{d}	8.43 ± 0.96 ^{d}	18.50 ± 4.22 ^{b}	12.57 ± 0.10 ^{d}	34.20 ± 3.17 ^{ab}
A1/A2	4.34 ± 0.12 ^{c}	4.57 ± 0.99 ^{de}	7.16 ± 2.32 ^{d}	17.89 ± 4.91 ^{b}	28.36 ± 0.94 ^{a}	37.68 ± 5.76 ^{a}
A2/A2	9.21 ± 3.96 ^{ab}	3.62 ± 0.92 ^{e}	17.17 ± 2.32 ^{c}	15.38 ± 1.05 ^{bc}	23.88 ± 2.02 ^{b}	30.74 ± 1.59 ^{b}
Peak area (%) - GE 1 phase						
A1/A1	0.23 ± 0.02 ^{d}	34.37 ± 0.45 ^{a}	1.30 ± 0.56 ^{e}	12.92 ± 1.79 ^{c}	12.09 ± 3.13 ^{d}	39.10 ± 2.58 ^{a}
A1/A2	4.80 ± 0.75 ^{c}	5.18 ± 1.54 ^{d}	8.47 ± 0.16 ^{d}	30.80 ± 0.66 ^{a}	12.77 ± 2.90 ^{d}	37.98 ± 2.19 ^{a}
A2/A2	13.46 ± 1.75 ^{a}	6.69 ± 1.49 ^{d}	14.26 ± 0.32 ^{cd}	19.01 ± 1.18 ^{b}	17.61 ± 0.03 ^{c}	28.97 ± 1.26 ^{b}
Peak area (%) - GE 2 phase						
A1/A1	2.39 ± 0.20 ^{c}	22.04 ± 3.49 ^{b}	22.06 ± 0.45 ^{bc}	3.68 ± 1.73 ^{e}	18.95 ± 2.27 ^{c}	30.88 ± 2.23 ^{b}
A1/A2	0.71 ± 0.43 ^{d}	22.03 ± 2.09 ^{b}	19.00 ± 0.20 ^{c}	13.52 ± 1.76 ^{c}	12.59 ± 2.32 ^{d}	32.16 ± 2.62 ^{ab}
A2/A2	5.36 ± 0.64 ^{c}	15.38 ± 1.42 ^{bc}	17.48 ± 2.07 ^{c}	10.25 ± 0.18 ^{cd}	20.98 ± 1.37 ^{b}	30.55 ± 1.67 ^{b}
Peak area (%) - GE 3 phase						
A1/A1	3.33 ± 0.56 ^{c}	7.13 ± 1.82 ^{d}	23.96 ± 5.26 ^{b}	15.31 ± 0.63 ^{bc}	27.36 ± 3.93 ^{ab}	22.91 ± 2.18 ^{c}
A1/A2	1.48 ± 0.85 ^{d}	8.21 ± 0.34 ^{d}	26.28 ± 2.55 ^{b}	16.27 ± 1.27 ^{bc}	20.88 ± 0.10 ^{c}	26.88 ± 0.88 ^{bc}
A2/A2	11.55 ± 0.18 ^{a}	6.87 ± 0.85 ^{de}	20.55 ± 1.72 ^{bc}	8.88 ± 0.82 ^{cd}	26.02 ± 2.23 ^{ab}	26.13 ± 1.15 ^{bc}
Peak area (%) - GE 4 phase						

A1/A1	7.58 ± 1.65 ^{ab}	6.82 ± 0.45 ^{d}	25.47 ± 0.62 ^{b}	3.79 ± 0.07 ^{e}	33.43 ± 3.73 ^{a}	22.92 ± 2.82 ^{c}
A1/A2	2.01 ± 0.24 ^{cd}	12.15 ± 0.01 ^{c}	31.89 ± 1.15 ^{a}	14.14 ± 0.83 ^{bc}	22.37 ± 2.16 ^{b}	17.45 ± 0.61 ^{d}
A2/A2	6.62 ± 0.07 ^{c}	9.52 ± 0.41 ^{c}	21.00 ± 0.00 ^{c}	9.08 ± 0.55 ^{cd}	29.03 ± 0.10 ^{a}	24.75 ± 0.18 ^{c}
Wavenumber (cm^{-1})	1,614 - 1,601	1,637 - 1,615	1,645 - 1,638	1,664 - 1,646	1,681 - 1,665	1,700 - 1,682

Mean values (± standard deviation, n = 3 measurements) within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$).

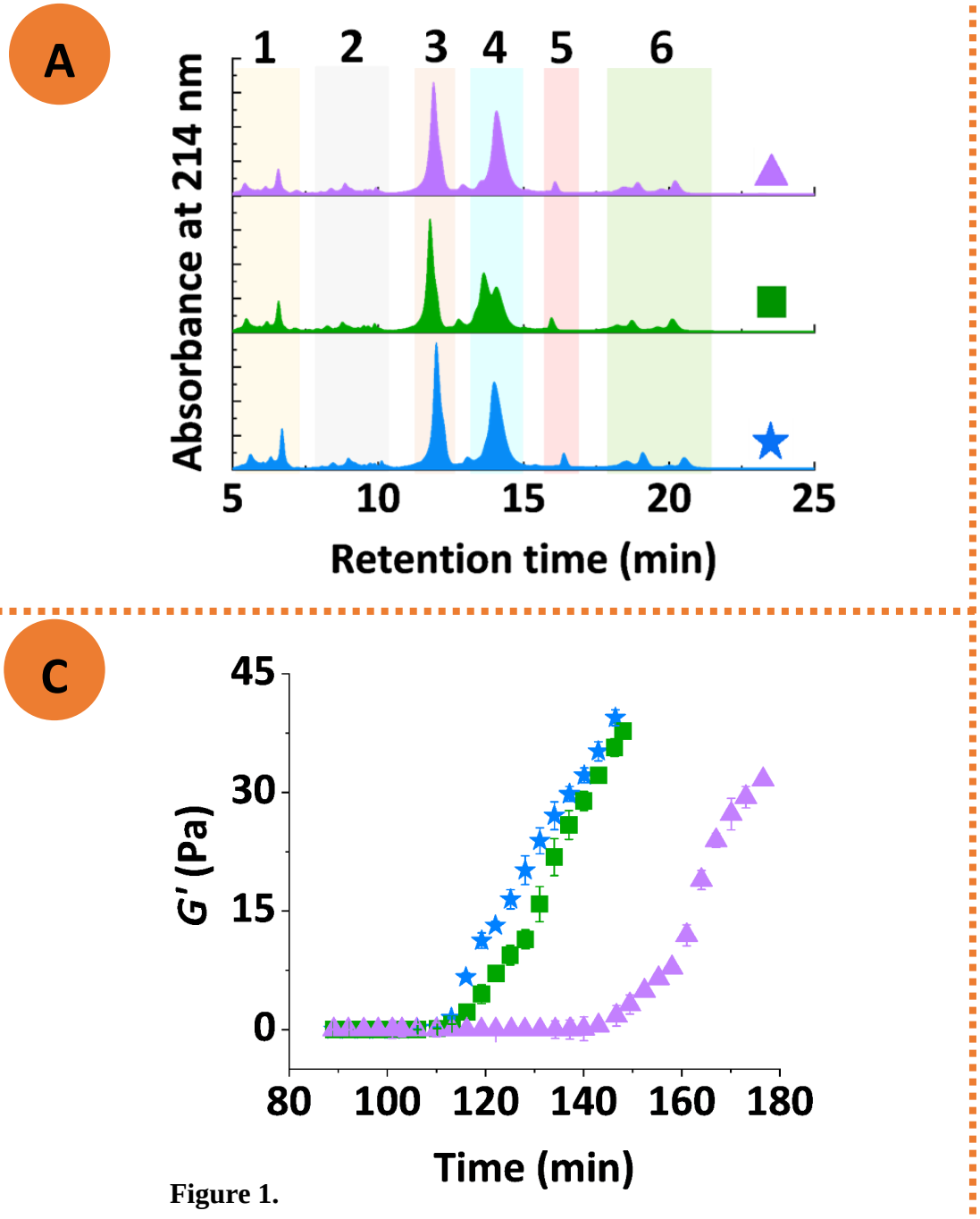
A
1
2
3
4
5
6
Absorbance at 214 nm
5
10
15
20
25
Retention time (min)
B
C
45
30
15
0
G′ (Pa)
80
100
120
140
160
180
Time (min)
D
2.8
2.4
2.0
1.6
1.2
G′ (Pa)
0.1
1
10
100
Frequency (Hz)

Figure 1.

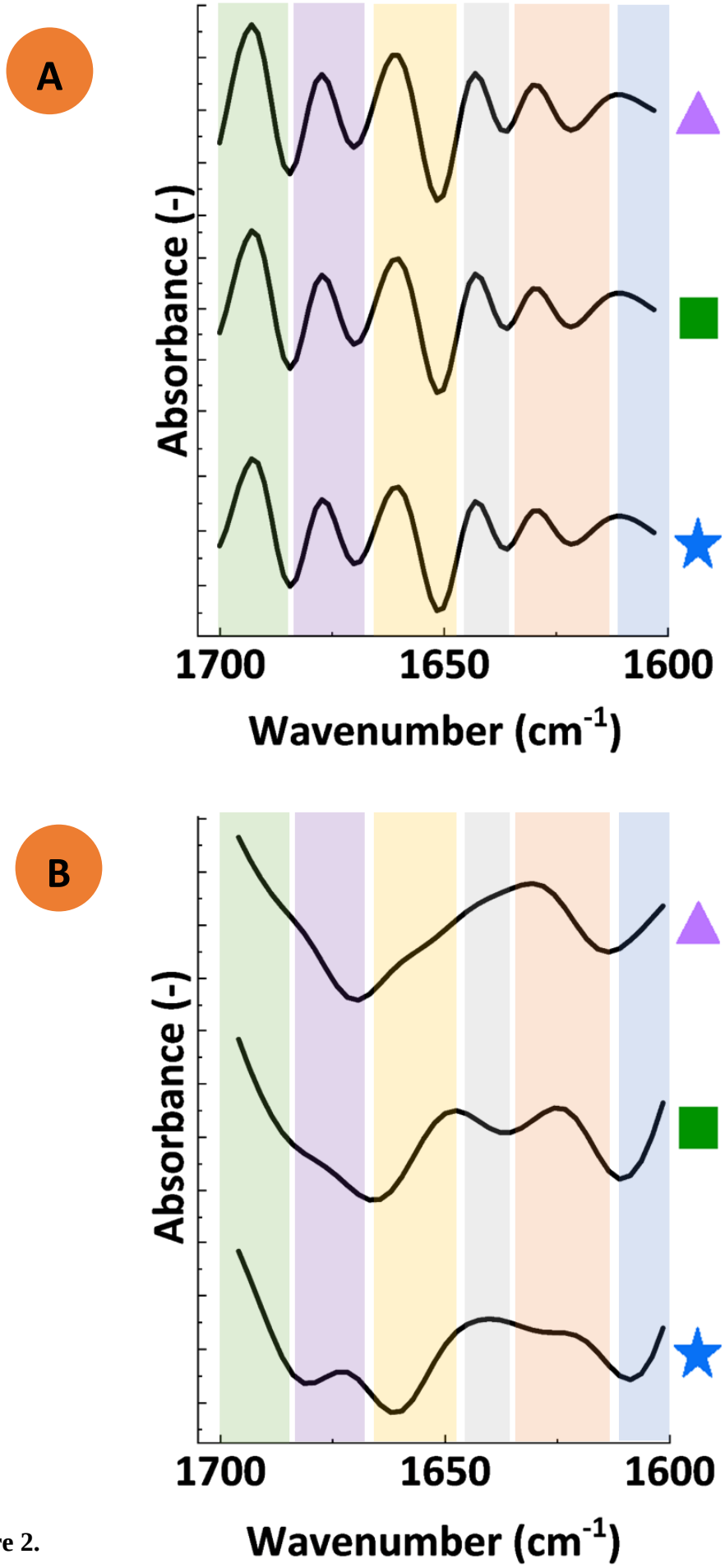
A
Absorbance (-)
1700
1650
1600
Wavenumber (cm-1)
B
Absorbance (-)
1700
1650
1600
Wavenumber (cm-1)

Figure 2.

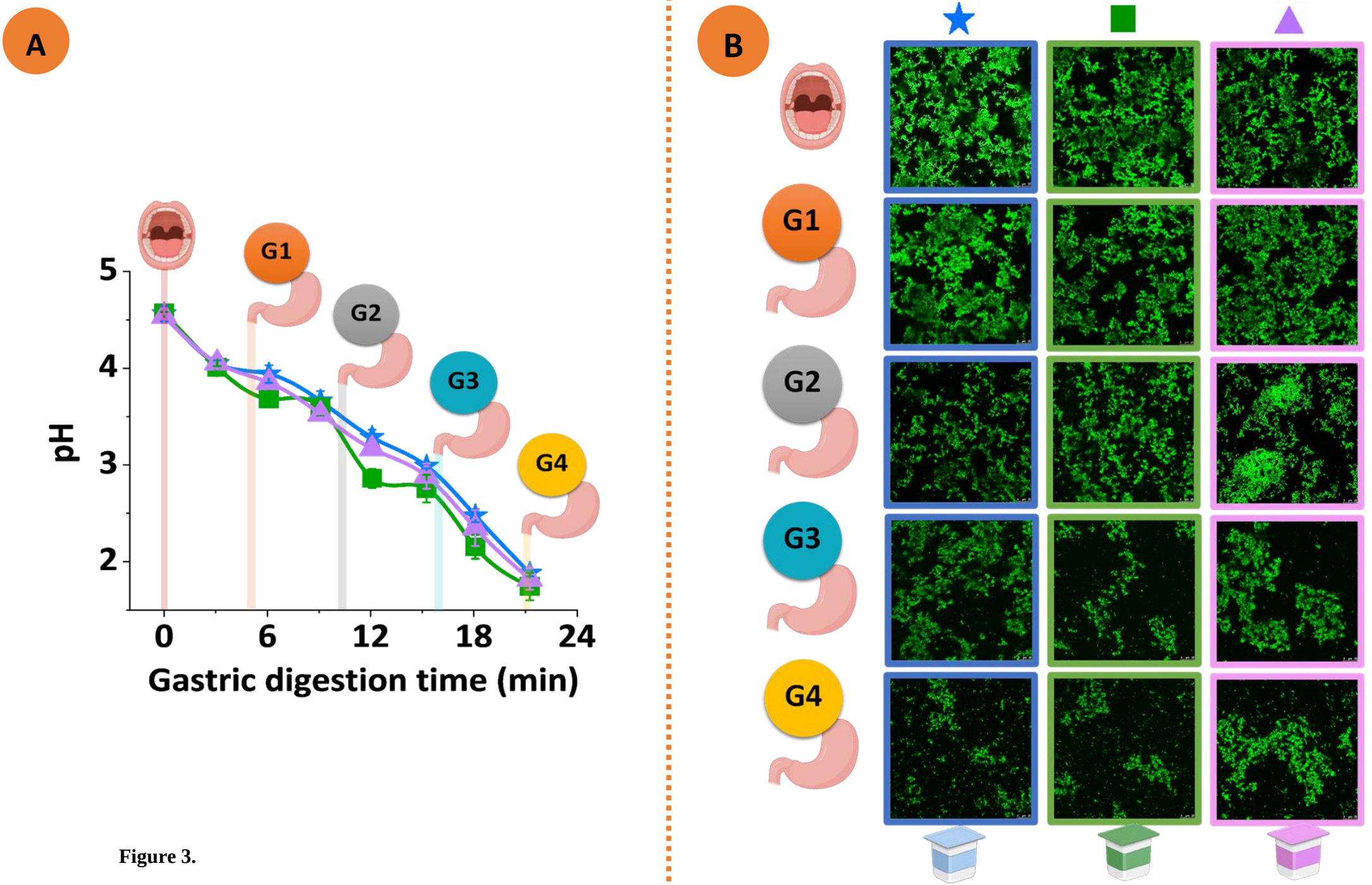
A
G1
G2
G3
G4
pH
5
4
3
2
0
6
12
18
24
Gastric digestion time (min)
B
G1
G2
G3
G4

Figure 3.

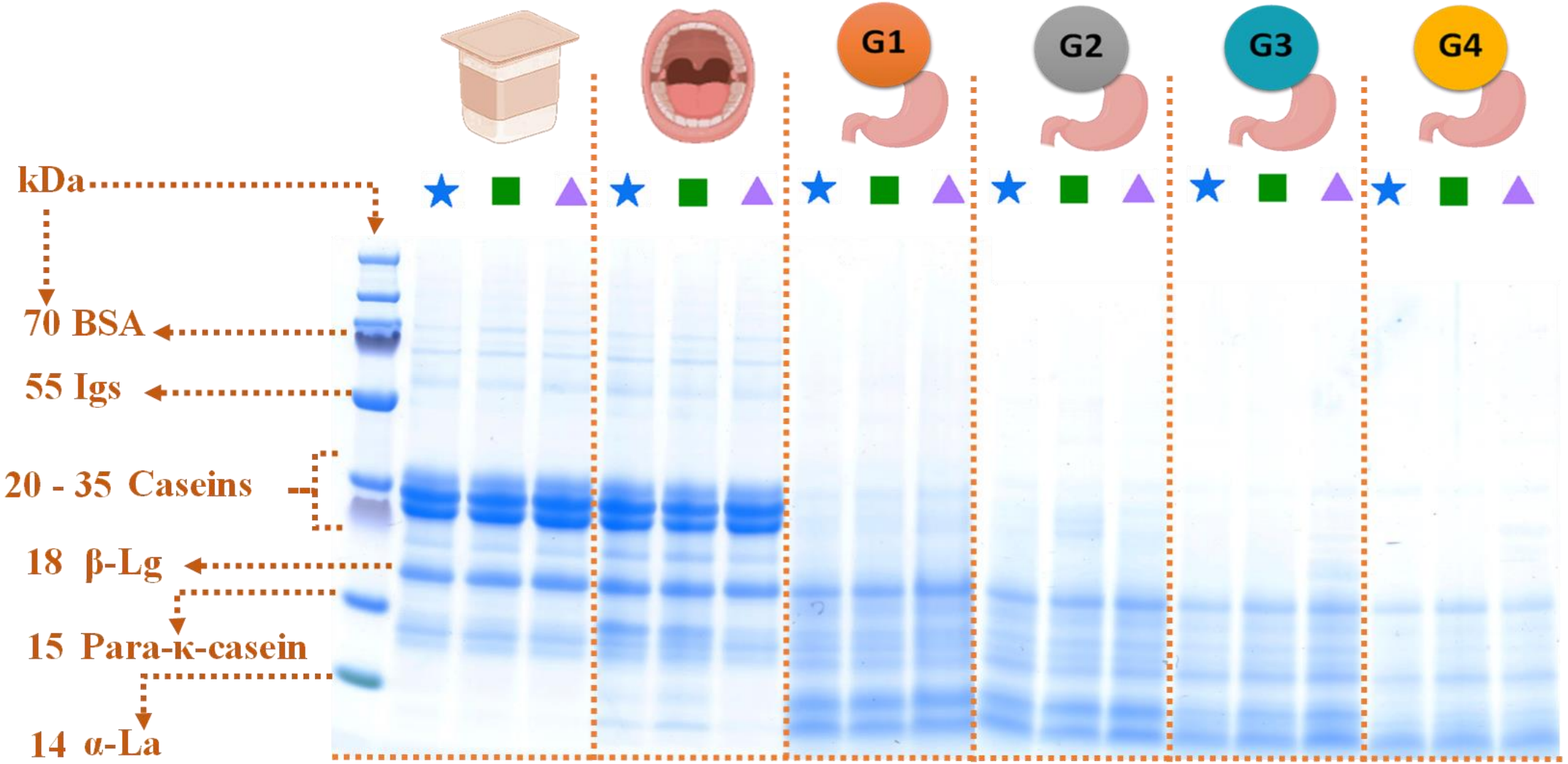
G1
G2
G3
G4
kDa
70 BSA
55 Igs
20 - 35 Caseins
18 β-Lg
15 Para-κ-casein
14 α-La

Figure 4.

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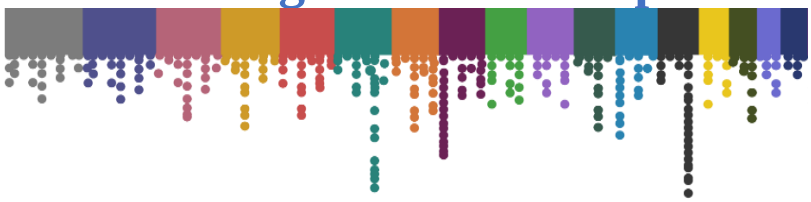

Cheddar cheese matrix and *in vitro* semi-dynamic gastric digestion: The role of β-casein phenotype

- Upon renneting, A2/A2 cheese milk required longer time to gel
- Cheeses with β-casein A1 after ripening were softer and less cohesive
- A1/A2 and A2/A2 cheeses had more aggregated β-sheets and less random coils
- A2/A2 cheese matrix influenced the slower protein breakdown during digestion
- Digesta from control and A1/A1 cheeses had more porous microstructure

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3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the work as follows:

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Name(s) of Co-Author(s)	Contribution (%)	Nature of Contribution	Signature	Date
Davor Daniloski	78	Designed and wrote the original draft, conceptualised, reviewed, edited, revised the manuscript, designed the experiments, the tables and the figures, and data analysis	[redacted]	17/03/2024
Richard Page	5	Concept development, manuscript editing, revision, and supervision		17/03/2024
Prabin Lamichhane	3	Concept development, manuscript editing and revision		17/03/2024
Conor J. Fitzpatrick	2	Concept development, manuscript editing and revision		17/03/2024
Mark Timlin	2	Concept development, manuscript editing and revision		17/03/2024
André Brodkorb	2	Concept development, manuscript editing and revision		17/03/2024
Todor Vasiljevic	3	Concept development, experimental design, manuscript editing and revision		17/03/2024
Noel A. McCarthy	5	Concept development, experimental design, manuscript editing, revision, submission, and supervision		17/03/2024

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Abstract

The objective of this study was to establish the impact of β-casein A1/A1, A1/A2 and A2/A2 phenotypes on cheese-making process, cheese matrix and their subsequent simulated semi-dynamic *in vitro* gastric digestion. The onset of rennet coagulation in cheese milk containing β-casein A2 was delayed and required longer time to gel. This resulted in a cheese that was almost 1.1 times harder and more cohesive compared to A1/A1 and A1/A2 cheeses at the end of the cheese ripening. The main structural motif differing among these cheeses included between 23 and 76 % higher levels of aggregated β-sheets in A2/A2 cheese. The protein breakdown of the cheese matrices during ripening resulted in a more porous microstructure in all cheese samples derived from β-casein A1 phenotype. Cheese samples took between 15 and 20 min to form the initial digesta coagulum when undergoing simulated gastric digestion which occurred in the pH range 4.0 - 4.3. The extent of the matrix degradation and protein breakdown was lower in A2/A2 cheese. The gastric digesta of A2/A2 cheese was characterised by a finer protein network containing approximately 20 % less random coils compared to all other digestas. This study highlights the differences in the cheese matrix structure and protein breakdown following gastric digestion as a result of β-casein phenotype and outlines how these results correlate with the initial cheese texture and (micro) structure.

Keywords:

Cheese; Rennet coagulation; β-caseins A1/A1, A1/A2, and A2/A2; cheese matrix; semi-dynamic *in-vitro* gastric digestion.

Introduction

Casein micelles are the basic building blocks of the gel formed during rennet coagulation of milk. Their destabilisation by enzymatic hydrolysis continues to be subject of studies as it is at the basis in curd firming processes and production of cheese (Dalgleish, 1979; Fox & McSweeney, 2017). The cheese curd is formed exclusively by combination of fat, some whey proteins, but mainly caseins (α-, β-, and κ-caseins) with appreciable quantities of micellar or colloidal calcium phosphate nanoclusters (Corredig & Salvatore, 2016). The manufacturing characteristics of cheese curd have been thoroughly studied for many years (Fox, 1989; Fox & McSweeney, 2017; Lucey, Johnson, & Horne, 2003; Lucey, 2022; Wright, 1924). Although it has been known for a while that cheese properties are affected by factors such as the traits of cows, the milk they produce, as well as the levels of protein, fat, and salt, these factors alone could not entirely account for the variations in how milk from different cows and breeds performs in cheese production (Fox, Cogan, & Guinee, 2017; Horne & Lucey, 2017).

The discovery of genetic variants in milk proteins has provided new insights into why milk properties, such as the rate of gel formation and curd firmness, differ among individual milk samples (Poulsen & Larsen, 2021). Recent research has demonstrated a weaker curd formation during cheese manufacture using milk with the κ-casein A phenotype compared to that of κ-casein B (Cendron, Franzoi, Penasa, De Marchi, & Cassandro, 2021; Jensen, Holland, Poulsen, & Larsen, 2012; Poulsen et al., 2013). A similar effect may be noticeable as a result of phenotype due to the correlation between β-casein content with cheese yield and milk coagulation (Marziali & Ng-Kwai-Hang, 1986). Among all caseins, β-casein accounts for approximately 40 % of the total casein in milk and has 17 different genetic variants (Daniloski, McCarthy, Huppertz, & Vasiljevic, 2022a), with β-caseins A1 and A2 being the most commonly found in bovine milk (Aschaffenburg, 1961). Variants A1 and A2 of β-casein, despite having an almost identical configuration, possess a different amino acid in position 67. Where proline is present at position 67 in the β-casein A2 variation, histidine is present in the β-casein A1 variation. This one substitution in the amino acid chain has demonstrated to significantly alter rennet coagulation time of cheese milk, the rate of proteolysis during cheese ripening, and the overall cheese yield (Bisutti et al., 2022; Gai et al., 2024; Marziali & Ng-Kwai-Hang, 1986).

The effect of β-casein A1/A1, A1/A2 and A2/A2 phenotypes on rennet coagulation time has, however, been noted by several researchers with an observation that milk carrying β-casein A1

is more favourable than milk with β-casein A2 for gel firmness and consequently cheese-manufacturing properties (Bisutti et al., 2022; Poulsen et al., 2016; Vigolo, Franzoi, Penasa, & De Marchi, 2022). Very recently during cheese-manufacture, Gai et al. (2024) reported poor rennet coagulation properties associated with A2/A2 milk compared to that of the β-casein A1 phenotype. Despite these properties, cheese produced from milk containing β-casein A2 was harder, more cohesive, and most fractural compared to the β-casein A1 derived cheese. It has also been suggested that the peptide bonds of A2/A2 cheese are more resistant to enzymatic cleavage by rennet during ripening compared to that of A1/A1 and A1/A2 cheeses (Jarmołowska, Kostyra, Krawczuk, & Kostyra, 1999; Stepaniak, Fox, Sorhaug, & Grabska, 1995).

Softer and less cohesive cheeses have been related to lower cheese elasticity and protein connectivity during digestion (Lucey et al., 2003), but also easier gastric digestibility or faster rate of gastric emptying (Mulet-Cabero, Mackie, Brodkorb, & Wilde, 2020b). For example, Lamothe, Corbeil, Turgeon, and Britten (2012) explained that the non-compact protein matrix with more space occupied by fat globules, reduced the Cheddar cheese hardness and decreased its cohesiveness. Additionally, this conformational property allows for greater permeation of pepsin within the cheese protein network, which can lead to increased protein digestibility in the stomach (Huppertz & Chia, 2021). Considering that the cheese matrix structure and strength may also influence the protein breakdown during gastric digestion, the current research aimed at investigating differences in cheese manufacture, and the resulting cheese texture and matrix structure as a result of the milk β-casein phenotype including A1 and A2. Additionally, this research aimed to investigate how these differences may have contributed to any potential variations in the digestion properties of the cheese.

2. Materials and methods

2.1. Milk collection and processing

Bovine milk (~ 1200 L) was obtained from a spring-calving herd (Irish Holstein-Friesian cows, Moorepark, Fermoy, Co. Cork, Ireland) in April 2023. All cows selected contained the same β-lactoglobulin, αs- and κ-casein variants, differing only in the β-casein A1/A1 (n_{cows} = 10), A1/A2 (n cows = 10), and A2/A2 (n cows = 10) phenotypes. A control sample was collected

from the bulk tank containing milk from the Moorepark herd (number of cows in the herd = 194). The cows' genotype data was obtained from the Irish Cattle Breeding Federation database (www.icbf.com). Milk protein genotype was verified using Reversed Phase - High Performance Liquid Chromatography (RP-HPLC), as shown in Figure 1 (Daniloski, Hailu, Brodkorb, Vasiljevic, & McCarthy, 2024). Milks were grouped into control (bulk milk), A1/A1, A1/A2, or A2/A2 samples and collected on the same day. Milk collection was carried out in triplicate with a total of 12 independent milks collected for the study over a 1-month period.

The composition of the individual raw milks was measured by MilkoScan (FOSS MilkoScan™ FT3, Hillerød, Denmark) for fat, protein, lactose, solid-non-fat and total solids (%, w/w) on the same day as collection. Milks were heated to 65 °C using a Microthermics tubular heat exchanger and cream separated using a centrifugal disk separator (Armfield Limited, Hampshire, England). The cheese milk was standardised to a protein to fat ratio of 0.95 by adding cream or skim milk to pasteurised whole milk. For standardisation purposes, the protein and fat content of the cream, skim milk and cheese milk were determined using a MilkoScan (FOSS MilkoScan™ FT3, Hillerød, Denmark), and 30 L of cheese milk from each group was subsequently pasteurised at 72 °C for 15 s using a pilot-scale tubular heat-exchanger (MicroThermics®, Raleigh, NC, USA). No statistically significant differences were found on raw or cheese milk composition between the four sample groups ($p > 0.05$, data not shown).

2.2. Cheddar cheese manufacture

Cheddar cheese production was performed according to Xia et al. (2020) and Lamichhane, Sharma, Kennedy, Kelly, and Sheehan (2019), with slight modifications. Briefly, the four standardised cheese milks (10 L) were transferred into cheese vats (Pierre Guerin Technologies, Mauze, France) and the temperature maintained at 32 °C and pH 6.55 (Mettler Toledo MP220; Mason Technology Ltd., Dublin, Ireland). The samples were inoculated with frozen direct vat starter cultures: containing 70 % *Lactococcus lactis* sub. *lactis* and *Lactococcus lactis* sub. *cremoris* (R-604, Chr. Hansen, Hørsholm, Denmark) and 30 % *Lactobacillus helveticus* (LH-B02, Chr. Hansen, Hørsholm, Denmark). After 40 min of pre-ripening of cheese milk, fermentation-produced bovine chymosin (CHY-MAX Plus, Chr. Hansen, Hørsholm, Denmark) was added at 40 international milk clotting units/kg of milk. Chymosin was diluted 10-fold with deionised water prior to addition.

All gels were cut at a constant firmness (G') value of 35 Pa using an AR-G2 rheometer (TA Instruments, New Castle, DE, USA) and the resultant curd whey mixture was stirred, and cooked until it reached a maximum scald temperature of 38 °C. Once the curd pH reached ~ 6.2, whey was drained and the curds were cheddared at 38 °C until the pH of curd was ≈ 5.4, milled, salted (2.7 % w/w) and pressed overnight (44 kPa). Three replicate trials were carried out, and the resultant cheese blocks (n = 12 [including repetitions], 4 groups x 3 samples from independent trials, with each block weighing ≈ 1.5 kg), were vacuum packed, ripened at 8 °C for 180 days, and were denoted as control, A1/A1, A1/A2, and A2/A2 cheeses.

2.3. Rheological properties of cheese milk and cheese yield

Viscoelastic properties were measured during renneting, whereby a 20 mL aliquot of cheese milk was transferred from the cheese vat 3 min after rennet addition and placed into the concentric cylinder on an AR-G2 rheometer (TA Instruments, New Castle, DE, USA) and time and frequency sweep measurements were subsequently carried out. The system was operating at 32 °C with a gap distance of 5,920 μm, strain amplitude of 2 % (bob diameter, 26 mm; cup diameter, 28 mm), and oscillation frequency 1 Hz. Changes in the elastic modulus (G') and the loss tangent ($tan\ \delta$) of the rennet-induced cheese milks were recorded every 30 s. The total time to reach a G' value of 35 Pa after rennet addition was recorded (at which time cutting of the gel in the cheese vat was initiated) (Everard et al., 2008). Since there were no significant differences in the protein, fat, and moisture contents among all cheese samples, the cheese yield (expressed as actual yield) was calculated as previously described (Guinee, O'Kennedy, & Kelly, 2006).

2.4. Physicochemical properties of cheese

Grated cheese samples (particle size of ≈ 1 mm) after 1, 30, 90 and 180 days of ripening were analysed in triplicate for protein content by the Kjeldahl method (ISO, 2014), fat content by rapid NMR fat analyser (Oracle, CEM Corp., Charlotte, NC, USA), and moisture and total solid contents by microwave and infrared moisture and total solids analyser (SMART 6, CEM Corp., Charlotte, NC, USA) (Xia et al., 2020). The pH of cheese was assessed by blending 20 g of grated cheese with 12 mL of deionised water and measuring using a standard pH meter (Mettler Toledo MP220; Mason Technology Ltd., Dublin, Ireland), according to the method of

the British Standards Institution (1976). Total mineral content analysis was performed by an Agilent 5110 synchronous vertical dual view ICP-OES analyser (Agilent Technologies, Santa Clara, CA, USA) (Daniloski et al., 2024). Colour measurements were taken from the surface of Cheddar cheese blocks after each ripening period. Five replications of L* (lightness - darkness), a* (green - red) and b* (blue-yellow) values were taken at random locations across the surface of the cheeses using a spectrophotometer (Konica Minolta Sensing Europe B·V., Nieuwegein, the Netherlands) (ISO, 2008).

2.5. Texture profile analysis (TPA) of Cheddar cheese

Cheese was prepared by cutting into cubes (25 mm^3) and analysed using a Texture Analyser TA-XT plus with a P75 probe and 50 kg load cell (Stable Micro Systems, Godalming, Surrey, UK) at 1, 30, 90, and 180 days of ripening. The hardness, fracturability, springiness, chewiness, and cohesiveness were recorded and calculated as described by Page et al. (2024).

2.6. Structural properties and microscopy analysis of cheese samples

For the conformational properties, cheese samples from every ripening time point were taken from the middle of each cheese wheel, kept in 50 mL Falcon tubes and immersed in liquid nitrogen. The frozen samples were immediately stored at - 80 °C overnight to prevent ice re-crystallisation, including any changes in their microstructure. The samples were then lyophilised over a 48 h period, using a pilot scale freeze dryer (Edwards Pirani 501 freeze dryer; Edwards Ltd, Crawley, UK). The freeze-dried cheese samples were then analysed for microstructural and secondary protein conformational changes. Scanning electron microscopy images of the freeze-dried cheese samples were captured using a Zeiss Supra 40P field emission scanning electron microscope (SEM) (Carl Zeiss SMT Ltd., Cambridge, UK) at 2.00 kV with a magnification of 4,000. Samples were prepared using double sided adhesive microscope stubs coated with chromium (K550X, Emitech, Ashford, UK). Additionally, the infrared spectra of the freeze-dried cheese samples were recorded using an Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectrometer (Bruker, BioATR II cell, INVENIO 100453, Billerica, MA, USA) controlled by a Haake K20/DC30 external water bath (Thermo Haake, Karlsruhe, Germany) at 37 °C. Before sample measurement, the background spectrum was scanned with a blank diamond ATR cell using the same instrumental conditions

as for the sample spectra acquisition. The freeze-dried cheese samples were scanned in the range of 4,000 to 900 cm^{-1}, a resolution of 4 cm^{-1}, and by averaging 100 scans of each spectrum. Fresh cheddar cheeses' microstructure was also examined at each ripening point using confocal laser scanning microscopy (CLSM) and labelling proteins by the green dye (Lamichhane et al., 2019).

2.8. Semi-dynamic *in vitro* gastric digestion

2.8.1. In vitro semi-dynamic gastric digestion of cheese

Digestion of the Cheddar cheese samples was conducted on the last day of ripening (Day 180) in triplicate using a procedure based on the semi-dynamic *in vitro* digestion protocol of INFOGEST consortium (Mulet-Cabero et al., 2020a), with some adaptations on the cheese bolus preparation, as described by Morzel, Ramsamy, and Le Feunteun (2023). The simulated electrolyte fluids, namely, Simulated Salivary Fluid (eSSF) and Simulated Gastric Fluid (eSGF at pH 7) were prepared as described in the INFOGEST standardised static *in vitro* digestion (Brodkorb et al., 2019). All enzymes' activities were assayed, according to Brodkorb et al. (2019).

Briefly, grated cheese samples (20 g) were mixed at a ratio of 1:1 with simulated salivary fluid (SSF) without amylase and macerated for 4 min in a stomacher (BagMixer 400P, Interscience, Saint Nom, France) to simulate mastication. The prepared samples were subsequently added to a double - wall 70 mL jacketed glass vessel (ref. 6.1418.250, Metrohm, Dublin, Ireland) maintained at 37 °C by circulating water. The vessel was equipped with a lid with openings to monitor the pH using a titration unit (800 Dosino, Metrohm, Zofingen, Switzerland). An overhead stirrer (OHS 200 Digital, VELP R Scientifica, Usmate Velate, Italy or CAT R 100 CT, Ingenieurbüro CAT M. Zipperer GmbH, Staufen, Germany) fitted with a 3D action shaker (Mini-gyro rocker, SSM3 Model, Stuart, Barloworld Scientific Ltd., Stone, Staffordshire, UK) operating at 20 rpm was used for agitation. The gastric phase was performed by adding 9.98 mL eSGF, porcine pepsin (4,000 U mL^{-1} of digesta, P-6887, Sigma-Aldrich, Arklow, Co. Wicklow, Ireland) and pancreatic lipase (60 U mL^{-1} of digesta, L3126, Sigma-Aldrich, Arklow, Co. Wicklow, Ireland) along with the slow addition of 0.5 M HCL (Sigma-Aldrich, Arklow, Co. Wicklow, Ireland) to gradually reduce the pH down to 2. Gastric emptying (GE) was

simulated by taking 4 samples, referred to as GE points in the text, based on the caloric value of the cheese and a gastric emptying rate of 2 kcal min^{-1}, as described in Mulet-Cabero et al. (2020a), which resulted in a total gastric emptying time of 63.16 min. The GE samples were removed as following: 15.79 min (GE 1), 31.58 min (GE 2), 47.28 min (GE 3) and 63.16 min (GE 4). The pH of the digesta emptied from the gastric vessel at an interval of 15-min was measured by a CyberScan pH meter 510 (Eutech Instruments, Singapore).

2.8.2. Properties of gastric digesta

The digested samples (10 mL) were taken from the bottom of the vessel using a serological pipette with a tip internal diameter of 2 mm as it resembles approximately the upper limit of the particle size that has been seen to pass through the pyloric opening into the duodenum (Mulet-Cabero et al., 2020a). The structure of the digesta (aliquot of 1 mL of fresh digest samples was kept on an ice for a very short time) was observed using CLSM with the labelling of fat and proteins. In the double stained samples, the fat phase was coded in red (Nile Red, $C_{20}H_{18}N_2O_2$, Sigma Aldrich, Merck Life Science Ltd., Cork, Ireland) and the protein phase was coded in green (Fast Green, FCF; $C_{37}H_{34}N_2Na_2O_{10}S_3$, Cayman Chemical, Ann Arbor, MI, USA) (Mulet-Cabero, Mackie, Wilde, Fenelon, & Brodkorb, 2019).

Digesta samples (6 mL per GE point) needed for protein structure fingerprinting and protein profiling, were snap frozen with liquid nitrogen, freeze-dried (Edwards Pirani 501 freeze dryer; Edwards Ltd, Crawley, UK) and stored at - 80 °C, until further analysis. The protein content of the freeze-dried cheese samples (section 2.4.) and freeze-dried digesta of all four GE points were determined by the Dumas method using a LECO FP628 Protein analyser (LECO Corp., St. Joseph, MI, USA). Also, the cheese samples obtained before and during simulated gastric digestion were analysed by sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 4 - 12 % tris-glycine polyacrylamide gels (Invitrogen, CA, USA) to observe the protein profiling and their subsequent breakdown during the gastric digestion, following the supplier instruction (Arranz et al., 2023). Fourier Transform Infrared Spectroscopy (see section 2.4.) operating at 37 °C evaluated the structural properties of the digested samples, taking into consideration the background (sSGF and pepsin) that was collected before every sample measurement (Daniloski et al., 2024).

2.9. Statistics and multivariate analysis

Triplicate trials were undertaken for Cheddar cheese manufacture from each milk type. The effect of the fixed factor (β-casein phenotype for both cheese and gastric digesta samples) was taken into consideration for all data. The data was analysed using a one-way ANOVA and when significant differences ($p < 0.05$) were found, the means were compared using Tukey's multi-comparison test. All analyses were performed using Minitab 20 (Minitab Inc., State College, Pennsylvania, USA). The Origin software (Origin Pro 2023, v. 95E, OriginLab Corporation, Northampton, MA, USA) quantified the second derivative FTIR spectra of both, freeze-dried cheeses and their digesta samples (Daniloski, McCarthy, O'Callaghan, & Vasiljevic, 2022b). All measurements were carried out in triplicate, on each cheese block across each ripening period.

3. Results

3.1. Compositional properties of cheese milk, cheese, and cheese yield

The composition of the control, A1/A1, A1/A2 and A2/A2 cheese milks varied only slightly ($p > 0.05$), with fat content ranging between 3.52 - 3.80, total protein between 3.31 - 3.53, casein content between 2.62 - 2.79, and lactose ranging from 4.65 - 4.83 (%, w/w). Also, both the calcium and phosphorus content were similar across all cheese milks and ranged between 30 and 33 mM ($p > 0.05$). These values were typical for Irish milk obtained in early lactation (Timlin et al., 2021). The chemical composition of the Cheddar cheese samples, produced from the control, A1/A1, A1/A2 and A2/A2 cheese milks, and measured after 180 days of ripening, were all very similar in terms of fat, total protein, casein protein, moisture, calcium, phosphorus, and cheese colour (Table 1), and were in-line with values previously shown for full-fat Cheddar cheese (Fenelon & Guinee, 2000). The similarity in composition between cheese samples is probably in part due to the standardisation of the protein-to-fat ratio in the cheese milks. Importantly, the genetic variant of β-casein had no effect on any compositional components in the cheese nor to the cheese yield (Table 1; $p > 0.05$).

3.2. Rheological properties of cheese milks

The development of the gel structure was measured by rheology and the values of the elastic modulus (*G'*) for all four milks are shown as a function of time (Figure 1). Each milk sample showed a coagulation point, defined as the point for the loss tangent (*tan δ*) = 1, between 15 and 30 min. Coagulation commenced in the control, A1/A1, and A1/A2 milks ≈ 16 min after rennet addition, compared to A2/A2 milk which started to coagulate at ≈ 20 min, reflecting the formation of a weaker gel with reduced elastic behaviour, compared to all other samples ($p < 0.05$, Figure 1 B). There was a significant increase in *G'* measured after 30 min of coagulation in all milks (Figure 1 B), but the fastest cutting point time (*G'* = 35 Pa) was assigned to both, control and A1/A1 milks (t ≈ 32 min), followed by A1/A2 (t ≈ 36 min), and finally A2/A2 milks (t ≈ 56 min). The *tan δ* profiles measured as a function of time (Figure 1 C) showed a significant difference among the samples, with the change in *tan δ* observed for control and A1/A2 milks being very similar, namely they decreased at the start of the coagulation and then plateaued with a maximum *tan δ* (0.13 for control milk and 0.17 for A1/A2 milk) at 31 min, before decreasing again by the end of the process. The *tan δ* at its maximum was significantly higher for A2/A2 milk (0.27), compared to all three milk samples containing β-casein A1 ($p < 0.05$) (Figure 1 C). It is also worth noting that the *tan δ* of A1/A1 milk plummeted at 22 min after which time it experienced a plateau period until 32 min and then again slightly decreased thereafter (Figure 1 C).

3.3. Texture of Cheddar cheeses throughout ripening

In general, the heterogeneous samples of the control and A1/A2 cheese on day 1 had the highest hardness, fracturability, and cohesiveness (Figure 2), and remained greater compared to those in the other cheese types ($p < 0.05$) throughout the ripening period. A significant decrease in the cheese hardness (27 - 48 %), fracturability (30 - 65 %), and cohesiveness (33 - 61%) was observed during ripening for all samples (Figures 2 A, B, and E). It is worth mentioning, the cheese hardness was significantly correlated with both cohesiveness ($r = 0.83$) and fracturability ($r = 0.95$) during all ripening times ($p < 0.05$). Even though springiness and chewiness decreased during cheese ripening, analysis in all four cheese types revealed only minor differences in these two TPA attributes between 90 and 180 days of cheese ripening ($p > 0.05$, Figures 2 C and D).

3.4. Microstructural of Cheddar cheeses throughout ripening

SEM images of cheese samples taken from each ripening point is shown in Figure 3 A. The microstructure of A2/A2 cheese on day 1 was clearly different from that of the other cheese samples. Its microstructure was more open which particularly resembled the impaired coagulation properties of its counterpart milk (Figures 1 B and C). The progressive aggregation of the casein throughout cheese-making (between days 30 and 90), to form an essentially homogeneous matrix containing entrapped fat, was most easily observed in the micrographs (note: within this study, only the importance of the protein matrix, but not the fat matrix on cheese-making, was taken into consideration). The protein network within all four cheese types became progressively coarser, but only until the first month of ripening where lumpy structures were observed (Figure 3 A). On the contrary, small gaps that became bigger, indicated by the black regions, were observed between 90 and 180 days of ripening (Figure 3 A). Better visualisation of the relative locations within the chesses, but also the effect of the protein matrix on samples' structure at any period of ripening, was observed by CLSM (Figure 3 B). Here again, after the first 30 days of ripening, the casein strands further fused together, closing most of the gaps in the curd microstructure. In contrast, as the ripening time progressed, the protein network was expected to break down. As viewed in the microstructure at day 90, the cheeses became more hydrated and expanded as the protein channels lengthened (Figure 3 B). Thus, the slower collapse of the casein matrix during cheese ripening probably occurred because of the thinner casein strands. The porosity visualised on the micrographs increased slightly with time in all cheese types, with the day 180 data being significantly higher than the other data points (Figure 3 B). Interestingly, the slightly more open structure and elongated protein channels in control and A1/A1 cheeses may have contributed to the significantly lower hardness and cohesiveness compared to the same texture attributes in other cheese types ($p < 0.05$, Figure 2). Cheeses made using A2/A2 milk had the densest protein matrix of all the cheeses, followed by A1/A2 cheese.

3.5. Protein conformational alternations of Cheddar cheeses throughout ripening

Quantification of changes in the secondary structure of the milk proteins in the cheeses within the Amide I band on the FTIR spectrum is shown in Table 2. During ripening, a marked variation in a band around 1640 cm^{-1} was evident, i.e., higher presence of random coils, indicating more unfolding of the protein secondary structure and therefore a softer and less cohesive cheese matrix ($p < 0.05$). These structural motifs increased between 20 - 37 % in all

samples over the ripening period of 180 days (Table 2). In contrast, as the samples matured, a decline in the proportion of α-helixes (20 - 50 %), and aggregated β-sheets (45 - 80 %), were observed in all four cheeses. Interestingly, on the last ripening point, A2/A2 cheese carried the highest amount of aggregated β-sheets in comparison to the other phenotypes ($p < 0.05$, Table 4). Thus, random coils and β-sheets in the present study seem to be involved, at least to some extent, in the structuring of the cheese matrices ($p < 0.05$). For instance, A2/A2 cheese had pronounced hardness and cohesiveness values at the end of the ripening period ($p < 0.05$, Figures 2 A and E) and also contained the lowest amount of random coils ($p < 0.05$, Table 2) in comparison to the other cheese variants. The secondary protein structure also exhibited a significant correlation with both, hardness, and cohesiveness of the cheeses ($p < 0.05$) with correlation coefficients ranging between 0.55 - 0.96 for all samples.

3.6. In vitro semi-dynamic gastric digestion of cheese samples

3.6.1. In vitro gastric pH profile

Changes in the pH profile was monitored during *in vitro* gastric digestion of the samples. The four cheeses, with initial mean pH around 5.20, showed no buffering differences in the oral phase ($p > 0.05$, Figure 4 A). Following this, the samples possessed slightly different pH profiles during different phases of the gastric digestion process, where the pH decreased continuously throughout digestion, reaching values ≈ pH 4 halfway through the digestion, and ≈ pH 2 by the end of the experiment ($p < 0.05$, Figure 4 A). The progressive pH decrease was related to the continuous secretion of gastric fluid containing acid as well as the reduction of buffering capacity of the digested cheese by gastric emptying, thus providing a more favourable environment for the pepsin activity. Although, the pH curves of control and A2/A2 cheeses were visually different compared to the other samples, only the curve of the A2/A2 cheese significantly differed from the other variants (Figure 4 A, $p < 0.05$). These observed variations in the pH curve among the samples might occur due to differences in the cheese buffering capacity, which were probably prompted by structural or kinetic alternations in the curd formation caused by the enzymatic coagulation ($p > 0.05$, Table 1).

3.7.2. Protein breakdown during gastric digestion of cheeses

The protein composition of the cheese types during the gastric phase was studied by SDS-PAGE (Figure 5). The bands corresponding to the cheese types in the oral phase did not differ among samples, but β-lactoglobulin was present in higher proportions in the control and A2/A2 cheeses based on the intensity of the associated band on the gels. Moreover, until GE 2 there were no visually significant differences among the samples. The digestion patterns of A1/A1, A1/A2, and A2/A2 cheeses were more similar than that of the control cheese until GE 3. At GE 4 the greatest protein breakdown was observed in the control and A1/A1 cheeses, followed by A1/A2 and finally A2/A2 cheeses. Casein bands were detectable between GE 1 and GE 3 but were almost completely faded at GE 4 (Figure 5). More specifically, both κ- and para-κ-casein bands (≈ 20 and 15 kDa, respectively) were almost completely absent in all four cheese samples at 48 min, however, their bands were still more intense in A1/A2 and A2/A2 cheeses. The intensities of all other protein bands including, α_S-casein (α_{S1}- + α_{S2}-caseins), β-casein, and β-lactoglobulin decreased gradually in the samples as digestion progressed (Figure 5). Towards the end of the digestion time (≈ 63 min), the digesta appeared to contain little to no intact protein and consisted mainly of peptides (Figure 5). In contrast to control and A1/A1 cheese types, at the end of digestion, some casein and β-lactoglobulin bands were still faintly visible in A1/A2 and A2/A2 samples.

3.7.3. Micro-structural characterisation of the cheese gastric digesta

Cheese matrix disintegration was visually low during the oral phase, but it was advanced during the gastric digestion (Figure 4 B). Interestingly, for all four samples, the oral digesta matrices were comprised of a high amount of α-helixes, but particularly in A1/A2 and A2/A2 cheeses, which might be due to the bigger cohesiveness of these cheese types ($p < 0.05$, Table 3). This observation was further supported by the moderate to high correlation coefficients between the chosen cheese textural property and α-helixes for all cheese types ($p < 0.05$, $r = 0.49 - 0.99$). The degradation profile of the matrix in the gastric phase varied markedly according to the type of cheese. At an early stage of the gastric digestion (15 min), a close-knit network of proteins was observed for all cheese types; nevertheless, during and after GE 1, the digesta matrix started its slow disintegration (Figure 4 B). Fat globules were distributed homogeneously in the protein matrix from all cheese samples, and some fat globules were also observed in the aqueous phase (note: fat globules were not further discussed, since the protein matrix was the scope of the current study). Although the knit protein network started breaking down in all

samples in a similar fashion (GE 2), at around 47 min of the gastric digestion, A2/A2 digesta possessed a protein matrix that was less open compared to the other digesta samples (GE 3, Figure 4 B). Herein, the A2/A2 digesta possessed the highest level of aggregated β-sheets ($p < 0.05$, 23 % larger than A1/A2, 78 % larger than A1/A1, and 76 % larger than the control GE 3 digests, Table 3). Once again, aggregated β-sheets within all digesta samples were strongly correlated to the cohesiveness of their counterpart cheeses ($p < 0.05$), with correlation coefficients ranging between 0.81 and 0.96.

The extent of protein matrix degradation and the more open curd structure as visualised in Figures 4 B and 5 at the final digestion times, was noticeably lower for A2/A2 cheese than for the other cheeses. The depicted structural differences might be attributable to the elastic, cohesive, and firmer texture of A2/A2 cheese in contrast to the other samples ($p < 0.05$, Figure 2). The visualised matrix degradation in the gastric phase was also lower for A1/A1 cheese. At the end of the digestion it reached similar levels to those observed for the control cheese. The A1/A1 digesta experienced a higher matrix breakdown than the one in A1/A2 digesta (Figure 4 B). The level of random coils, between GE 3 and GE 4 in all four digesta samples, increased significantly ($p < 0.05$, between 30 and 80 %), with their content in the following order: control > A1/A1 > A1/A2 > A2/A2 ($p < 0.05$, Table 6). Thus, the protein breakdown shown in Figure 5, along with the opening of the protein matrix (Figure 4 B) and high level of random coils (Table 3), appeared to be bigger in the digesta samples carrying either β-casein A1 or a mixture of both β-caseins, but lower in A2/A2 digesta, likely due to the greater protein breakdown by pepsin. Also, cheese cohesiveness and hardness were significantly correlated to all three, α-helixes, aggregated β-sheets, and random coils derived from the oral digesta and all four gastric digesta points of the samples ($p < 0.05$, $r = 0.44 - 0.98$).

4. Discussion

The general properties of rennet-induced gels important for cheese production are now well established. The mechanism by which the casein molecules are destabilised by the addition of rennet in milk has attracted much attention during the last century and there is an extensive knowledge in the literature (Berridge, 1942; Horne & Lucey, 2017; Lucey et al., 2003; Lucey, 2022; Wright, 1924). One of the important topics investigated, has been the impact of caseins, but particularly κ-casein, and its genetic profile on the structure and the physicochemical characteristics of rennet gels (Hansson, Pettersson, & Schaar, 1985; Jensen et al., 2012; Poulsen

& Larsen, 2021; Schaar, 1984). Nevertheless, associating the rennet gel properties and cheese production to either homozygous β-caseins A1 and A2 or the mixture of both, has received less attention.

In this regard, Hallén, Allmere, Näslund, Andrén, and Lundén (2007) and Poulsen et al. (2013) found that the milk with the κ-casein A, but especially its B variant, were associated with a higher amount of κ-casein, smaller casein micelles, increased whey protein expulsion, decreased curd firming time, firmer rennet gels and improved cheese-making traits. This might be the case in the present study, since the control milk had both κ-casein variants and gelled faster than all other milks, but not compared to A1/A1 sample (Figure 1, $p < 0.05$). Despite that A1/A1, A1/A2, and A2/A2 milks had the same genetic variant of κ-casein (A) and insignificant differences in the levels of κ-casein and total proteins ($p > 0.05$), A2/A2 milk took longer to reach the onset of coagulation gel strength ($p < 0.05$, Figures 1 B and C). Therefore, it is difficult to assign effect on milk coagulation to a certain casein variant, because the casein molecules are known to be tightly linked (Huppertz, Fox, & Kelly, 2018). Hence, it is important to be considered the impact of cow aspects, such as health status, lactation stage, and parity, as well as the content and relative proportions of caseins and minerals in milk, pH and temperature, that are known to correlate well with the casein genotype and milk coagulation properties (Ikonen, Morri, Tyrisevä, Ruottinen, & Ojala, 2004).

Initially, A2/A2 cheese had the lowest hardness and cohesiveness (Figure 2), but at the end of the cheese maturation this cheese was firmer, more cohesive, and carried a higher level of aggregated β-sheets, compared to all other cheese types ($p < 0.05$, Figures 2 and 3, Table 2). This significant change in the cheese properties might happen due to the origin of the time-dependent behaviour of cheese that lies in its structure, which is not static; the majority of bonds between structural elements, particularly among caseins are not permanent. These bonds occasionally break and reform due to Brownian motion, thus mainly leading to protein unfolding during cheese maturation (Lucey et al., 2003; Van Vliet, Van Dijk, Zoon, & Walstra, 1991). Therefore, the random coils in all three cheeses carrying β-casein A1 increased during the cheese ripening ($p < 0.05$). These conformational motifs resemble open, highly hydrated state presented by the molecules in solution, exposure of hydrophobic groups and weakening hydrogen bonds, breakage of α-helixes and aggregated β-sheets, and are predominately related to impaired firmness of a gel (Daniloski, McCarthy, Gazi, & Vasiljevic, 2022c; Daniloski et al., 2022b; Farrell, Qi, Wickham, & Unruh, 2002; Farrell, Wickham, Unruh, Qi, & Hoagland, 2001). The study of Wang et al. (2023) found that cheese with higher levels of random coils,

but lower levels of ordered secondary structure, were less cohesive and softer, which corroborates with the current study's results (Figure 2 and Table 2).

During cheese ripening, proteolysis of milk proteins plays an important role on chemical, physical and sensorial properties of cheese (Lamichhane, Kelly, & Sheehan, 2018). The addition of rennet into milk, as explained elsewhere, is responsible of inducing the formation of a milk gel due to destabilisation of the casein micelles (Dalgleish, 1979). Rennet activity still occurs during the whole cheese-making process and ripening time, leading to the breakdown of αs- and β-caseins during primary proteolysis (Fox, 1989). Proteolysis of the Cheddar cheese matrix during ripening decreases both, cheese firmness and cohesiveness that might be the case in the present study (Fenelon & Guinee, 2000). Very recently, Gai et al. (2024) explained that cheeses with β-casein A1 had significantly higher levels of primary proteolysis compared to A2/A2 cheese by the end of the first month of cheese ripening, nevertheless, at the end of cheese maturation, its levels were comparable among the samples ($p > 0.05$).

Additionally, the starter cultures, including *Lactococcus lactis* sub. *Lactis* and *cremoris,* commonly used in cheese manufacture, also utilised within this study, possess a principal role of lactic acid production, causing a decrease in pH and breaking down the milk proteins in cheese into shorter peptides (Fox, McSweeney, & Lynch, 1998). Even though these lactic acid bacteria are weakly proteolytic, they have a cell envelope-associated proteinase, such as X-prolyl dipeptidyl aminopeptidase (PepX) that has affinity toward proteins and peptides containing proline residues. To be more specific, peptides containing proline residues are known to be hydrolysed by a bacterially derived PepX enzyme (Muehlenkamp & Warthesen, 1996; Sousa, Ardö, & McSweeney, 2001). Namely, β-casein is found to carry the highest amount of proline residues compared to all other caseins (17 %) evenly distributed along its polypeptide chain (34 and 35 proline residues are found in β-casein A1 and A2, respectively) (Huppertz et al., 2018). Importantly, β-casein A2 has an additional proline residue in its 67 position that protects it from hydrolysis at the N-terminal amino acid residue by most bacterial aminopeptidases (Nguyen, Johnson, Busetti, & Solah, 2015). However, this peptide is still susceptible to hydrolysis by PepX activity, although to a less extent as compared to β-casein A1 (De Noni, Stuknytė, & Cattaneo, 2015). It has been postulated that the peptide bond in β-casein A1 between isoleucine^{66} and histidine^{67} was more susceptible to enzymatic cleavage compared to that in β-casein A2 between isoleucine^{66} and proline^{67} (De Noni et al., 2015; Muehlenkamp & Warthesen, 1996; Nguyen et al., 2015). This might give an indication behind

the reason of the significantly lower hardness and protein network incoherence of control, A1/A1 and to lesser extent in A1/A2 than in A2/A2 cheeses (Figures 2 and 3).

Furthermore, Markoska (2023) by using molecular modelling evaluated the tertiary structure of two peptides with 11 amino acids from both, β-casein A1 (Tyr-ProPhe-Pro-Gly-Pro-Ile-His-Asn-Ser-Leu) and β-casein A2 (Tyr-ProPhe-Pro-Gly-Pro-Ile-His-Asn-Ser-Leu). The author found that the conformation of β-casein A1 peptide was more specious and not packed into the tertiary structure as the β-casein A2 peptide (Markoska, 2023). Namely, the close packing of the molecule that hid the isoleucine[66] and proline[67] bond internally in β-casein A2, possibly restricts access to proteases, thus lowers the extent of cleavage of β-casein A2, and consequently rendering firmer cheese, but also more cohesive (Figure 2). Raynes, Day, Augustin, and Carver (2015) explained that due to the higher hydrophobic environment of β-casein A2, the interactions of this protein with other caseins would be enhanced, thus leading to tighter packing within the casein micelle, which supports the observations of Markoska (2023). Although these structural changes are noted in peptides (Markoska, 2023) or isolated β-caseins (Raynes et al., 2015), it must be noted that cheese is a system composed of different types of milk proteins and their genetic variants, each that might affect the structural arrangements of the molecule, and subsequently the structure and functionality of the cheese.

Numerous studies have explained the importance of the cheese matrix on subsequent gastric digestibility (Fang, Rioux, Labrie, & Turgeon, 2016; Lamothe et al., 2012; Tran Do & Kong, 2018; Žolnere, Arnold, Hull, & Everett, 2019). Most of them commonly accepted the theory that harder and more cohesive cheeses are emptied slower as they need more time to disintegrate during the gastric digestion, which is in line with the present study (Figures 2 and 3). Low cohesion of the cheese matrix could explain the behaviour of control and A1/A1 cheeses in the gastric phase. Indeed, these cheese matrices were more fragile and underwent higher protein breakdown within 63.16 min of gastric digestion ($p < 0.05$, Figures 4 and 5). Hence, control and A1/A1 samples were more susceptible to degradation by pepsin. One possible explanation might be the unrevealed peptide bond within the structure of β-casein A2 that can lower the pepsin accessibility, which leads to lowered protein breakdown in A1/A2 and A2/A2 digesta samples (see above) (Markoska, 2023). Additionally, Thorn et al. (2005) revealed that β-casein, which contains a relatively large quantity of proline, may manipulate the inherently unstable monomers of native κ-casein by binding to and shielding their hydrophobic surfaces, which might be the case in the current A2/A2 cheese examined in this study. If that is the scenario, the binding will lower the interactions of κ-casein with other

molecules (pepsin), that would lead to an increased quantity of κ-casein in the digesta, still faintly visualised in the SDS-PAGE, between GE 3 and GE 4 point of A1/A2 and A2/A2 cheeses digesta. The bigger protein breakdown was also attributed to weakly cross-linked protein structure and altered secondary protein conformation with larger quantities of random coils observed in the control and A1/A1 cheese digesta, particularly in their GE 4 phases ($p < 0.05$, Table 3). In this regard, increased quantities of random coils are related to poor structural stability in the cheese digesta, which may be due to the breakdown of the proteins as a result of the pepsin activity (Daniloski et al., 2024).

5. Conclusion

The manufacture of Cheddar cheese from standardised milk containing only the β-casein A2/A2 variant led to a longer onset time for rennet coagulation with control, A1/A1, and A1/A2 samples having greater gel strength in a shorter period compared to A2/A2 milk. Although during ripening the cheeses had similar composition with insignificant differences ($p > 0.05$), control and A1/A1 cheeses were less firm and less cohesive compared to A1/A2, but specifically to A2/A2 samples. The associated findings of the A2/A2 cheese may also be related to the increased content of aggregated β-sheet structures (protein motif related to a packed state and greater connection among caseins, but also cheese with improved hardness and cohesiveness). Even though the textural properties decreased, and the cheese matrix experienced greater disintegration during ripening in all cheeses, the denser protein structure and compact matrix formed in A2/A2 cheese probably impeded diffusion of gastric juice containing pepsin and acid, resulting in slower protein breakdown as opposed to all other samples. This suggests that the lower protein degradation is attributable to the characteristics of the cheese matrix, which partially resists the gastric environment. To what extent the higher protein breakdown in all cheeses carrying β-casein A1 might influence liberation of peptides during cheese ripening or during their intestinal digestion, requires further evaluation in future studies. Overall, the production of Cheddar cheese from A2/A2 milk will affect current manufacturing processes, but it is important to note that delays in rennet coagulation time may be rectified by addition of processing aids. However, this was not part of the current study.

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Author Contributions

Davor Daniloski conceived the study and research question; designed and wrote the original draft, conceptualised, reviewed, edited the manuscript, designed the tables and the figures. **Davor Daniloski** and **Richard Page** prepared the methodology, formal analysis and investigation. **Prabin Lamichhane, Conor J. Fitzpatrick, Mark Timlin and André Brodkorb** gave critical feedback and analysis, reviewed and edited the manuscript. **Todor Vasiljevic** and **Noel A. McCarthy** provided critical feedback and analysis, secured funding, reviewed and edited the manuscript and supervised the study. All authors have contributed to the manuscript and reviewed the final version.

Figures

Figure 1. A) RP-HPLC chromatographic profiles used for identification of different milk samples (1 = κ-casein A/A; 2 = αs_2-casein A/A; 3 = αs_1-casein B/B; 4 = β-casein [A1/A1, A1/A2, and A2/A2]; 5 = α-lactalbumin; 6 = β-lactoglobulin A/B). **B)** Elastic modulus (*G'*) as a function of time during coagulation of cheese milks. **C)** Loss tangent (*tan δ*) as a function of time during coagulation of cheese milks. Control (orange hexagon), A1/A1 (blue star), A1/A2 (green square), and A2/A2 (purple triangle) cheese milks. Data shown are average values of data from three collections. Error bars represent standard deviation.

Figure 2. Cheese texture: **A)** hardness; **B)** fractuability; **C)** springiness; **D)** chewiness; **E)** cohesiveness at different ripening times. Control Cheddar cheese (orange Hexagon); A1/A1 Cheddar cheese (blue star); A1/A2 Cheddar cheese (green square); A2/A2 Cheddar cheese (purple triangle). Error bars represent standard deviation.

Figure 3. A) SEM and **B)** CLSM micrographs of Cheddar-type cheeses during ripening times. Control Cheddar cheese (orange Hexagon); A1/A1 Cheddar cheese (blue star); A1/A2 Cheddar cheese (green square); A2/A2 Cheddar cheese (purple triangle). Scale bar for SEM is 100 μm (magnification = 200 X) and for CLSM is 75 μm.

Figure 4. A) Change in pH of the Cheddar cheese during gastric digestion. Error bars represent standard deviation. **B)** CLMS micrographs of the clots obtained during the gastric digestion of cheeses at different times, scale bars represent 250 μm. Control Cheddar cheese (orange Hexagon); A1/A1 Cheddar cheese (blue star); A1/A2 Cheddar cheese (green square); A2/A2 Cheddar cheese (purple triangle).

Figure 5. SDS-PAGE patterns under reducing conditions of oral and GE 1 - 4 phases obtained during the gastric digestion of Cheddar cheeses at different times. BSA (Bovine Serum Albumin); Igs (Immunoglobulins); β-Lg (β-lactoglobulin), α-lactalbumin (α-La). Control Cheddar cheese (orange Hexagon); A1/A1 Cheddar cheese (blue star); A1/A2 Cheddar cheese (green square); A2/A2 Cheddar cheese (purple triangle).

Table 1. Composition and physicochemical properties of Cheddar cheeses (180-day of ripening).

Parameter	Cheddar cheeses			
	Control	A1/A1	A1/A2	A2/A2
Protein (%)	26.19 ± 0.52 [a]	25.63 ± 0.13 [a]	25.62 ± 0.73 [a]	25.69 ± 0.17 [a]
Fat (%)	33.05 ± 0.39 [a]	33.68 ± 0.22 [a]	33.18 ± 0.20 [a]	33.06 ± 0.34 [a]
Moisture (%)	35.09 ± 0.25 [a]	35.42 ± 0.43 [a]	35.76 ± 0.33 [a]	35.66 ± 0.86 [a]
Total calcium (mg/100 g cheese)	821.67 ± 30.75 [a]	787.19 ± 56.11 [a]	829.29 ± 36.05 [a]	780.12 ± 25.69 [a]
Total phosphorus (mg/100 g cheese)	577.94 ± 11.91 [a]	512.69 ± 35.57 [a]	539.75 ± 25.02 [a]	514.86 ± 13.74 [a]
pH	5.30 ± 0.01 [a]	5.30 ± 0.01 [a]	5.27 ± 0.01 [a]	5.21 ± 0.01 [a]
Cheese yield (%)	10.13 ± 0.49 [a]	9.82 ± 0.09 [a]	10.03 ± 0.52 [a]	9.91 ± 0.23 [a]
Colour - L	76.32 ± 0.71 [b]	77.50 ± 0.80 [a]	77.29 ± 1.01 [a]	77.22 ± 0.67 [a]
Colour - a*	- 4.21 ± 0.09 [a]	- 4.31 ± 0.11 [a]	- 4.33 ± 0.22 [a]	- 4.36 ± 0.15 [a]
Colour - b*	28.45 ± 1.03 [ab]	28.50 ± 0.77 [ab]	29.18 ± 0.64 [a]	27.79 ± 0.84 [b]
Cutting point time, *G'* = 35 Pa (min)	32.26 ± 0.05 [c]	32.14 ± 0.11 [c]	36.28 ± 0.32 [b]	55.98 ± 0.18 [a]

Mean values (± standard deviation, n = 3 measurements) within a row that do not share a common superscript letter are significantly different ($p \leq 0.05$).

Table 2. Total percentage areas of different secondary structures in Amide I in Control, A1/A1, A1/A2, and A2/A2 Cheddar cheeses depicted with FTIR spectroscopy.

Sample	Peak area (%) - Day 1					
	Side chain	Intramolecular β-sheet	Random coil	α-helix	β-turn	Aggregated β-sheet
Control	0.76 ± 0.07 ^{d}	0.19 ± 0.08 ^{f}	26.81 ± 4.17 ^{c}	33.86 ± 7.95 ^{a}	5.92 ± 1.01 ^{d}	42.46 ± 2.62 ^{a}
A1/A1	1.21 ± 0.41 ^{cd}	6.02 ± 2.35 ^{de}	25.17 ± 0.27 ^{c}	25.85 ± 3.02 ^{c}	5.47 ± 1.30 ^{de}	36.28 ± 2.65 ^{b}
A1/A2	0.68 ± 0.06 ^{d}	4.98 ± 0.26 ^{e}	23.29 ± 5.20 ^{d}	32.22 ± 6.15 ^{a}	7.47 ± 3.89 ^{c}	31.36 ± 1.47 ^{c}
A2/A2	1.49 ± 0.40 ^{c}	6.07 ± 0.67 ^{de}	23.18 ± 3.99 ^{d}	32.03 ± 0.98 ^{a}	4.91 ± 0.00 ^{e}	32.32 ± 3.27 ^{cd}
Peak area (%) - Day 30						
Control	0.80 ± 0.16 ^{d}	3.61 ± 0.42 ^{e}	29.57 ± 6.99 ^{b}	26.56 ± 2.05 ^{b}	5.88 ± 0.80 ^{d}	33.58 ± 0.12 ^{bd}
A1/A1	2.26 ± 0.30 ^{b}	8.88 ± 2.47 ^{cd}	31.01 ± 2.50 ^{b}	27.39 ± 2.98 ^{b}	6.74 ± 1.30 ^{c}	23.72 ± 3.01 ^{de}
A1/A2	1.46 ± 0.03 ^{c}	10.92 ± 0.21 ^{c}	31.03 ± 1.06 ^{b}	26.23 ± 0.39 ^{b}	6.00 ± 0.22 ^{d}	24.36 ± 0.28 ^{de}
A2/A2	1.42 ± 0.06 ^{c}	13.63 ± 2.27 ^{b}	26.65 ± 2.26 ^{c}	25.31 ± 0.82 ^{c}	5.82 ± 0.27 ^{d}	27.17 ± 3.50 ^{cd}
Peak area (%) - Day 90						
Control	0.35 ± 0.01 ^{de}	11.29 ± 0.45 ^{c}	24.73 ± 1.23 ^{d}	31.01 ± 1.40 ^{cd}	7.16 ± 1.08 ^{c}	25.45 ± 1.11 ^{d}
A1/A1	1.37 ± 0.38 ^{c}	7.89 ± 0.53 ^{d}	34.58 ± 3.23 ^{c}	24.47 ± 1.10 ^{a}	5.23 ± 0.61 ^{de}	26.46 ± 0.61 ^{d}
A1/A2	0.72 ± 0.05 ^{d}	12.91 ± 1.48 ^{b}	29.81 ± 1.41 ^{c}	26.21 ± 0.50 ^{b}	6.03 ± 0.17 ^{cd}	24.31 ± 2.61 ^{de}
A2/A2	2.60 ± 0.09 ^{b}	13.62 ± 1.15 ^{b}	22.90 ± 0.22 ^{de}	26.00 ± 0.80 ^{d}	6.35 ± 1.17 ^{cd}	28.52 ± 0.52 ^{cd}
Peak area (%) - Day 180						

Control	1.78 ± 0.39 ^{c}	25.15 ± 0.04 ^{a}	33.23 ± 1.33 ^{de}	24.73 ± 0.05 ^{ab}	17.01 ± 0.41 ^{ab}	18.09 ± 0.54 ^{ef}
A1/A1	2.14 ± 0.90 ^{b}	15.36 ± 3.39 ^{b}	35.46 ± 2.01 ^{de}	14.77 ± 0.16 ^{a}	18.97 ± 2.24 ^{a}	17.29 ± 1.06 ^{f}
A1/A2	3.13 ± 0.53 ^{a}	23.52 ± 5.66 ^{b}	33.69 ± 3.14 ^{d}	25.00 ± 1.35 ^{ab}	17.34 ± 5.23 ^{ab}	17.32 ± 1.17 ^{f}
A2/A2	1.97 ± 0.31 ^{b}	25.05 ± 0.40 ^{a}	29.01 ± 0.69 ^{e}	26.05 ± 0.15 ^{b}	17.50 ± 0.17 ^{a}	20.42 ± 1.42 ^{e}
Wavenumber (cm^{-1})	1,614 - 1,601	1,637 - 1,615	1,645 - 1,638	1,664 - 1,646	1,681 - 1,665	1,700 - 1,682

Mean values (± standard deviation, n = 3 measurements) within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$).

Table 3. Total percentage areas of different secondary structures in Amide I of Control, A1/A1, A1/A2, and A2/A2 digesta depicted with FTIR spectroscopy.

Sample	Peak area (%) - Oral phase					
	Side chain	Intramolecular β-sheet	Random coil	α-helix	β-turn	Aggregated β-sheet
Control	0.55 ± 0.26 ^{e}	11.77 ± 0.82 ^{cd}	32.97 ± 0.24 ^{c}	27.41 ± 1.65 ^{c}	16.29 ± 1.79 ^{b}	11.01 ± 3.12 ^{d}
A1/A1	1.19 ± 0.48 ^{de}	22.79 ± 1.52 ^{a}	31.76 ± 6.08 ^{c}	24.00 ± 1.98 ^{cd}	7.51 ± 0.60 ^{e}	12.75 ± 2.46 ^{cd}
A1/A2	1.69 ± 0.39 ^{d}	8.03 ± 0.52 ^{e}	22.50 ± 2.85 ^{d}	33.28 ± 0.03 ^{b}	16.85 ± 2.59 ^{ab}	17.67 ± 0.13 ^{b}
A2/A2	3.63 ± 0.38 ^{b}	9.46 ± 1.31 ^{e}	16.60 ± 4.44 ^{e}	30.05 ± 2.82 ^{bc}	19.35 ± 0.18 ^{a}	20.90 ± 0.36 ^{a}
Peak area (%) - GE 1 phase						
Control	0.73 ± 0.09 ^{e}	1.60 ± 0.21 ^{f}	24.72 ± 1.80 ^{d}	40.12 ± 0.77 ^{a}	21.20 ± 1.64 ^{a}	11.63 ± 3.20 ^{cd}
A1/A1	1.44 ± 0.17 ^{d}	9.53 ± 1.59 ^{e}	35.69 ± 1.59 ^{b}	33.06 ± 1.84 ^{c}	9.75 ± 0.30 ^{de}	10.53 ± 0.54 ^{d}
A1/A2	2.30 ± 0.06 ^{c}	9.85 ± 0.25 ^{de}	25.17 ± 0.63 ^{d}	24.99 ± 2.96 ^{cd}	16.92 ± 2.22 ^{ab}	20.76 ± 1.07 ^{a}
A2/A2	2.90 ± 0.31 ^{c}	10.77 ± 0.09 ^{d}	23.51 ± 2.51 ^{d}	24.32 ± 2.30 ^{cd}	18.22 ± 2.23 ^{ab}	20.27 ± 2.04 ^{a}
Peak area (%) - GE 2 phase						
Control	0.77 ± 0.15 ^{e}	9.41 ± 0.98 ^{e}	31.99 ± 1.42 ^{c}	31.67 ± 1.26 ^{b}	16.07 ± 1.56 ^{b}	10.09 ± 0.29 ^{de}
A1/A1	5.56 ± 1.84 ^{a}	26.70 ± 1.37 ^{a}	42.14 ± 1.82 ^{a}	11.98 ± 0.67 ^{f}	3.03 ± 0.46 ^{f}	10.60 ± 0.22 ^{d}
A1/A2	2.45 ± 0.19 ^{c}	11.00 ± 0.79 ^{d}	31.12 ± 3.31 ^{c}	20.81 ± 2.94 ^{d}	19.18 ± 0.98 ^{a}	15.44 ± 0.01 ^{bc}
A2/A2	2.37 ± 0.02 ^{c}	12.92 ± 0.55 ^{c}	31.67 ± 0.53 ^{c}	18.46 ± 1.95 ^{de}	16.14 ± 1.16 ^{b}	18.45 ± 1.70 ^{b}
Peak area (%) - GE 3 phase						

Control	3.69 ± 0.02 ^{b}	14.28 ± 0.17 ^{c}	36.90 ± 2.67 ^{b}	24.64 ± 3.28 ^{cd}	11.19 ± 0.73 ^{d}	9.30 ± 0.03 ^{de}
A1/A1	1.99 ± 0.37 ^{d}	13.07 ± 1.27 ^{c}	43.69 ± 2.24 ^{a}	18.15 ± 0.68 ^{de}	15.37 ± 2.07 ^{bc}	7.73 ± 0.39 ^{e}
A1/A2	2.44 ± 0.47 ^{c}	10.08 ± 0.05 ^{de}	34.44 ± 0.36 ^{b}	24.16 ± 0.68 ^{cd}	14.90 ± 0.11 ^{c}	13.99 ± 0.83 ^{c}
A2/A2	1.84 ± 0.21 ^{d}	12.20 ± 0.49 ^{cd}	32.63 ± 0.39 ^{c}	15.98 ± 1.10 ^{e}	19.64 ± 0.59 ^{a}	17.71 ± 0.56 ^{b}
Peak area (%) - GE 4 phase						
Control	0.83 ± 0.77 ^{e}	12.15 ± 0.12 ^{cd}	44.86 ± 1.68 ^{a}	19.59 ± 0.98 ^{d}	14.63 ± 1.97 ^{c}	7.94 ± 0.38 ^{e}
A1/A1	2.18 ± 0.53 ^{cd}	10.74 ± 0.30 ^{d}	44.51 ± 1.49 ^{a}	19.14 ± 0.71 ^{d}	16.38 ± 0.72 ^{b}	7.06 ± 0.32 ^{e}
A1/A2	2.55 ± 0.92 ^{c}	22.80 ± 0.41 ^{a}	37.26 ± 0.78 ^{b}	11.25 ± 0.05 ^{f}	14.18 ± 0.31 ^{c}	11.97 ± 0.04 ^{cd}
A2/A2	1.25 ± 0.43 ^{de}	17.54 ± 0.90 ^{b}	37.19 ± 2.91 ^{b}	14.31 ± 1.75 ^{ef}	17.69 ± 0.65 ^{ab}	12.01 ± 1.34 ^{cd}
Wavenumber (cm^{-1})	1,614 - 1,601	1,637 - 1,615	1,645 - 1,638	1,664 - 1,646	1,681 - 1,665	1,700 - 1,682

Mean values (± standard deviation, n = 3 measurements) within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$).

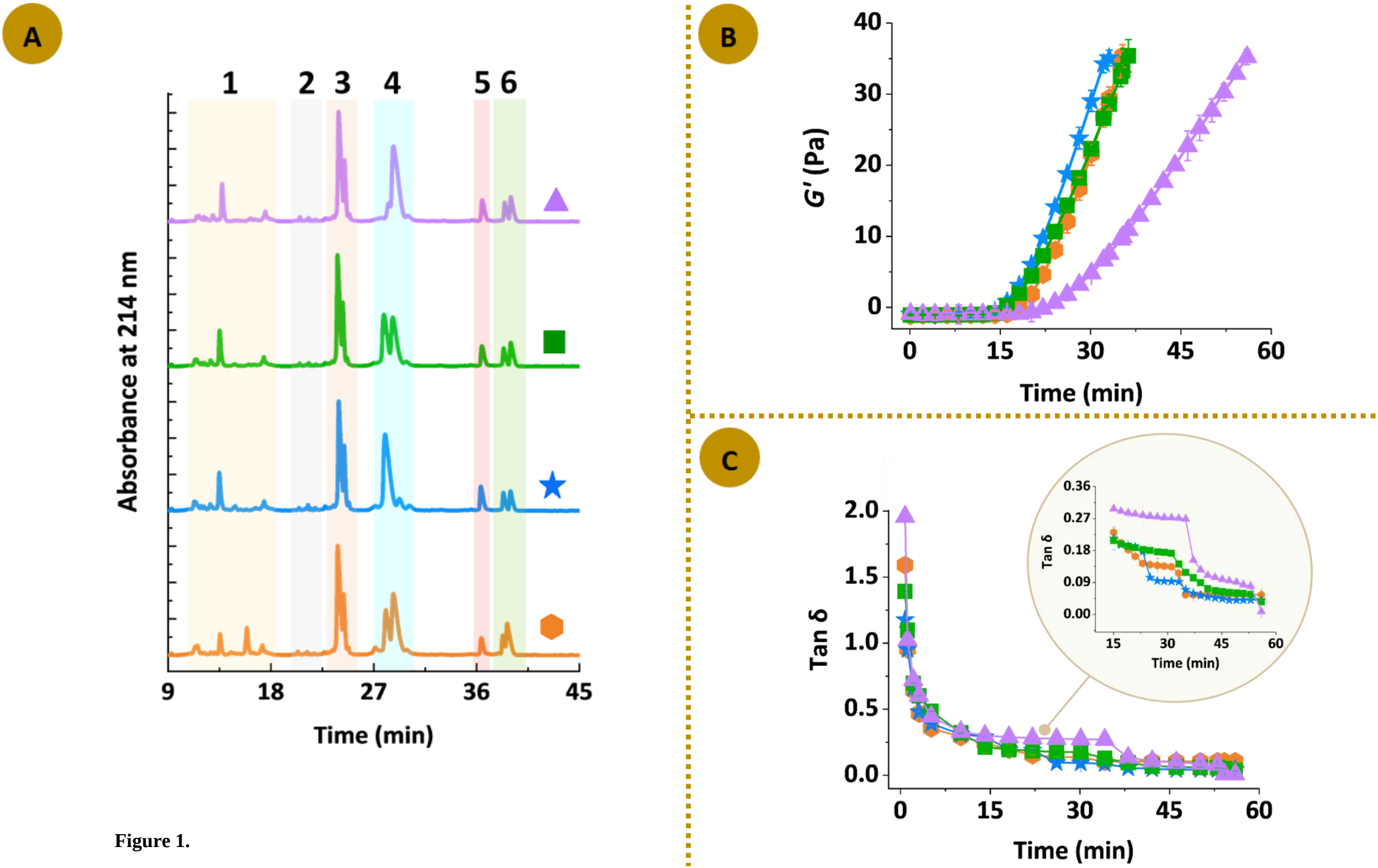
A
1
2
3
4
5
6
Absorbance at 214 nm
9
18
27
36
45
Time (min)
B
G' (Pa)
0
10
20
30
40
0
15
30
45
60
Time (min)
C
Tan δ
0.0
0.5
1.0
1.5
2.0
0
15
30
45
60
Time (min)
Tan δ
0.00
0.09
0.18
0.27
0.36
15
30
45
60
Time (min)

Figure 1.

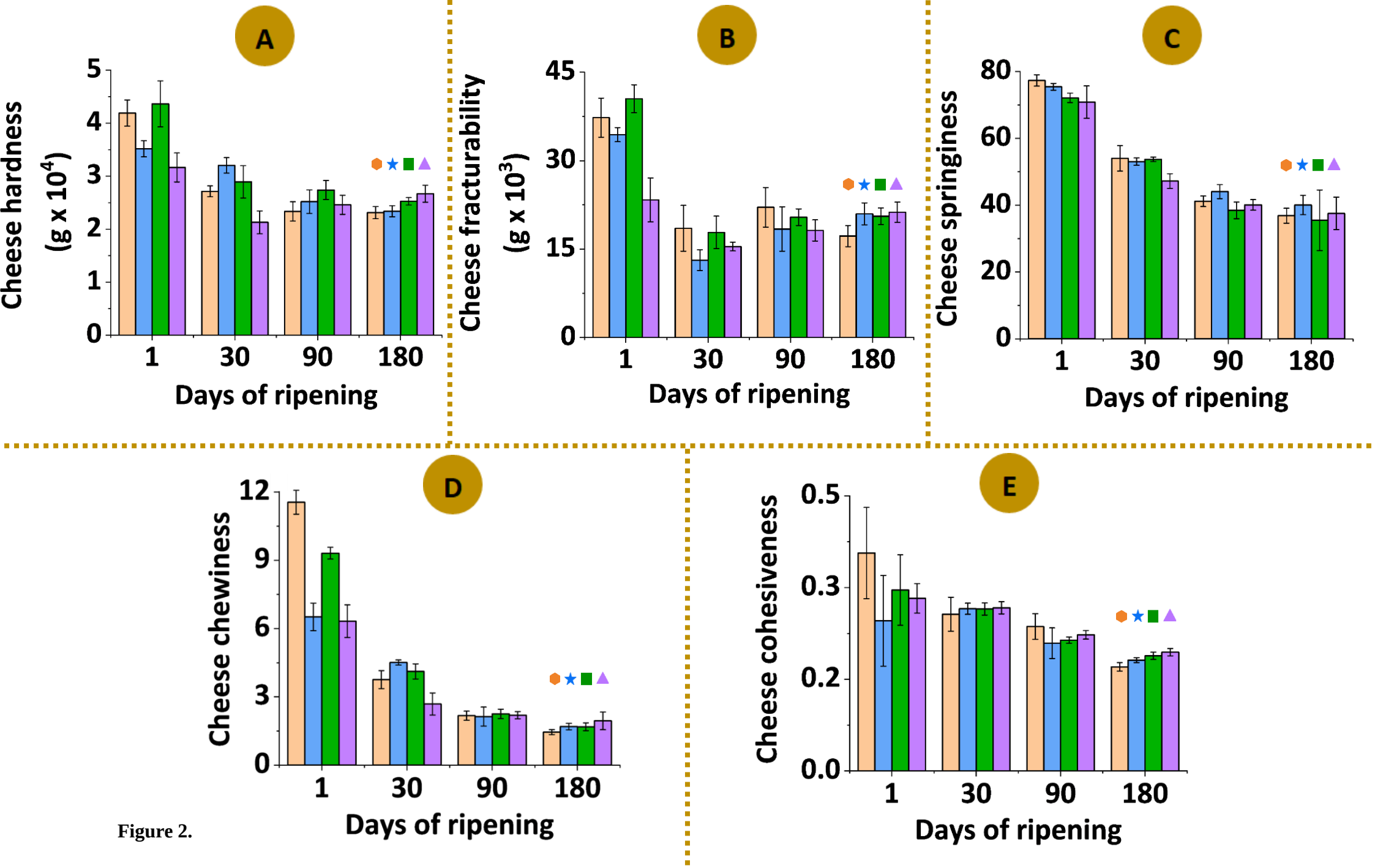
A
Cheese hardness
(g x 10^4)
5
4
3
2
1
0
1
30
90
180
Days of ripening
B
Cheese fracturability
(g x 10^3)
45
30
15
0
1
30
90
180
Days of ripening
C
Cheese springiness
80
60
40
20
0
1
30
90
180
Days of ripening
D
Cheese chewiness
12
9
6
3
0
1
30
90
180
Days of ripening
E
Cheese cohesiveness
0.5
0.3
0.2
0.0
1
30
90
180
Days of ripening

Figure 2.

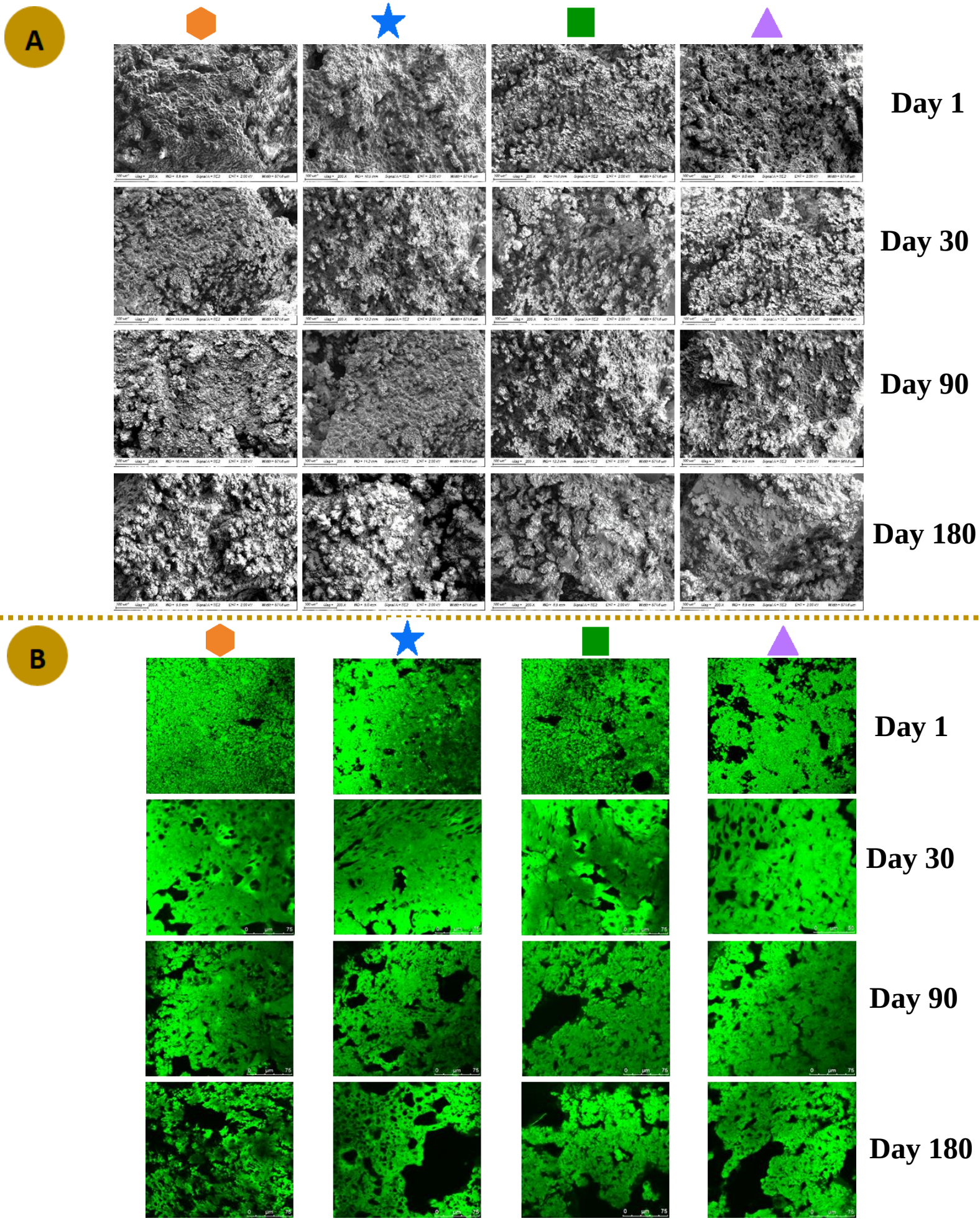

Figure 3.

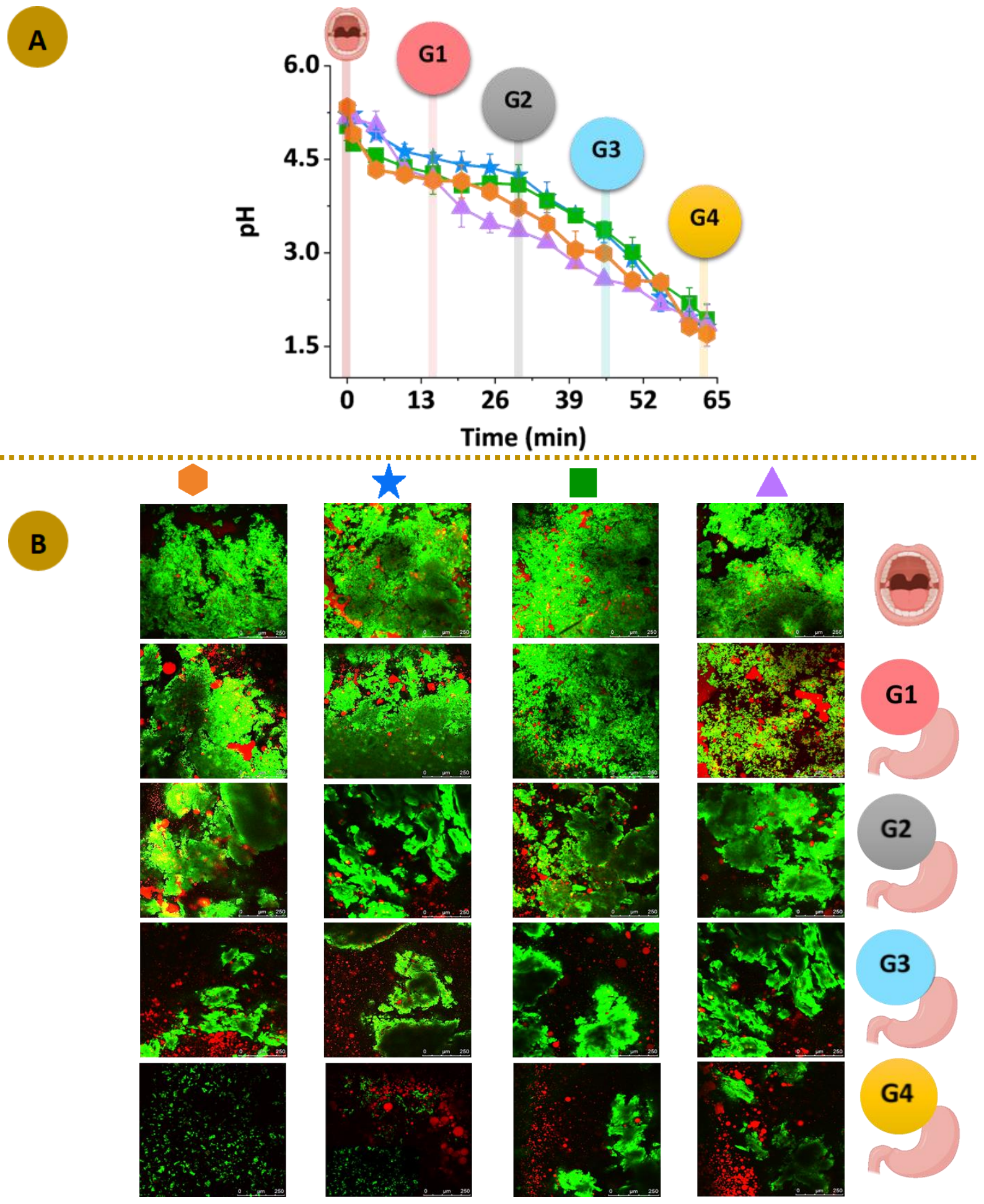

Figure 4.

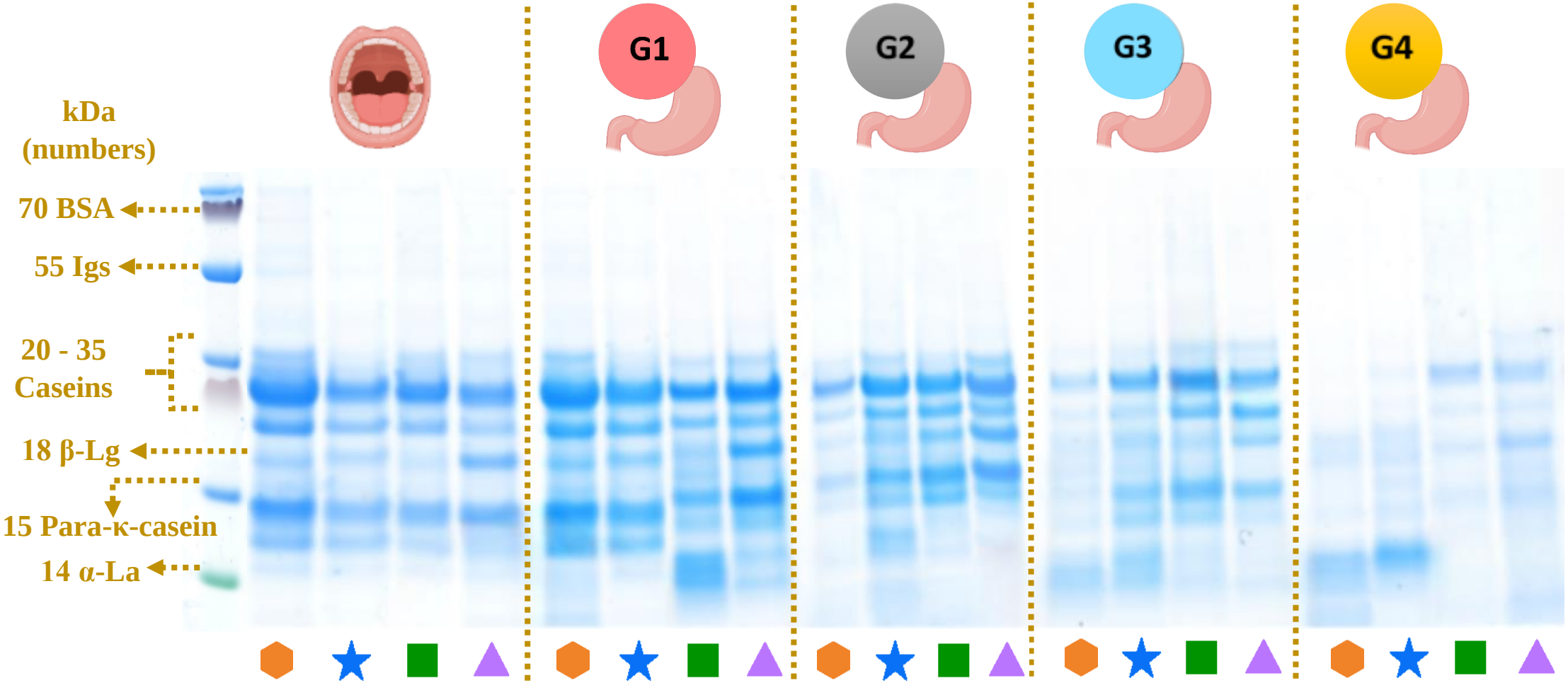
kDa
(numbers)
70 BSA
55 Igs
20 - 35
Caseins
18 β-Lg
15 Para-κ-casein
14 α-La
G1
G2
G3
G4

Figure 5.

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Future of the thesis: Chapter 13.

Bovine β-Casomorphins: Friends or Foes? A comprehensive assessment of evidence from *in vitro* and *ex vivo* studies

- Identification and potential effects of bovine β-casomorphins on human and animal organisms were systematically analysed
- Bovine β-casomorphins may possess potential beneficial and possible negative implications for human and animal health
- Bovine β-casomorphins may be transported into the human blood only if the permeability of the gut barrier is compromised
- Serum enzymes may degrade and eliminate bovine β-casomorphins from the blood stream, before accessing the internal organs

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Bovine β-Casomorphins: Friends or Foes? A comprehensive assessment of evidence from *in vitro* and *ex vivo* studies

Davor Daniloski [a,b], Noel A. McCarthy [b], Todor Vasiljevic [a,*]

[a] Advanced Food Systems Research Unit, Institute for Sustainable Industries and Liveable Cities and College of Health and Biomedicine, Victoria University, Melbourne, Victoria, 8001, Australia
[b] Food Chemistry and Technology Department, Teagasc Food Research Centre, Moorepark, Fermoy, P61 C996, Cork, Ireland

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ABSTRACT

Background: Complex polymorphisms in the polypeptide chain of bovine β-casein are responsible for the genetic variants that give rise to different bioactive peptides during *in vitro* and *ex vivo* digestion, or food fermentation. One specific group of bioactive peptides, known as β-casomorphins, are opioid-agonists for μ-receptors and have been suggested to assume an active role in the development of various non-communicable diseases, including diabetes mellitus, cardiovascular diseases, neurological disorders, pulmonary inflammation, to name a few. Their potential bioactivity and role in human health is dependent on their release from the latent form within the primary structure of β-casein, which can occur during the manufacture of dairy products or during gastric and intestinal digestion. Consequently, β-casomorphins can be either completely hydrolysed or absorbed in the gut or be transferred into the blood stream and internal organs in their intact form. Their biological function as opioid agonists is expressed in the gut, thus upon epithelial translocation they may affect various physiological states, such as causing gastrointestinal issues, bloating, and lactose intolerance.

Scope and approach: This review evaluated the possible disadvantages and potential beneficial effects of β-casomorphins on human health, within the scope of *in vitro* and *ex vivo* studies. Applying a systematic approach, a literature search was performed across four electronic databases (Scopus, Web of Science, PubMed, and Cochrane) to identify suitable studies.

Key findings and conclusions: The data mined from *in vitro* and *ex vivo* trials on the health impact of β-casomorphins is both inconclusive and limited to completely support the possible adverse or potential beneficial health effects of β-casomorphins. These peptides are usually further cleaved in the gut, which prevents their migration across the gut-blood-brain barrier. Nevertheless, in some individuals that are immunocompromised, their condition increases permeability of the gut barrier often referred to as a "leaky gut" condition. Thus, the absorption of β-casomorphins appears possible. This may indicate that the presence of β-casomorphins can affect gastrointestinal functions only. However, since the overall concern with β-casomorphins appears debatable and not well defined, more experimental trials are required to investigate the metabolic pathways of these identified peptides, their release during digestion, and subsequent fate after the digestion process. Consequently, repeatability of the findings under a number of other laboratory conditions is required before the data can be fully substantiated. Due to the rapidly evolving nature of the issue and emerging studies in this field, further exploration into the bioactivity of β-casomorphins is warranted.

1. Introduction

Bovine milk is an excellent source of nutrients, containing relatively high levels of proteins, lipids, carbohydrates, and minor components, such as minerals and vitamins, making it widely used in human nutrition (Thiruvengadam, Venkidasamy, Thirupathi, Chung, & Subramanian, 2021). Milk protein can be mainly divided in to two fractions, caseins (CNs) and whey proteins (WPs), present at a ratio of approximately 80:20 in mature bovine milk (Daniloski, Petkoska, Lee, Bekhit, Carne, Vaskoska, & Vasiljevic, 2021). The CN group of proteins can be further classified in to four different types, namely αs_1-CN, αs_2-CN, β-CN, and κ-CN, in a ratio of 4:1:4:1, respectively (Bijl, Holland, & Boland, 2020).

* Corresponding author.
E-mail address: todor.vasiljevic@vu.edu.au (T. Vasiljevic).

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Approximately 40% of the total CN and one third of the total protein content in bovine milk is β-CN. However, β-CN can be further sub-divided according to the changes in its amino acid composition, which is encoded for by the CSN2 gene on chromosome 6.

Based on the amino acid substitution arising from the cleavage site in the polypeptide chain, twelve to fifteen different genetic variants of β-CN exist, with variants A1 (His^{67}) and A2 (Pro^{67}) most abundant in modern cattle, which frequency depends on cow genetics (Daniloski, Cunha, et al., 2021; Nguyen, Busetti, Smolenski, Johnson, & Solah, 2021). The distinction between these two forms is a mutation in the amino acid polypeptide chain at position 67 (Asledottir et al., 2018; Nguyen, Solah, Busetti, Smolenski, & Cooney, 2020). Furthermore, the β-CN variants can be separated into two of the most prevalent families, namely A1 family, including A1, B, and F variants; and A2 family, including A2, A3, and I variants (Asledottir et al., 2018). As a result of the single nucleotide polymorphism in modern European cattle a few thousand years ago, the A1 β-CN variant (His^{67}: 155.2 g/mol) started to predominate, even though, at first, all cattle originated from the A2 β-CN variant (Pro^{67}: 115.1 g/mol) renowned as the oldest β-CN proteoform (Sebastiani et al., 2020). Aside from the composition of the β-CN polypeptide chain, very little information exists on the structure and functional differences between β-CN proteoforms. The differences between A1 and A2 β-CN genetic variants have opened a debate regarding the genetic selection of Holstein-Friesian cattle (Kamiński, 2021; Olenski, Kamiński, Szyda, & Cieslinska, 2010), the effects of A1 and A2 β-CN on the physiochemical properties of milk and dairy products (Day, Williams, Otter, & Augustin, 2015), on milk yield and gross composition (Ristanić et al., 2020), and the retail market (Bentivoglio, Finco, Bucci, & Staffolani, 2020). Results from numerous studies have clearly shown that selecting to breed cows with the A2 allele has an advantageous effect on milk protein yield, which is a desirable and profitable production trait in current dairy breeding programs (Bech & Kristiansen, 1990; Lv et al., 2020; Nilsen et al., 2009; Olenski et al., 2010). Additionally, the genetic profile of bovine β-CN has an important effect on dairy processing and product manufacture, particularly, during rennet- or acid-induced gelation for cheese and yoghurt manufacture (Day et al., 2015; Poulsen et al., 2013). Contrary to the benefits of cow's breeds containing A2 β-CN on the milk protein yield, it has been found that protein from A2 cows is a contributory factor in non-coagulating and poor-coagulating milks. This has been attributed to the lower total CN and specifically κ-CN content, and lower mineral (Ca, Mg, and P) concentration (Frederiksen et al., 2011; Jensen, Holland, Poulsen, & Larsen, 2012; Vallas et al., 2012). Hence, A2 β-CN milk used for manufacturing of yoghurt (Nguyen, Schwendel, Harland, & Day, 2018) and cheese (Oliveira Mendes, Ferreira de Morais, & Ferreira Rodrigues, 2019) resulted in structures (gels) that were more porous, contained thinner protein strands and had a lower gel strength than gels produced from A1 β-CN or A1/A2 β-CN milk.

Despite the benefits of the A1 β-CN genetic variant in improving the curd consistency and milk coagulation properties, what has garnered more interest is the lower digestibility of this variant compared with the A2 β-CN and the greater amount of released bioactive peptides during its digestion that may have a possible impact on human health (Sebastiani et al., 2020). In fact, due to the mechanism of the gastrointestinal enzymes and peptidase of the brush border membrane (microvillus membrane at the luminal surface of intestines) in the digestive tract, milk proteins can be extensively hydrolysed. This activity leads to the release of peptides and free amino acids (Sanchón et al., 2018). A number of comprehensive studies examined the mechanism and functional properties of opioid-like bioactive peptides liberated during the hydrolysis of β-CN. One group of peptides that may be released are known as β-casomorphins (BCMs), identified as active binders to μ-receptors in the digestive, nervous, and immune systems and all contain an identical N-terminal sequence, namely the same first three amino acids within their structure (Tyr-Pro-Phe) (Table 1). Although they are encrypted within the β-CN polypeptide chain in a non-functioning state, once the gastrointestinal digestion process starts they can be liberated in the human gut (Asledottir et al., 2019; Cieślińska, Kamiński, Kostyra, & Sienkiewicz-Szłapka, 2007; De Poi et al., 2020; Jianqin et al., 2015). Daniloski, Cunha, et al. (2021) explained that β-casomorphin7 (BCM7) may also be released from A2 β-CN, albeit at lower levels compared to A1 β-CN and A1/A2 β-CN, and which was recently demonstrated by Lambers, Broeren, Heck, Bragt, and Huppertz (2021). Additionally, F and B variants of β-CN as part of the family of A1 β-CN, and A3 β-CN and I β-CN as part of the A2 β-CN family have also been reported to release BCMs during digestion (Asledottir et al., 2018). Therefore, upon proteolysis of β-CN from either family, short BCM opioid peptides are released (Asledottir et al., 2017). The European Food Safety Authority (EFSA) previously reviewed the published data related to the possible health effects of bovine milk and its liberated BCMs (EFSA, 2009). Having followed a rigorous review of the available literature, EFSA found no strong and conclusive evidence of a cause and effect association between BCMs and the occurrence of such chronic diseases (Summer, Di Frangia,

Table 1
The structure of β-casomorphin peptides released from bovine β-casein.

β-casomorphins	β-casein location	Structure	Source
β-casomorphin3	β-casein f (60–62)	**Tyr-Pro-Phe**	De Noni et al. (2015)
β-casomorphin4	β-casein f (60–63)	**Tyr-Pro-Phe**-Pro	Nguyen, Johnson, Busetti, and Solah (2015a)
β-casomorphin5	β-casein f (60–64)	**Tyr-Pro-Phe**-Pro-Gly	Nguyen, Solah, Johnson, Charrois, and Busetti (2014)
β-casomorphin6	β-casein f (60–65)	**Tyr-Pro-Phe**-Pro-Gly-Pro	Asledottir et al. (2018)
β-casomorphin7	β-casein f (60–66)	**Tyr-Pro-Phe**-Pro-Gly-Pro-Ile	Summer et al. (2020)
β-casomorphin8	A1 β-casein f (60–67)	**Tyr-Pro-Phe**-Pro-Gly-Pro-Ile-**His**	Brooke-Taylor et al. (2017)
β-casomorphin8	A2 β-casein f (60–67)	**Tyr-Pro-Phe**-Pro-Gly-Pro-Ile-**Pro**	Brooke-Taylor et al. (2017)
β-casomorphin9	A1 β-casein f (60–68)	**Tyr-Pro-Phe**-Pro-Gly-Pro-Ile-**His**-Asn	Nguyen, Busetti, and Solah (2017)
β-casomorphin9	A2 β-casein f (60–68)	**Tyr-Pro-Phe**-Pro-Gly-Pro-Ile-**Pro**-Asn	
β-casomorphin10	A1 β-casein f (60–69)	**Tyr-Pro-Phe**-Pro-Gly-Pro-Ile-**His**-Asn-Ser	
β-casomorphin10	A2 β-casein f (60–69)	**Tyr-Pro-Phe**-Pro-Gly-Pro-Ile-**Pro**-Asn-Ser	
β-casomorphin11	A1 β-casein f (60–70)	**Tyr-Pro-Phe**-Pro-Gly-Pro-Ile-**His**-Asn-Ser-Leu	Haq (2020a)
β-casomorphin11	A2 β-casein f (60–70)	**Tyr-Pro-Phe**-Pro-Gly-Pro-Ile-**Pro**-Asn-Ser-Leu	
β-casomorphin13	A1 β-casein f (60–72)	**Tyr-Pro-Phe**-Pro-Gly-Pro-Ile-**His**-Asn-Ser-Leu-Pro-Gln	
β-casomorphin13	A2 β-casein f (60–72)	**Tyr-Pro-Phe**-Pro-Gly-Pro-Ile-**Pro**-Asn-Ser-Leu-Pro-Gln	
β-casomorphin21	A1 β-casein f (60–81)	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-**His**-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro- Pro-Leu-Thr-Gln-Thr	
β-casomorphin21	A2 β-casein f (60–81)	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-**Pro**-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro- Pro-Leu-Thr-Gln-Thr	

Ajmone Marsan, De Noni, & Malacarne, 2020). Nevertheless, numerous studies indicated that BCMs, especially β-casomorphin5 (BCM5) and BCM7, are of a possible concern as they may contribute to the development of non-transmissible conditions, such as different types of cancer, autism, obesity, diabetes, and cardiovascular diseases (Cieślińska et al., 2015; Fiedorowicz et al., 2014; Jarmołowska et al., 2019). Conversely, some studies have found possible beneficial effects of BCMs (particularly BCM7) on human health and deterioration of chronic conditions induced by ingestion of bovine milk originated from different β-CN; however, this phenomena, similar to the previously mentioned negative impacts, remains unclear and therefore requires further evidence (Thiruvengadam et al., 2021).

Ideally, experimental work on the gastrointestinal digestion of β-CN should be conducted *in vivo*, however, this is not always scientifically, ethically, financially practical, or even possible (e.g., infant digestion in relation to the consumption of bovine-based infant formulae). As a result, numerous *in vitro* and *ex vivo* digestion systems have been developed, from static mono-compartmental to dynamic multi-compartmental models, used to simulate milk protein digestion within the gastrointestinal tract (Brodkorb et al., 2019; Minekus et al., 2014; Mulet-Cabero, Egger, et al., 2020; Sanchón et al., 2018). *In vitro* digestion models are typically performed using well-defined, single enzymes of porcine or bovine origin. These commercial enzymes offer a straightforward approach to digestion studies. Nevertheless, human gastrointestinal juices contain a combination of various enzymes, enzyme inhibitors and salts, which could be more practical when examining the human digestive environment. This digestion model is also known as the *ex vivo* approach (Asledottir et al., 2018; Islam, Ekeberg, Rukke, & Vegarud, 2015). Despite the effectiveness and rapid screening of both methods, it remains difficult to resolve either the significant variance between the individual parameters used in these methodologies, or specific individual differences in gut permeability (Asledottir et al., 2017; Li, Yu, Wu, & Chen, 2020).

This review investigates *in vitro* and *ex vivo* human and animal based

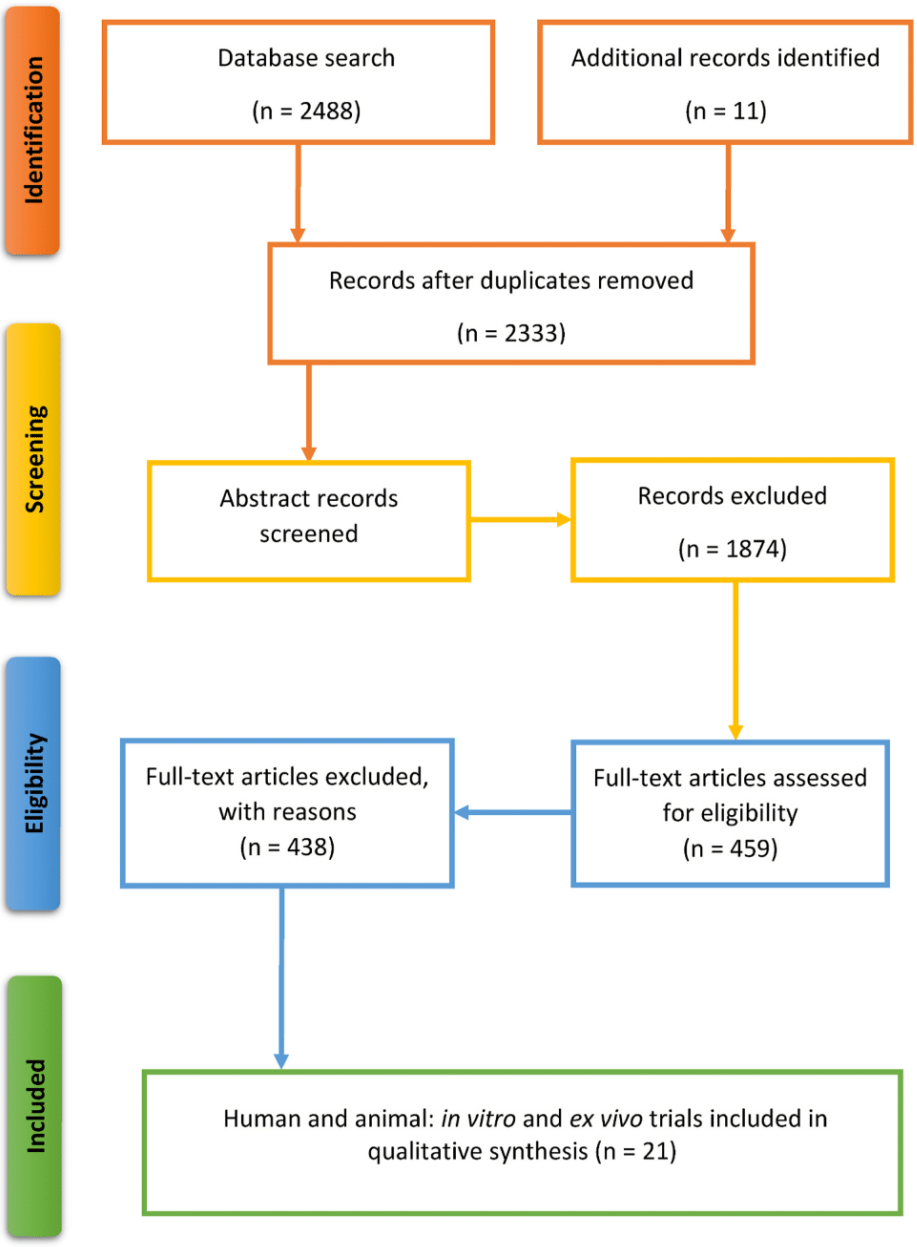

Fig. 1. A flow chart outlining the article search and inclusion process.

published studies, providing some insights into the possible negative and potential positive effects of natural (hydrolysis or digestion of naturally extracted β-CN) and synthetic bovine BCMs (prepared in a laboratory extracted from β-CN or supplied by the pharmaceutical industry]). Currently, the strength of evidence in regards to the impact of BCMs is relatively weak and focused mainly on the fate of BCM5 and BCM7 upon *in vitro* digestion (Asledottir et al., 2019; Dalziel et al., 2014). The importance of *in vitro* and *ex vivo* conducted trials that can lead to well-informed and logical discernment for the quality of the results of different BCMs liberated from bovine milk of various β-CN variants will be elaborated. This comprehensive review provides a general description of the effects of BCMs (pro-inflammatory consequences, incidence of chronic metabolic diseases, or possible health benefits) liberated after digestion of bovine β-CN on human and animal *in vitro* and *ex vivo* trials. The main aim of this review was to provide a critical evaluation of the advances and limitations of existing *in vitro* and *ex vivo* digestion systems on BCMs. Specifically, the release of BCMs in the human gut, their migration into the bloodstream (an important step to carry out biological activities outside the intestinal tract), and the final fate of BCMs.

2. Study selection, eligibility, and extraction criteria

The literature search was performed using PubMed, Scopus, Web of Science, and Cochrane in September 2020 with the following search terms: *in vitro* and *ex vivo* examination of β-CN, BCMs, BCM5, or BCM7; or cow's milk genetic variants; or A1 milk and A2 milk; or β-casein dairy products. No limits were imposed on the publication dates of results, and additional relevant reports not captured in this search were identified by manually searching on Google Scholar. The reference lists of all identified manuscripts were also searched (Fig. 1). The authors excluded all studies where the intervention was not related to bovine milk genetic variants, β-CNs, BCMs and where the studies were not performed *in vitro* or *ex vivo*.

2.1. Studies selection

In total, 2488 publications were identified throughout the search of the online databases. An additional 11 publications were found from other sources. Following the procedure of duplicate elimination, 2333 articles were screened for eligibility. From these studies, 1874 articles were excluded after title and description screening, and another 438 articles were excluded after the final text screening. Out of all identified studies, 21 original *in vitro* and *ex vivo* studies met the requirements for inclusion and were involved in the analysis (Fig. 1). Studies meeting the following criteria were included in this comprehensive review: only *in vitro* and *ex vivo* studies; written in the English language; published in peer-reviewed journals; performed and tested on humans and animals (cells, tissues, and/or organs); involved only bovine milk and dairy products; and examined the impact of BCMs on human or animal health. Moreover, both *in vitro* and *ex vivo* studies, which examined numerous bovine milk peptides or peptides derived from other animals' or human's milk proteins were not taken into consideration, but only the relevant results related to bovine milk BCMs. Thus, studies assessing the effects of bovine milk, β-CN, and BCMs in *in vivo* conditions; conference abstracts, reviews and patents; letters to editors; and not original performed studies; were excluded. Studies where a combination of *in vitro, ex vivo,* and *in vivo* were examined, only the results from *in vitro* and *ex vivo* were reviewed.

2.2. Characteristics of the selected studies

All 21 original studies included in this review differed in their research approach. In the first part, 14 trials reported the negative effect of BCMs on human (Asledottir et al., 2019; Cieślińska et al., 2015; Fiedorowicz et al., 2011, 2014; Jarmołowska et al., 2019; Kurek, Przybilla, Hermann, & Ring, 1992; Trivedi et al., 2014, 2015) and animal (Brantl, Teschemacher, Henschen, & Lottspeich, 1979; Chia et al., 2018; Dalziel et al., 2014; De Ponti et al., 1989; Hautefeuille, Brantl, Dumontier, & Desjeux, 1986) organs, tissues, or cells within *in vitro* studies (Table 2). Additionally, 2 of the human trials (Kampa et al., 1997; Zhu, Li, Wu, & Li, 2018), 4 of the animal trials (Sheng, Zhang, Li, & Sun, 2020; Trompette et al., 2003; Zhang, Miao, Wang, & Zhang, 2013; Zhang, Song, Liu, Liu, & Zhang, 2015), and 1 study examined both animal and human data (Zoghbi et al., 2006), depicting the protective and therapeutic mechanisms of BCMs; thus, were considered within the inclusion criteria (Table 3). Tables 2 and 3 describe the exposure, intervention, data analysis, and final outcomes from each of the studies mentioned above. Table 1 presents the currently examined BCMs liberated and found in bovine milk originating from different β-CN phenotypes. Additionally, Fig. 2 shows only the BCMs included and described in this study.

3. β-casomorphins: Release, mechanism of action, transport, and breakdown

3.1. Digestion of milk proteins and liberation of β-casomorphins

In order to observe and determine the physiological properties of milk derived peptides, such as BCMs, milk protein digestion is necessary. In cases where *in vivo* trials are not possible, the key principles of bioaccessibility, bioavailability, and bioactivity of peptides should ideally only be explored through *in vitro* or *ex vivo* models to successfully simulate protein digestion (Giromini, Cheli, Rebucci, & Baldi, 2019). The first step of protein digestion begins under acidic condition in the stomach, namely, gastric acid, which denatures and unfolds proteins making them more accessible to the enzymatic action of pepsin, initiating the hydrolysis of proteins into polypeptides (Summer et al., 2020; Ye, 2021). Owing to the fact that only a few peptides are liberated in the stomach, the digestion process continues in the duodenum whereby enzymes released by enterocytes at the brush border membrane hydrolyse most of the polypeptides into shorter peptides and amino acids (Xu, Hong, Wu, & Yan, 2019). These short chain peptides and amino acids can potentially be absorbed by the jejunum and ileum by penetrating through the intestine epithelial membrane, via co-transport with potassium and hydrogen ions, before entering the circulatory blood system (Asledottir et al., 2019; Giromini et al., 2019; Parada & Aguilera, 2007) (Figs. 3 and 5). From all the renowned peptides derived from β-CN, BCMs have been received much attention from both the scientific community and the wider public (Brooke-Taylor, Dwyer, Woodford, & Kost, 2017; Saadi, Saari, Anwar, Hamid, & Ghazali, 2015; Summer et al., 2020). As stated in the literature, the digestive enzymes (proteases), such as pepsin, trypsin, and chymotrypsin, which can be commercially purchased or extracted from human or porcine juices, are frequently included during *in vitro* and *ex vivo* studies, respectively (Fig. 3) (Daroit & Brandelli, 2021). These proteases alone or in conjunction with pancreatic elastase and leucine aminopeptidase may release BCMs from β-CN and thus may be involved in the subsequent further hydrolysis of BCMs (Edwards, Dawson, Keenan, & Day, 2021; Yoshikawa, 2013). Once in the intestines, brush border enzymes, particularly, dipeptidyl peptidase IV (DPP-4: EC 3.4.14.5) produced by enterocytes, selectively removes the N-terminal dipeptide from peptides, which contains proline (Pro) or alanine (Ala) residue at position 2. This N-terminal cleavage results in the formation of different length peptides (Barnett, McNabb, Roy, Woodford, & Clarke, 2014; Summer et al., 2020). Thus, DPP-4 can hydrolyse BCMs into their derived C-terminal shorter BCMs, dipeptides and X-proline dipeptides, or amino acids prior to being observed in the human blood plasma (Osborne et al., 2014) (Fig. 4). There are three known locations where DPP-4 is active. Specifically, DPP-4 is found on the surface of numerous cell types, including enterocytes, endothelial, and immune cells (membrane bound). It can be also found as part of the human-associated microbiome where DPP-4-like activity can be

expressed by the gut microbiota. Finally, DPP-4 may also be found in a soluble form in the circulatory blood system (Klemann, Wagner, Stephan, & von Hörsten, 2016; Mulvihill & Drucker, 2014; Olivares, Schüppel, et al., 2018) (Fig. 4). When BCMs are transferred across the epithelial barrier in the gut, the enzymatic activity and expression of DPP-4 increases; hence, the DPP-4 peptidase enzyme can trigger an immune (activating Th2 pathway) and non-specific inflammatory response. Its reduced function is usually associated with compromised immune status (Jarmołowska et al., 2019; Uematsu, Urade, Yarnaoka, & Yoshioka, 1996).

3.2. Opioid-acting role of β-casomorphins: Presence in the gastrointestinal system

The β-casomorphin peptides may be released from β-CN during enzymatic digestion in the gastrointestinal tract, during proteolysis, or during certain types of milk processing (e.g., cheese production, industrial milk protein hydrolysis, etc.). These peptides, classified as morphine-like exorphins, are released exogenously from various bovine milk genetic variants and have been shown to have opioid activity, which can activate μ-opioid receptors (Pica, Stuknytė, Masotti, De Noni, & Cattaneo, 2021). μ-Opioid receptors are expressed by selected central and peripheral neurons, and are involved in several physiological processes, including motor activity, analgesia, gastrointestinal transit; they can control the opioid peptide motility, secretion, and regulation of their digestion, absorption, and immunomodulation (Brantl, Teschemacher, Bläsig, Henschen, & Lottspeich, 1981; Zuffa et al., 2021). Previous studies (Chang, Lillian, Hazum, Cuatrecasas, & Chang, 1981; Clare & Swaisgood, 2000) have shown that opioid receptors can recognise peptides that have amino acid sequences containing Tyr at the N terminus, such as BCM peptides. Therefore, BCM peptides can affect a number of physiological processes, such as increased gastrointestinal tract transit time, activated colon myeloperoxidase [MPO] activity, elevated levels of DPP-4 concentration in blood and increased levels of blood glucose (Duraffourd et al., 2012; Tyagi, Daliri, Kwami Ofosu, Yeon, & Oh, 2020; Zoghbi et al., 2006).

It has been proposed that if BCMs are released during the digestion of β-CN in the gastrointestinal tract, particularly in the small intestine, they will bind to the μ-opioid receptor (present on the enteric nervous system located in the gastrointestinal wall). The μ-opioid receptors are G-protein coupled receptors (GPCRs), composed of α, β, and γ subunits. After the activation of the opioid receptor by morphine-like agonists (such as BCMs), Gα and Gβγ subunits of the μ-opioid receptor dissociate from one another, and can control different intracellular effects/pathways, including adenylcyclase C (AC), phospholipase (PLC), cyclic adenosine monophosphate (cAMP [formed by the action of adenosine triphosphate - ATP]), potassium and calcium channels, inositol triphosphate (IP 3), triglyceride (DAG), protein kinase A (PKA), transcription of inflammatory cytokines (nuclear factor - kB [NF-kB]). This can lead to changes in biological activity, such as mucin production, gastrointestinal permeability, lowering blood pressure, immunomodulation, and enhanced cell proliferation (Kodukula & Zeng, 2018; Listos et al., 2019; Tyagi et al., 2020) (Fig. 5). Subsequently, BCMs may be transferred through the epithelium, where it is thought they can exhibit opioid activity in the blood and initiate either beneficial or possibly detrimental physiological and immunological responses (Haq, 2020a). The beneficial impact can include mucin production, inhibiting the proliferation of cancer cell lines, and decreasing oxidative cell stress. Alternatively, negative effects caused by BCMs' presence in cells or tissues involve lymphocyte proliferation, allergies, intolerances, skin reactions, etc. (Haq, Kapila, & Kapila, 2015; Jarmołowska et al., 2019; Kampa et al., 1997; Kurek et al., 1992; Tyagi et al., 2020; Zhang et al., 2013). However, to date BCM fractions have not been found or identified in plasma obtained from adult humans. Nonetheless, as a result of the so called "leaky gut" condition presented in certain population cohorts, such as older adults, children with an immunocompromised system or neonates, the absorption of larger peptides and the presence of BCMs in their plasma may be possible (Asledottir et al., 2019; Miner-Williams, Stevens, & Moughan, 2014; Wasilewska et al., 2011).

During post gastric digestion within immunocompromised individuals, BCMs can be found in the small intestine, before being degraded by the intestinal brush border peptidases (microbial or membrane bound DPP-4) or else attached to μ-opioid receptors, where they may be biologically active in the intestine or colon (Asledottir et al., 2017). BCMs, particularly BCM7, may cause delayed gastrointestinal transit by inhibiting intestinal peristalsis and motility, modulate ion transport, regulate various endocrine responses (secretion of insulin, somatostatin, and gastrin), which appears to be associated with modification of target gastrointestinal endocrine cells, inflammation and gastrointestinal discomfort, including bloating, constipation, and diarrhoea, symptoms related to lactose intolerance (Barnett et al., 2014). This might also show knock-on effects on other aspects of gut function; it may possibly exacerbate existing hypolactasia (lactose intolerance), resulting in longer gut transit times and prolonged lactose fermentation by gut microflora which can cause or worsen abdominal pain and distension (Romero-Velarde et al., 2019). This process in conjunction with physiological factors (age, gender, and pregnancy) and pathological factors (viral infections, neoplasms, autoimmune diseases, diabetes, hypertension, skin diseases, and neuro disorders) can initiate suppression of brush border DPP-4 and microbial DPP-4-like activity (Olivares, Schüppel, et al., 2018; Wasilewska et al., 2011). Hence, it is possible that the half-life of BCM7 in the intestinal tract may increase, causing localised issues in the small intestine or colon, (Mulvihill & Drucker, 2014). Furthermore, while DPP-4 has been found to be produced all along the intestinal tract, its activity is higher in the ileum compared to the caecum and colon (Olivares, Neyrinck, et al., 2018). As a result of its uneven distribution in the intestines, colonic inflammation may occur, which subsequently affects the break-down of malabsorbed lactose, possibly due to changes in the gut microbiota (Pal, Woodford, Kukuljan, & Ho, 2015), leading to progressive lactose fermentation and manifestation of lactose malabsorption symptoms in some individuals (Haq, 2020b).

3.3. Transfer of β-casomorphins in blood and internal organs

Numerous *in vitro* and *ex vivo* studies confirmed that opioid peptides that have been bound to μ-opioid receptors and which have initiated intracellular changes to α, β, and γ subunits (Fig. 5, section 3.3.2.) may penetrate the intestinal barrier using a number of different pathways (Horner, Drummond, & Brennan, 2016). Penetration is usually through either carrier-mediated permeation or passive transcellular diffusion, allowing for the transport of BCM opioid peptides between the intestinal brush-border membrane and the blood stream (Daniloski, Cunha, et al., 2021; Matsui, 2018; Xu et al., 2019) (Fig. 5). Therefore, BCMs could potentially be transported around the body *via* the blood stream, inducing physiological responses in the internal organs. However, the transport capacity of BCMs has been found to be limited to tripeptides and dipeptides only (Fiedorowicz et al., 2014; Jarmołowska et al., 2019; Sokolov et al., 2014; Vij, Reddi, Kapila, & Kapila, 2016; Wasilewska et al., 2011).

Therefore, it is worth mentioning that in the healthy adult population, excluding infants and the elderly, the normal function of the intestinal mucosal barrier ensures that bioactive peptides and microorganisms are prevented from entering the blood stream, maintaining its capacity to absorb micronutrients only (Lázaro, Pondé, & Rodrigues, 2016). The mucosal barrier layer of the intestine is protected by the presence of mucins (secreted by the epithelial cells), immunoglobulin A, and antimicrobial peptides (Sánchez de Medina, Romero-Calvo, Mascaraque, & Martínez-Augustin, 2014). Further protection at the mucosal barrier comes in the form of a number of immune cells, such as neutrophils, monocyte/macrophages, dendritic cells, mast cells, innate lymphoid cells, B cells, and T cells (Sánchez de Medina

et al., 2014). Also, an additional protective effect is provided by the fact that the DPP-4 enzyme is present in the blood system and can rapidly hydrolyse BCMs that may cross the intestinal barrier, (Kreil, Umbach, Brantl, & Teschemacher, 1983) (Fig. 4). The smaller peptides and free amino acids formed as a result of BCM hydrolysis are then removed from the blood *via* the renal and hepatic system (Mulvihill & Drucker, 2014) (Fig. 4). Therefore, if BCMs are potentially hydrolysed, inactivated, and removed from the blood plasma, how is it proposed that they can allegedly migrate to different internal organs and tissues (other than the blood tissue), and initiate physiological effects? Therefore, there seems to be a significant number of unknowns surrounding the biological pathways involved in BCM breakdown, transport and effects on human health. In-line with existing literature, Figs. 3–5 illustrate a number of the theoretical scenarios involved in the physiological pathways of BCM production, transport, and fate.

3.4. Human and animal in vitro and ex vivo trials

3.4.1. Possible negative health effects of β-casomorphins

The unfavourable effects of BCMs on human and animal cell cultures, tissues, or organs based on the data gathered from *in vitro* and *ex vivo* studies are summarised in Table 2. The most prominent of all BCMs are BCM5 and BCM7, which represent fragments f60–64 (Tyr-Pro-Phe-Pro-Gly) and f60–66 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) of β-CN, respectively (De Noni, Stuknytė, & Cattaneo, 2015). Four decades ago, Brantl et al. (1979) showed that BCM5 in *mouse vas deferens* had a greater affinity for μ-opioid receptors than BCM7. The same research group supported their previous findings by once again showing that BCM5 had a higher binding affinity for the μ-opioid receptors in the ileum of guinea pigs compared to BCM7 (Brantl et al., 1981). Additionally, in the isolated short distal colon (DC) segments and whole large intestine (LI), the opioid agonist activity of BCM5 that altered the colonic motility was detected (*in vitro* in rats). With application of BCM5 (20 μM) diluted in Krebs buffer immediately prior to use, the tension in the DC increased by 71 ± 17% and contractions in rectum increased by 83 ± 11% (Dalziel et al., 2014). Although BCM5 showed higher activity and potency in opioid activity assays (affinity for specific opiate binding sites), compared to all other BCMs, most studies have focused on the biological and physiological properties of BCM7 from the ingestion of dairy products and possible reflection on the human health (Brantl et al., 1981; Liu & Udenigwe, 2019). This was likely driven by two primary reasons. Firstly, bovine BCM7 was speculated to be implicated in the development of several human diseases, particularly coronary diseases, metabolic diseases, and conditions involving the nervous system (Summer et al., 2020). Secondly, BCM7 appeared to be more resistant to the proteolytic cleavage by digestive enzymes comparably to BCM5, which limits the occurrence of the latter in digested milk and dairy products (Haq, 2020b). One of possible reasons for such observations could be due to presence of an additional Pro in BCM7 compared to BCM5, which may hinder proteolytic hydrolysis, hence the generation of BCM5 seemed unlikely (Kim, Birtwhistle, & Kim, 1972). Notably, an important characteristic of BCMs which apparently governs their activity is the existence of Tyr^{60} at the N-terminal end which renders these peptides bioactive, including BCMs, to express a strong affinity for the active site of μ-opioid receptors (Tyagi et al., 2020). The opioid mechanism of BCMs is likely initiated by the electrostatic attraction found in the vicinity of the Tyr^{60} phenolic hydroxyl group. Chang et al. (1981) found that all BCMs' bioactivity can be lost by the removal of the Tyr^{60} residue at the N-terminal end. In comparison, even though some bovine α-CN peptides lack Tyr at the amino terminus, they have been shown to still possess opioid activity (Teschemacher, Koch, & Brantl, 1997; Tyagi et al., 2020). To date, this phenomenon has not been completely explained and the original *in vitro, ex vivo,* and *in vivo* studies are discussed and elaborated on assumptions. Thus, if the previously explained scenario is taken into consideration, since all bovine BCMs (liberated from β-CN) have a common structural feature, e.g. Tyr at the amino terminal end, and the existence of another aromatic residue in the third position (Phe^{62}), they possess the same structural motif to bind to μ-opioid receptor (Meisel & Fitzgerald, 2000). Moreover, Pro^{61} was found to be an important ligand of Tyr^{60} and Phe^{62} that provides a specific orientation of the side chains. These side chains were found to be essential for the biological function of the opioid peptides (BCMs) (Mierke, Nößner, Schiller, & Goodman, 1990). Accumulating evidence suggests that the behaviour of BCMs, especially BCM7 may be found in its conformational changes at different pH, in particular at pH values found across the human intestinal tract (pH = 5.7–7.4). Very recently, Markoska, Huppertz, and Vasiljevic (2021), using one dimensional proton nuclear magnetic resonance (^{1}H NMR), reported four isomers of BCM7 at pH 2.3 and eight isomers of the same peptide at pH 6.7. The authors stated that at pH 2.3, Gly^{64} - Pro^{65} bond of BCM7 was generally in the *trans* position, however, on increasing the pH to 6.7, the bond isomerises to the *cis* conformation (Markoska et al., 2021). Hence, the authors suggested the existence of *trans-trans-cis* (Tyr H_α-^{1}Pro $H_{\delta\delta}$ > Phe H_α-^{2}Pro $H_{\delta\delta>}$Gly H_α-^{3}Pro$_\alpha$), *trans-cis-cis* (Tyr H_α - ^{1}Pro $H_{\delta\delta>}$Phe H_N - ^{2}Pro $H_{\alpha>}$Gly H_α - ^{3}Pro$_\alpha$), *cis-trans-cis* (Tyr H_α - Pro $H_{\alpha>}$Phe H_α - ^{2}Pro $H_{\delta\delta>}$Gly H_α - ^{3}Pro$_\alpha$), and *cis- cis-cis* (Tyr H_α - Pro $H_{\alpha>}$Phe H_N - ^{2}Pro $H_{\alpha>}$Gly H_α - ^{3}Pro$_\alpha$) isomers at pH 6.7 (Markoska et al., 2021). Thus, the Gly^{64} - Pro^{65} bond of BCM7 appears highly pH dependent. Similarly, Ivanova, Yakimova, Angelova, Stoineva, and Enchev (2010) revealed that the process of *cis-trans* isomerisation of dipeptides was strongly pH dependent. Notably, the *cis-trans* isomerism of peptides has been prone to hydrolysis initiated by the activity of the digestive enzymes, particularly trypsin and pepsin (Lin & Brandts, 1985). This only opens another query, whether isomers of BCM7 can be uniquely digested by enzymes and may possess a completely dissimilar fate in the human gut, serum, and/or internal organs (Markoska et al., 2021; Vance, LeBlanc, & London, 1997).

Kurek et al. (1992) reported that human volunteers exhibited a direct histamine release reaction upon ingestion of BCM7 in a dose-dependent manner, and the authors classified the BCM7 as non-cytotoxic, direct stimulus for liberation of histamine *in vitro*, and a possible trigger for food allergies. The amount of liberated histamine depended on the added amount of BCM7 attached to immune cells, particularly the peripheral leukocytes (Kurek et al., 1992); hence, those cells are equipped with μ-opioid receptors and are involved in signal transmission by exogenous opioids, such as BCMs. When morphine-like peptides, including BCMs, bind to immune cells and activate them, the immune cells start releasing pro-inflammatory cytokines in the blood (Eisenstein, 2019). Therefore, activating signals from immune cells can increase opioid receptor expression, and consequently cause an immune activation, suggesting that BCMs might induce upregulation of the immune system (McCarthy, Wetzel, Sliker, Eisenstein, & Rogers, 2001; Roy, Wang, Kelschenbach, Koodie, & Martin, 2006). In contrast, Park and Haenlein (2021) reported the downregulation of the immune system by BCMs, specifically BCM7. Within the study, BCM7 was called an immune suppressant that might inhibit the proliferation of intestinal lymphocytes and may influence the development of intestinal immune tolerance, decreasing the intestine defence towards enteroviruses (Park & Haenlein, 2021). Therefore, these immunomodulatory effects of BCM7 on the human immune system might be of clinical importance in the case of allergic outcomes and development of numerous gastrointestinal disorders in infants and toddlers (Fiedorowicz et al., 2011). Fiedorowicz et al. (2011) found that the effect of incubating bovine BCM7 (10^{-12} mol/L) with human leukocytes resulted in a 110–130% increase in the proliferation of peripheral blood mononuclear cells (PBMCs), compared to the control (100%).

Fiedorowicz et al. (2014) in their research treated PBMCs with BCM7 originating from bovine milk of three different genetic β-CN variants (A1/A1, A1/A2, and A2/A2). The analysis indicated that BCM7 derived from all genetic variants of milk induced a reduction in the expression of the membrane-bound DPP-4 gene, resulting in significantly lower DPP-4 enzyme activity in children suffering from atrophic dermatitis (AD)

compared to the healthy children group (HC) (Fiedorowicz et al., 2014). Therefore, could the decline in DPP-4 activity in immunocompromised individuals (AD) be the reason for skin inflammation and presence of allergic symptoms? A previous study by Ansorge et al. (2009) stated that the presence of DPP-4 can limit inflammatory activity and allergic reactions caused by some pro-inflammatory peptides, with decreased DPP-4 activity known to worsen skin related allergies (Forssmann et al., 2008), which might be the case for BCMs.

Furthermore, in a previous study by Jarmołowska et al. (2019) it was shown that even though DPP-4 gene expression was not significantly different between genders, it had a tendency to increase among girls with autism spectrum disorder (ASD). The authors observed a higher DPP-4 concentration in the blood serum phase of children with ASD than in children without ASD. In addition, significantly higher concentrations of serum BCM7 also occurred among ASD children compared to non-ASD children (Jarmołowska et al., 2019) (Table 2). One possible explanation for this action is that the immunocompromised system of ASD children may cause a "*leaky gut*" disorder resulting in increased levels of BCM7 and DPP-4 in the blood serum (section 3.3.) (Asledottir et al., 2019; Wasilewska et al., 2011). However, one might expect that the higher levels of DPP-4 in ASD children relative to non-ASD children might have also increased the incidence of BCMs' hydrolysis, which seems not to be the case. Proportionally, with increased levels of BCMs in the intestines or blood stream, more DPP-4 enzyme would be expressed. Cieślińska et al. (2015) also found significant differences in the expression of DPP-4 or the expression of μ-opioid receptors in PBMCs of non-ASD and ASD children. However, Jarmołowska et al. (2019) explained that even though DPP-4 levels were higher in ASD children, their enzymatic activity was lower especially in the ASD female group. The same authors related the decreased enzymatic activity of DPP-4 in serum with the amount of non-hydrolysed milk proteins, the level of sex hormones, and the ability of DPP-4 to inactivate glucagon-like and glucose-dependent insulinotropic peptides (Jarmołowska et al., 2019). Additionally, inhibition of DPP-4 enzymatic activity in immunocompromised patients or patients with diabetes mellitus can be triggered by the presence of certain bioactive dipeptides, such as f60-61 (Tyr^{60} - Pro^{61}) (Asledottir et al., 2019), or antidiabetic agents (sitagliptin, saxagliptin, vildagliptin, linagliptin, and alogliptin), renowned as DPP-4 inhibitors. Consequently, DPP-4 inhibitors, despite their ability to decrease the enzymatic activity of DPP-4, theoretically could increase the levels of detectable BCMs in the blood stream (Kim, Yu, & Lee, 2014). Sokolov et al. (2014) suggested that although high levels of DPP-4 were found in the blood stream, the immune system (immunoglobulins M and G) of autistic children might be an additional factor that could suppress DPP-4 enzymatic activity. Furthermore, Asledottir et al. (2019) also observed and identified by mass spectrometry that BCM7 was partially degraded into f60-65 (YPFPGP), f61-66 (PFPGPI), and f62-66 (FPGPI) by porcine brush border membrane vesicles. The authors stated that if the f62-66 (FPGPI) peptide is released it can produce bioactive dipeptide f60-61 (YP), found as a potent DPP-4 inhibitor, thus the degradation of BCM7 will be hindered (Asledottir et al., 2019; Nongonierma & FitzGerald, 2014).

Ultimately, there are a number of other factors that can also contribute to increased intestinal permeability, such as stress, stomach ulcers, ulcerative colitis, autism, diabetes, Parkinson disease, allowing other peptides to cross the gut barrier and enter the blood stream (Daniloski, Cunha, et al., 2021). Hence, αs_1-casomorphin, casoxin, and αs-exorphin (originating from α_1-CN), casoxin A, B, and C (from κ-CN), α-lactorphin (from α-La), β-lactorphin (from β-Lg), lactoferroxins (from lactoferrin), serorphin and albutensin A (from bovine serum albumin) are a number of milk derived peptides which may cross through the gut barrier and therefore exert physiological effects in the intestines, blood, or other internal organs (Miner-Williams et al., 2014). As an interpretation of all these bioactive compounds is beyond the scope of this review, the focus has been placed on those potential health benefits and disorders that have been linked to BCMs.

The transfer of BCMs across the intestinal membrane has also been previously shown by Hautefeuille et al. (1986), who examined the ability of β-casomorphin4 (BCM4), BCM5, and their analogues (β-[D-Ala^2]CM-4-NH_2 and β-[D-Ala^2,Met^5]CM-5-NH_2) to transfer electrolytes across the ileum barrier, obtained from rabbit specimens via an *in vitro* study. The analogues of BCM5 were able to transfer electrolytes effectively across the intestine and were present on the mucosal side, despite other BCMs which were found only on the serosal intestinal membrane, without showing the ability to transfer electrolytes through the mucosal membrane. The authors hypothesised that some of the BCMs could possibly be absorbed intact by the intestine (Hautefeuille et al., 1986). Similarly, De Ponti et al. (1989) under *in vitro* conditions examined the impact of bovine BCM4 and BCM5 on the peristaltic reflex of the colon (neurally mediated reflex in the intestines that causes caudad propulsion of chyme) obtained from guinea pigs. The results showed that BCM4 had no influence of the efficiency of the peristaltic reflux, however, as previously mentioned, BCM5 proved its higher potency in comparison with the other BCMs by acting at opioid receptors and inhibiting the peristaltic reflex; thus, controlling the intestinal motor responses (De Ponti et al., 1989).

The enteric nervous system that is comprised of a network of neuros and glial cells, including the enteric neuroblasts that can migrate from the vagal level to the gut level, are found to be responsible for coordinating aspects of the gut function (Le Douarin & Teillet, 1973; Nagy & Goldstein, 2017). Some milk-derived peptides, particularly BCMs, once associated with the endogenous opioids, which serve as neuromodulators, produced and secreted by nerve cells neurons, may initiate the activation of opiate receptors (μ-opioid receptor) (section 3.3.2.) (Froehlich, 1997; Mohanty, Mohapatra, Misra, & Sahu, 2016; Tyagi et al., 2020). It has been found that this interaction might affect the central or peripheral nervous systems that are involved in maintaining the hypotension, fluctuating body temperature, lack of appetite, alteration of sexual behaviour, to name a few (Molina & Abumrad, 1994). In this regard, endogenous opioid agonist peptides may influence the development and the function of central nervous system's cells, whilst BCMs might be transported through neonatal mucosal membranes, and consequently regulate physiological responses in infants, resulting in relaxation and sleep (Mohanty et al., 2016). Additionally, BCMs were found to control electrolyte transport, exert anti-secretory action (Daniel, Vohwinkel, & Rehner, 1990), stimulate analgesic behaviour (Matthies et al., 1984), and endocrine responses (secretion of insulin and somatostatin) (Meisel & Schlimme, 1990). This process might happen through changing the epigenetic mechanisms (i.e., how cells control gene activity) in the global DNA methylation patterns (i.e., how methyl groups are added to DNA) or with the production of reactive oxygen species (ROS), and might be related to gastrointestinal issues, discomfort and inflammation, which suggests a "gut - brain" epigenetic relationship (Trivedi et al., 2014, 2015). The systemic oxidative stress and DNA methylation changes have been previously reported in patients suffering of schizophrenia and autism, as a result of tremendously low levels of antioxidant glutathione which synthesis is limited by cysteine (Cys) availability (Chang et al., 2019; Cruz et al., 2021; Frustaci et al., 2012). The epigenetic changes affecting gene pathways in cultured human neuroblastoma cells (i.e., cancer cells) showed that bovine BCM7 was involved in the alteration of epigenetic mechanisms; the authors postulated that those changes were associated with inflammation and gut disease-related metabolic pathways (Trivedi et al., 2015). The way of understanding this finding is a defective positive feedback mechanism, by which the BCMs suppress or decrease the absorption of Cys from the gastrointestinal tract inducing inflammation and gastrointestinal discomfort (Trivedi et al., 2015). Similar results on inflammation and systemic oxidation were suggested by Trivedi et al. (2014) where BCM7 via an opioid receptor-mediated modulation of human colorectal adenocarcinoma (Caco-2) cells affected cellular redox status and abnormal DNA methylation patterns (Trivedi et al., 2014). The redox imbalance caused by increased production of reactive oxygen species

Table 2
Possible disadvantageous effects of β-casomorphins found in human and animal trials *(in vitro* and *ex vivo)*.

Study	Exposure	Intervention (Dosage)	Results	Outcome
Brantl et al. (1979)	β-casomorphin's (BCM's) peptides isolated from β-casein (β-CN) after digestion. Presented: the opioid activity in guinea pig ileum (GPI) and mouse vas deferens preparations (MVD).	Lyophilised bovine casein: purified by adsorption/ desorption with activated charcoal and Amberlite adsorption, gel filtration and HPLC. Chloroform methanol extract prepared. Liberated fractions: tested for opiate activity.	GPI (isolated carboxypeptidase ϒ [CPY]) digestion products, normorphine and methionine-enkephalin, IC_{50} - μmol/L): BCM5 (GPI): 5.0 μmol/L. BCM7 (GPI): 55.00 μmol/L. MVD (isolated carboxypeptidase ϒ [CPY]) digestion products, normorphine and methionine-enkephalin, IC_{50} - μmol/L): BCM5 (GPI): 35.0 μmol/L. BCM7 (GPI): > 500 μmol/L.	The activity of BCM5 and BCM7 was different. It was unclear, whether there was any relationship between the BCMs and other pronase-resistant opioids.
Brantl et al. (1981)	Examination of the opioid activity of BCMs (4, 5, 6: synthetic; and 7 extracted from casein) in *in vitro* tests for an association with opioid receptor. Binding assay (BA) was performed. 1. Isolated mouse vas deferens (MVD: δ receptors vs ε receptors). 2. Guinea-pig ileum longitudinal muscle myenteric plexus preparation (GPI). 3. Naloxone-reversible analgesia in rat vas deferens (RVD): Suppress of ^{3}H-naloxone connecting to opiate receptors in rat brain homogenate.	Determination of opioid activity in isolated organ preparation. 1. MVD (measured IC_{50}): inhibition of electrically induced contractions: proved antagonizable by the opiate antagonist (-)-naloxone, but not by its optical isomer naloxone. 2. GPI: Preparation, mounting and electrical stimulation (frequency: 0.1 Hz, pulses: 60 V, 0.5 ms). 3. RVD: Observation of opioid activity in Opiate receptor binding assays *in vitro*.	Binding assay (BA: Inhibition of naloxone, IC_{50} = μmol/L): BCM4: 2.70. BCM5: 1.10. BCM6: 3.20. BCM7: 14.0. MVD (Inhibition of naloxone, IC_{50} = μmol/L): BCM4: 84.3 ± 12.8. BCM5: 42.1 ± 5.90. BCM6: > 150. BCM7: > 200. GPI (Inhibition of naloxone, IC_{50} = μmol/L): BCM4: 21.9 ± 2.60. BCM5: 6.50 ± 0.60. BCM6: 27.4 ± 1.70. BCM7: 57.0 ± 7.50. RVD (Inhibition of naloxone, IC_{50} = μmol/L): BCM4: < 0.29. BCM5: < 0.18. BCM6: 0.15. BCM7: 0.26.	BCM5 was the most potent in all the assays observed. Each BCM was more potent on the GPI than on the MVD. The first knowledge that BCMs probably represent μ-type opiate receptor agonists.
Hautefeuille et al. (1986)	Examination of the *in vitro* ion transport mechanism of BCM4 and BCM5 in a rabbit ileum and their influence on short-circuit current (I_{sc}) and stimulate intestinal electrolyte absorption.	White rabbits weighing 2.5-3.5 kg were included and segments of distal ileum were used. The serosal and external muscular layers were stripped with fine forceps. Pieces of stripped tissue were mounted between the two halves of a Lucite chamber and bathed on each side by 10 mL of isotonic Ringer solution. Measurement of net sodium (Na) and chlorine (Cl) fluxes between mucosa and serosa.	The serosal external muscular layers Serosal addition: 1. BCM4: up to 10^{-3} M. 2. BCM5: up to 10^{-5} M did not reduce I_{sc}. 3. BCM4: 10^{-4} M caused a transitory enhancement of delivered short circuit current that was not inhibited by (-) naloxone. Serosal administration of both BCM4 and BCM5 indicated a dose dependent, (-)naxlone inhibited reduction in I_{sc}. Serosal addition of the same analogue stimulated absorption of Na and Cl +2.90 ± 0.95 and +2.12 ± 0.60 μeq · h^{-1} · cm^{-2}, respectively, and inhibited residual flux (-1.80 k 0.57 μeg · h^{-1} · cm^{-2}). The data for mucosal addition of BCM4 and BCM5 was not reported.	BCM4 and BCM5 stimulated intestinal absorption of electrolytes by an opioid mechanism. The intestine might absorb certain milk-related opioid peptides.
De Ponti et al. (1989)	The effect of bovine BCM4 and BCM5 on the peristaltic reflex in the guinea pig isolated colon.	10 cm segment of the distal colon was removed and mounted in a 100 mL organ bath, perfused with Tyrode solution (interluminal balloon, every 10 min). The compounds were added to the bath 3 min before distending stimulus. Repetition: 4 times.	BCM4 (IC_{50}): No activity shown on the peristaltic reflux. 1. BCM5 (IC_{50}): 5.21 μM. 2. BCM5 (potency ratios vs morphins): 0.06 and 0.96 μM. 3. Blockade of the peristaltic reflex by BCM5: reversed by the naloxone.	Certain BCMs inhibited intestinal propulsion and cholinergic neurotransmission in the guinea-pig colon, probably by acting at opioid receptors.
Kurek et al. (1992)	Influence of bovine BCM7 of human peripheral leukocytes to liberate histamine, indicating an allergic reaction.	1. 20 mL of peripheral blood was sedimented. 2. Cells incubation: 30 min at 37 °C. 3. 0.1 mM, 1 mM, and 10 mM of BCM7 was applied; determination of % of histamine. 4. Samples were frozen: -70 ^{0}C. 5. Pre-incubation of the cells with naloxone: 10 min at 37 °C.	Histidine release, LDH secretion and trypane blue staining. 1. Histamine (%): BCM7 (10 mM) = 29.3 ± 5.1; BCM7 (1 mM) = 11.9 ± 2.4; BCM7 (0.1 mM) = 8.5 ± 1.6. 2. Stained cells (%): BCM7 (10 mM) = 17.0 ± 5.6; BCM7 (1 mM) = 3.3 ± 0.9; BCM7 (0.1 mM) = 15.5 ± 3.4. Histamine liberation when BCM7 affected the cells (%).	BCM7 can be regarded as noncytotoxic, direct stimulus for liberation of histamine *in vitro*.

(*continued on next page*)

Table 2 (*continued*)

Study	Exposure	Intervention (Dosage)	Results	Outcome
		6. Finally, samples were treated with 0.2 mM, 2 mM, and 20 mM of BCM7: 30 min at 37 °C. n (samples) = 6.	1. BCM7 (20 mM): 69.3 ± 9.3. 2. BCM7 (2 mM): 55.2 ± 12.8. 3. BCM7 (0.2 mM): 44.3 ± 6.3. Histamine liberation when naloxone (0.1nM) was introduced in the cells (%). 1. BCM7 (20 mM): ≈ 69.66 ± 12.5. 2. BCM7 (2 mM): ≈ 44.94 ± 11.0. 3. BCM7 (0.2 mM): ≈ 24.72 ± 13.4.	
Fiedorowicz et al. (2011)	Determination the influence of μ-opioid receptor agonist peptides: bovine BCM7.	Proliferation and cytokine secretion of human peripheral blood mononuclear cells (PBMCs) when treated with BCM7: incubated for 12 h. 1. WST-1 proliferation test. 2. BrdU proliferation test. 3. Determination of cytokines.	WST-1 test (PBMCs): No significant increase of the cellular proliferation resulting from the PBMCs incubation with any of the examined opioids was observed. BrdU test (PBMCs): Significant changes = BCM7 (10^{-12} mol/L) vs control, $p < 0.05$. Cytokines secreted by PBMCs (IL-4; Il-8; IL-13; IFN - γ) = BCM7: 10^{-6}; 10^{-9}; 10^{-12} mol/L vs control, $p < 0.01$. 1. The inhibited level of INF - γ secreted by the PBMCs. 2. An increased level of IL - 4 secreted by the PBMCs ($p < 0.05$). 3. Significant increased level of IL - 8 secreted by the PBMCs ($p < 0.01$). 4. A decreased level of IL - 13 secreted by the PBMCs ($p < 0.05$).	Immunomodulatory effects of BCM7, which may be of clinical significance especially in the case of allergic diseases in newborns.
Dalziel et al. (2014)	The effect of BCM5 on spontaneous contraction. To amplify an *in vitro* muscle contraction model system to assess the effects of BCM5 on intestinal motility.	Distal colon (DC): Influence of BCM5 on spontaneous contractions. DC segment results: mean ± SEM from ≥ 5 preparations, from ≥ 3 animals). Isolated intact whole large intestine (LI): Influence of BCM5 on propagating contractions along isolated whole intestine. LI results: mean ± SEM from 3 - 8 preparations.	DC = BCM5 (20 μM): tension increased by 71 ± 17 % and the frequency doubled (n = 9). These activities were decreased by addition of (-)naloxone (n = 7). LI = BCM5 (20 μM): contractions in rectum increased by 83 ± 11 % (n = 5). These activities were decreased by addition of (-)naloxone.	Short distal colon segments and whole large intestine are able to detect opioid agonist activity that alters colonic motility. These may include gastrointestinal-related problems.
Trivedi et al. (2014)	Examination of the BCM7 mechanism to make alterations in cellular redox status, DNA methylation and transcription by altering cysteine. Repetition: 4 times.	Monolayers cultures 1. SH-SY5Y human neuroblastoma cell lines. 2. Caco2 human colon carcinoma cells lines. Cysteine: 600μl media containing [35 S]-cysteine (1 μCi/1 mL) and 10 μM unlabelled cysteine for 5 min; thiol metabolite assay; DNA methylation analysis.	1. Pre-treatment of SH-SY5Y with BCM7 for 30 min inhibited the uptake of radiolabelled (35 S) - cysteine in a concentration - dependent manner. BCM7 (IC_{50}) = 1.31 nM. Cysteine uptake was reduced with lower potency in Caco2: BCM7 (IC_{50}) = 15.95 nM. 2. Cysteine inhibition by BCM7 was fully developed at 4, 24, and 48 h. 3. BCM7: Transiently decreased methylation, with a 2-3-fold reduction at 24 h ($p < 0.01$). 4. BCM7: Increase genome-wide methylation at the TSS peak to 17 %. 5. BCM7 significantly decreased methylation status: 3891 genes in this TSS region.	Restricted antioxidant capacity, caused by casein (bovine milk) - derived opioid peptides, may predispose susceptible individuals to inflammation and systemic oxidation.
Fiedorowicz et al. (2014)	Cow's milk based diet originating from three different genetic variants: A1/A1, A1/A2, & A2/A2 β-casein. The experiment lasted for 5 days.	Peripheral blood mononuclear cells (PBMCs) from the studied subjects were treated with the peptide extracts of raw BCM7 (1mL solution) extracted from the β-casein genetic variants and incubated for 48 h. 1. Group 1: Healthy children (HC). 2. Group 2: Children with severe form of Atrophic Dermatitis (AD).	μ-opioid receptor gene expression μ-opioid receptor increased levels have been observed in children with severe form of Atrophic Dermatitis (AD) in all examined samples. BCM7 concentration of 1 μg/mL & 1 ng/mL from the three genetic variants of milk presented 2.7 & 5.5 times higher μ-opioid receptor gene expression in AD patient's PBMCs in comparison with HC PBMCs ($p < 0.001$). DPP-4 gene expression The analyses indicated decrease in the DPP-4 gene expression in the AD group in contrast with HC group. BCM7 concentration of 1 μg/mL & 1 ng/mL from all genetic variants presented 1.5 & 2.5 times lower DPP-4 gene expression in AD patient's	The results from this study showed the importance μ-opioid receptor and DPP-4 genes expression by consummation of BCM7 liberated from β-CN and the occurrence of AD symptoms' exacerbation and possibility for further food allergy initiation. In regards of gene expression, no difference between A1/A1, A1/A2, & A2/A2 β-casein has been shown.

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Table 2 (*continued*)

Study	Exposure	Intervention (Dosage)	Results	Outcome
			PBMCs in comparison with HC ($p < 0.05$; $p < 0.001$).	
Trivedi et al. (2015)	Examination of the BCM7 mechanism to make alterations in transcriptional changes of human RNA and DNA.	Monolayers cultures 1. SH-SY5Y human neuroblastoma cell lines treated for 4 h with 1 μM BCM7 and untreated control samples prior to RNA or DNA extraction. DNA and RNA isolation; Examination of differentially expressed transcripts (DETs) and differentially methylated transcripts (DMTs); Site-specific CpG methylation: fragmentation and MBD-capture; Microarray assays; Gene ontology.	Gene expression was analysed by genome-wide microarray; methylated transcripts (DMTs and DETs) 1. BCM7 (DMTs): ~ 197 promoter region DMTs (inflammatory disease). 2. BCM7 (DETs): ~ 1467 promoter region DETs (inflammatory disease and response). 3. Inhibition of cysteine uptake. 4. BCM7: 22 % increase in DNA TSS methylation.	BCM7 caused epigenetic changes affecting gene pathways related to gastrointestinal disease, discomfort and inflammation.
Cieślińska et al. (2015)	Polymorphism of DPP-4 and μ-opioid receptor genes in human cells as a result of the activity of BCM7.	Peripheral blood mononuclear cells (PBMCs) from the studied subjects were isolated and treated with custom synthetised BCM7 (1 ng/mL). Frequency of A118G SNP (single nucleotide polymorphism) in the μ-opioid receptor gene and rs7608798 SNP of the DPP-4 (AG) gene were examined. Three genetic variants: AA, AG, GG within both groups. 1. Group 1: Healthy children (HC). 2. Group 2: Children diagnosed with autism spectrum disorder (ASD). Differences between ASD and HC in the distribution of genetic variants and resultant influence of the analysed SNPs on gene expression.	Gene expression of DPP-4 in PBMCs under the influence of BCM7 1. HC: ≈ 1.07. 2. ASD: ≈ 1.14. Similar level of DPP-4 gene expression in PBMCs: HC & and ASD: $p > 0.05$. Gene expression of DPP-4 in PBMCs under the influence of BCM7 and the correlation with rs7608798 SNP - HC (AA): ≈ 0.98. - HC (AG): ≈ 1.11. - HC (GG): ≈ 1.08. - ASD (AA): ≈ 0.95. - ASD (AG): ≈ 1.32. - ASD (GG): ≈ 1.14. There was only slight increase expression in ASD-affected children with genotype AG for SNP: $p > 0.05$. Gene expression of μ-opioid receptor in PBMCs under the influence of BCM7 1. HC: ≈ 1.83. 2. ASD: ≈ 1.85. Similar level of DPP-4 gene expression in PBMCs: HC & and ASD: $p > 0.05$. Gene expression of the μ-opioid gene receptor in PBMCs under the influence of BCM7 and the correlation with a polymorphic site A118G at AG genotype - HC (AA): 1.70. - HC (AG): 2.49. - ASD (AA): 1.57. - ASD (AG): 1.84. There was an increased expression in HC with genotype AG for SNP: $p < 0.01$.	The study provided new insights into the role of single nucleotide polymorphisms, developed by BCM7, on gene expression that can possibly affect, develop, and enhance the autism symptoms.
Chia et al. (2018)	Five generations of mice were treated with cow's milk powder (A1 or A2 β-casein, 60.53 g/100 g). Duration: 30 weeks.	Four generations, out of F0 generation have been included in the study: F1, F2, F3, & F4 and were fed either A1 or A2 β-casein supplemented diets. a) Peptide extraction from whole blood, lymph tissues and mass spectrometry. b) Bacterial DNA preparation. c) Histological Staining.	Peptide Extraction No BCM7 peptide was detected in either sample type. Intestinal Microbial Communities A1 β-casein: increased the level of several bacterial species (some associated with diabetes, including *Streptococcus pyogenes* and *Streptococcus suis*. In contrast, *Enterobacter cloacae, Enterobacter hormaechei* and *Klebsiella oxytoca*: decerased by A1 β-casein supplemented diet. Gastrointestinal Integrity Neither an A1 nor A2 supplemented	During the *in vitro* A1 β-casein: it slightly influenced the homeostasis and type 1 diabetes incidence. This process requires generations in order to be indicated.

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Table 2 (*continued*)

Study	Exposure	Intervention (Dosage)	Results	Outcome
			diet affected the gastrointestinal integrity of the mice.	
Jarmolowska et al. (2019)	Pasteurized cow's milk based diet (No info on the genetic variant).	PBMCs (5 - 10 mL), serum (2 mL), & urine (15 mL) from the studied subjects were treated with the peptide extracts of raw BCM7 extracted from the milk (20 mL). 1. Group 1: Healthy children (HC). 2. Group 2: Children diagnosed with autism spectrum disorder (ASD).	BCM7 Concentration in patients' serum and urine (mean ± SEM) Higher concentrations of serum BCM7 ($p < 0.001$) occurred among ASD (42.96 ± 5.52 ng/mL) than in HC (26.42 ± 1.63 ng/mL). ASD - boys: 39.52 ± 2.26 ng/mL. HC - boys: 25.5 ± 1.9 ng/mL. ASD - girls: 59.2 ± 9.04 ng/mL. HC - girls: 27.9 ± 2.97 ng/mL. Urine BCM7 did not show any important differences. DPP-4 concentration in serum Higher concentrations of serum DPP-4 ($p < 0.01$) occurred among ASD (1089 ± 44.3 ng/mL) than in HC (934 ± 52.7 ng/mL). ASD - boys: 1050 ± 36.86ng/mL. HC - boys: 922.2 ± 65.04 ng/mL. ASD - girls: 1336 ± 144.0 ng/mL. HC - girls: 953.4 ± 92.14 ng/mL. Urine BCM7 did not show any important differences. DPP-4 Gene Expression in PBMCs Tendency to increase the DPP-4 gene expression among girls with autism has been observed.	The research proved that BCM7 released from cow's milk & DPP-4 are potentially factors in determining the pathogenesis of autism among children.
Asledottir et al. (2019)	The effect of BCM7 on human GI juices and porcine jejunal membrane peptidases. Commercial enzymes (pepsin and trypsin) were used.	*In vitro* digestion (INFOGEST). Parrale digestion: 0.5 mL of BCM7 dissolved in distilled H_2O (4 mg/mL). Incubation: 180 min - 24 h. Gastric phase sampling: after 60 min. Intestinal phase sampling: 120 min.	Prediction of bioactive peptides based. 1. BCM6 (Score): 0.948. 2. BCM7 (Score): 0.917. Peptides with scores above 0.5 were considered bioactive. BCMs: ACE inhibitor = angiotensin-converting enzyme inhibitor; DPP-4 inhibitor = DPP-4 inhibitor. After 2h brush border membrane digestion, more than 40 % of BCM7 was degraded, and only small amounts (5 %) of BCM7 were still intact after 24-h brush border membrane digestion.	Not concluded if the small amount of intact BCM7 detected after *in vitro* digestion was carried via active transceptors in the human intestinal epithelial cells the blood system.

was found to induce oxidative stress and disruption of redox signalling, thus leading to a potential cause of highly specific acute and chronic changes in the cell phenotype. Contrarily, DNA methylation is a significant contributor to epigenetic alterations in gene expression (Kim, Ryan, & Archer, 2013). In this regard, the abnormal DNA methylation patterns have been linked to numerous neurological conditions, such as schizophrenia, autism, immunodeficiency, facial abnormalities syndrome, which might affect the barrier properties of intestines (Trivedi et al., 2014). While these findings showed that BCMs might interact with the mechanisms that can regulate DNA methylation, it is still unknown how these bovine milk-derived peptides target specific genes or, alternatively, if these changes are even possible or realistic in a human gut under *in vivo* conditions.

The scientifically proven evidence from *in vitro* and *ex vivo* studies provided to date and discussed as part of this review supports a likelihood that BCMs might be found in the intestinal barrier. However, their possible transport into the blood circulatory system through the action of the brush border enzymes and passive and active translocation systems is still inconclusive and no evidence has been shown in the literature. This may lead to an indirect judgement that the presence of BCMs may only have a localised effect on gastrointestinal function.

3.4.2. *Potential health benefits of β-casomorphins*

Despite reports of the possible link between BCMs and an increased prevalence in chronic diseases, their positive effects on animal and human cells, such as lowering oxidative stress or modifying intestinal mucins, have been reported in the literature (Table 3). Indeed, after intestinal absorption, BCMs appear to be involved in improving gastrointestinal protection by regulating intestinal mucus discharge (Trompette et al., 2003). Additional mucin secretion initiated by BCMs, especially BCM7, could potentially protect the gut against enteric pathogens (Trompette et al., 2003). Furthermore, Zoghbi et al. (2006) found that BCM7 may also act directly on the intestinal goblet cells by increasing the expression of the mucin gene, leading to increased mucin secretion. This secretion of mucin may be a protective response initiated in the intestinal goblet cells to defend or preserve the intestine against an inflammatory reaction (Zoghbi et al., 2006). Another study (Zhang et al., 2013) showed that BCM7 enhanced the antioxidant status in proximal tubular epithelial cells by removing free radicals and preventing high glucose-induced renal oxidative stress. Additionally, BCM7 significantly reduced angiotensin II (Ang II) in cell culture lines and led to increase in insulin excretion and suppression of glucagon, which is the main regulator of hyperglycaemia and diabetic heart disease (Zhang et al., 2013). BCM7 also has the potential to inhibit epithelial-mesenchymal transition

Table 3
Potential beneficial effects of β-casomorphins found in human and animal trials *(in vitro* and *ex vivo)*.

Study	Exposure	Intervention (Dosage)	Results	Outcome
Trompette et al. (2003)	The effect of BCMs on rat mucus discharge after luminal or arterial administration in isolated vascularly perfused rat jejunum. Examination of mucus production.	Isolated vascularly perfused rat jejunum in isotonic saline: filled with a bolus of 0.8 mL of pre-warmed isotonic saline (control) or a solution (pH 7-7.5, 5 osmolality 300 mosmol/kgH_20) containing $1.2 \cdot 10^{-4}$ mol/L BCM3, BCM4, BCM6, and BCM7 at 37 °C, incubated for 30 min. n (samples) = 6.	1. BCM7: induced a strong mucin secretion = 555 ± 54 % of control. The effect was reversed by intra-arterial administration of (-) naloxone (≈ 170 %). 2. BCM4 and BCM6: Mucin secretion was not induced, neither by naloxone nor intraluminal administration. 3. Intra-arterial BCM3: Mucin production was not increased. 4. Intra-arterial BCM4: Mucin production increased. 5. Intra-arterial BCM7: Large stimulatory effect on mucus secretion ($p < 0.05$).	BCMs after absorption modulate intestinal mucus discharge. They can be involved in defence against noxious agents and could have dietary and health applications.
Kampa et al. (1997)	The importance of BCM in the inhibition of the proliferation of human prostate cancer cell lines.	Three human prostate cancer cell lines, LNCaP, PC3 and DU145 with different receptor profile for a series of hormones and growth factors were used. In the whole prostate cancer cells ligand binding assays were performed.	LNCaP cell line BCM 1 - 5: produce a dose dependent and partially reversible inhibition of cell growth. BCMs: produce a dose-dependent inhibition of cell growth, which was potentiated by the addition of the antagonist. PC3 cell line BCMs 1 - 5: antiproliferative action was bolstered by the antagonist. BCMs: produce a reversible inhibition of cell growth. DU145 cell line BCMs 1 - 5: no data on its mechanism. BCMs: Antiproliferative action was reversed by diprenorphine.	BCMs inhibited cell proliferation of human prostate cell lines, also by the action partly involving opioid receptors.
Zoghbi et al. (2006)	Influence of BCM7 on *in vitro* mucin producing cell lines (elaboration of the function of intestinal goblet cells). Included control cells (CC) and treated cells (TC). Repetition: 3 times.	Rat and human colon cell culture lines 1. DHE (mucin - producing rat colon adenocarcinoma line). 2. HT29-MTX (human colon carcinoma-derived mucin-secreting goblet cell line). Treatment 1. CC: not treated with BCM7. 2. TC: Addition of BCM7 10^{-6} to 10^{-4} M to the cell cultures. Incubation: 30 min - 24h. Identification and measurements: μ-Opioid receptors - visually in the cell lines; mucin-like glycoproteins; rat and human mucins; mucins and μ-opioid receptor cDNAs; cyclophilin. Blockage of μ-opioid receptor: cyrodime.	DHE 1. BCM7 = 10^{-4} M (2 - 8 h incubation): increased mucin-like glycoprotein secretion from = 227 ± 12 % of control. 2. BCM7 = 10^{-4} M (8 h incubation): the level of gene expression was approximately 200 % of control. 2. BCM7 = 10^{-4} M (24 h incubation): Increase in rMuc2 (183 ± 14 % of control) and rMuc3 (172 ± 8 % of control) mRNA levels; not modify rMuc1, rMuc4 or rMuc5AC levels. HT29-MTX 1. BCM7 = 10^{-4} M (8 h incubation): induced mucin-like glycoprotein secretion (163 ± 9 % of controls). 2. BCM7 = 10^{-4} M: secretion of mucin MUC5AC (169 ± 3 % of controls). 3. BCM7 = 10^{-4} M (24 h incubation): secretion of mucin MUC5AC (176 ± 14 % of control).	Possibility: BCM7 can modulate intestinal mucins through a direct effect on goblet cells. BCM7 can possibly improve gastrointestinal protection in the neonate but also in the adult.
Zhang et al. (2013)	The effect of BCM7 on the oxidative stress in proximal tubular epithelial cells (NRK-52E) exposure to high glucose (HG) by using biochemical methods: HBCM7: (high glucose plus 10^{-5} mol/L BCM7). MBCM7(high glucose plus 10^{-7} mol/L BCM7). LBCM7(high glucose plus 10^{-9} mol/L BCM7).	1. NRK-52E cell line: Dulbecco's modified Eagles medium; 37 °C in a humidified atmosphere of 5 % CO_2. 2. High Glucose media: 30 mmol/L D glucose. 3. Normal glucose DMEM media: 24.5 mM mannitol. 4. BCM7 (10^{-5}/10^{-7}/10^{-9} mol/L): added to the cell cultures 30 min prior to changing the medium to a high glucose medium. 5. Losartan: added to cell cultures 15 min prior to changing the medium to a high glucose medium. Repetition: 3 times.	BCM7 reduced HG-induced oxidative stress in NRK-52E Treatment of the cells with BCM7 (10^{-5}/10^{-7}/10^{-9} mol/L): blunted the changes of SOD, GPx, and MDA of NRK-52E cells in a concentration-dependent manner. Superoxide dismutase HBCM7 (10^{-5} mol/L): ≈ 5.90 U/mg protein. MBCM7 (10^{-7} mol/L): ≈ 4.76 U/mg protein. LBCM7 (10^{-9} mol/L): ≈ 4.57 U/mg protein. Glutathione peroxidase HBCM7 (10^{-5} mol/L): ≈ 223 units/mg protein.	BCM7 would alleviate high glucose-induced renal oxidative stress *in vitro*: May be associated with down regulation of the concentration of Ang II partly.

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Table 3 (*continued*)

Study	Exposure	Intervention (Dosage)	Results	Outcome
			MBCM7 (10^{-7} mol/L): ≈ 187 units/mg protein. LBCM7 (10^{-9} mol/L): ≈ 133 units/mg protein. Malondialdehyde HBCM7 (10^{-5} mol/L): ≈ 17.1 μmol/mg protein. MBCM7 (10^{-7} mol/L): ≈ 21.1 μmol/mg protein. LBCM7 (10^{-9} mol/L): ≈ 25.2 μmol/mg protein.	
Zhang et al. (2015) Effect of BCM7 on HG-induced changes of TGF-β1 BCM7 was able to suppress the expression of TGF-β1 mRNA.	The effect of BCM7 on the oxidative stress in proximal tubular epithelial cells (NRK-52E) exposure to high glucose (HG).	1. Epithelial-mesenchymal transition (EMT) of NRK-52E cell line: Dulbecco's modified Eagles medium; 37 °C in a humidified atmosphere of 5 % CO_2, 24h. 2. High Glucose media: 30 mmol/L D glucose. 3. Normal glucose DMEM media: 24.5 mM mannitol. 4. BCM7 (10^{-5}/10^{-7}/10^{-9} mol/L): added to the cell cultures 30 min prior to changing the medium to a high glucose medium. 5. Losartan: added to cell cultures 15 min prior to changing the medium to a high glucose medium. 6. Naloxone (10^{-5} mol/L was added to cell cultures and treated for 72 h. Repetition: 3 times.	Changes of Ang II in the culture medium BCM7 treatment reduced Ang II significantly in the culture medium of NRK-52E cells exposure to HG. Ang II HBCM7 (10^{-5} mol/L): ≈ 76.2 pg/mL. MBCM7 (10^{-7} mol/L): ≈ 78.1 pg/mL. LBCM7 (10^{-9} mol/L): ≈ 80.0 pg/mL. BCM7 has the potential to inhibit high glucose-induced renal proximal tubular EMT partly by modulating AngII-TGF-β1 pathway, but not by opioid receptor (proven by the naloxone).	Effect of BCM7: HG-induced changes of gene about EMT Treatment of the cells with BCM7 (10^{-5}/10^{-7}/10^{-9} mmol/L) obviously blunted the phenotypic conversion of NRK-52E cells in a concentration-dependent manner. Effect of BCM: HG-induced changes of protein about EMT BCM7 reversed the decrease in E-cadherin and increase in α-SMA in a concentration-dependent manner in NRK-52E cells treated with high glucose. Effect of BCM7: HG-induced change of Ang II BCM7 treatment reduced the concentration of Ang II significantly in the culture medium of NRK-52E cells exposure to HG.
Zhu et al. (2018)	Protective effect BCM7 in oxidative stressed human lens epithelial cells (HLECs).	1. HLECs (SRA01/04): prepared in 1 % antibiotic solution; at 37 °C and atm. 5 % CO_2 and 95 % air. 2. Glucose: 5, 10, 20, 30, 40, and 50 mM, for 12, 24, and 48 h; 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolimol/ L bromide (MTT) to each well; Incubation: 4 h at 37 °C. 3. BCM7 (10^{-4}/10^{-5}/10^{-6}/10^{-7} /10^{-8} mol/L): added to the cell cultures 30 min and cultivated for 48 h. 4. MTT: each well and incubated the cells for 1 h at 37 °C.	ROS and MDA (amount of ROS and MDA to the same protein content of the same cells) Pre-treatment with BCM7 (10^{-5} mol/L) significantly decreased the levels of: ROS ≈ 68.7 compared to HG (40 mM) ≈ 107. MDA ≈ 6.76 compared to HG (40 mM) ≈ 18.0. SOD (amount of SOD to the same protein content of the same cells) SOD increased when BCM7 (10^{-5} mol/L) increased: SOD (BCM7) ≈ 59.8 compared to SOD (HG) ≈ 20.3. Foxo1 total (FT) and Foxo1 nuclear (FN) (expression of relative proteins) FT (BCM7) vs FT (HG) = slight increase ($p < 0.05$). FT (BCM7) ≈ 5.31 compared to FT (HG) ≈ 0.23.	BCM7 protects HLECs against oxidative stress by reducing the expression of ROS and MDA, strengthening the expression of Foxo1 nuclear transfer. Increased level of Sp1 and the decreased mechanism of SOD, and GSH-px.
Sheng et al. (2020)	The influence of BCM7 on myocardial hypertrophy (MH) in hyperthyroidism-induced cardiomyopathy (hyperthyroid heart disease - HHD).	H9c2 cells, digested with trypsin and cultured for 1-2 days. Control group (CG): 10 % fetal bovine serum (FBS). Model group (MG): treated with 2 μM of L-Thy for 24 hours.	CG vs MG: Expressions of p65 and P-p65 in the MG drastically increased; expression of IkBα decreased. MG vs BG: Expressions of p65 and P-p65 in the BG significantly lower than	BCM7 may decline cell damage by blocking the over-activation of NF-κB pathway, which may be an important target for HHD.

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Table 3 (*continued*)

Study	Exposure	Intervention (Dosage)	Results	Outcome
		BCM7 group (BG): H9c2 cells were previously cultured with 10^{-6} mol/L BCM7 for 6 h, and then co-cultured with L-Thy-containing medium for 24 h.	those in MG; expression of IkBα drastically increased. NF-kB mRNA relative level CG: ≈ 0.98. MG: ≈ 2.08. BG: ≈ 1.62.	
IKB-α mRNA relative level CG: ≈ 1.01. MG: ≈ 0.35. BG: ≈ 0.72.				

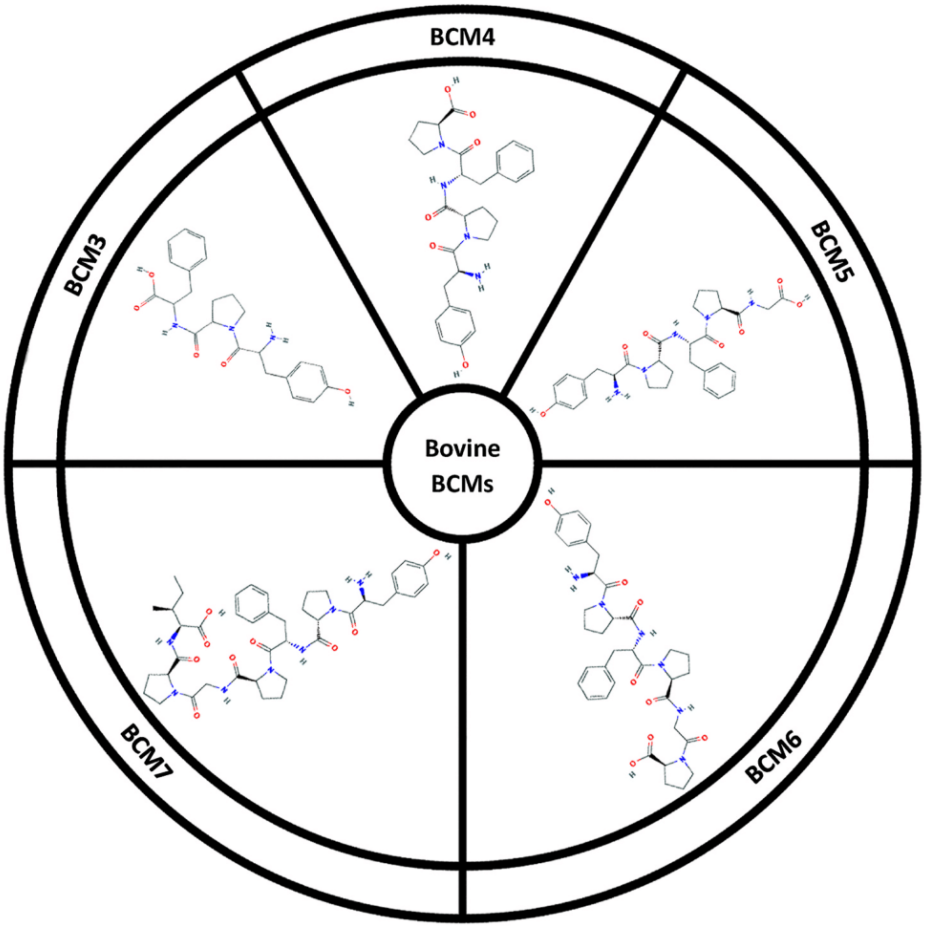

Fig. 2. The chemical structure of the bovine β-casomorphins (BCMs) included in this study.

partly by modulating the Ang II pathway and inhibiting the expression of the transforming growth factor β1 (TGF-β1). This mechanism indicates the likelihood of BCM7 to protect against the incidence of hyperglycaemia and renal fibrosis in diabetic kidney cell lines (Bosco et al., 2013; Zhang et al., 2015). The therapeutic effect of BCM7 has also been shown on human lens epithelial cells by protecting them against oxidative stress (Zhu et al., 2018). A decrease in cell expression of ROS and oxidant malondialdehyde (MDA) was observed, while a concomitant increase was seen in the expression of Foxo1 total nuclear transfer, which is vital for cellular function (Zhu et al., 2018). Recently, BCM7 has been shown to have potential in preventing and treating myocardial hypertrophy by blocking the over-activation of the Nuclear Factor (NF-κB) mediated signalling pathway in H9c2 cells (derived from embryonic rat cardiomyocytes), which may be an important target for hyperthyroid heart disease, likely by stimulating the μ-receptors found in the aforementioned cells (Sheng et al., 2020). Previously Kampa et al. (1997) outlined the receptor-mediated action of BCM5 derived from bovine β-CN which inhibited the cell proliferation of three human prostate cancer cell lines. The maximum inhibition of the prostate cancer cell lines initiated by the action of BCM5 alone or coupled with an opioid antagonist (diprenorphine) and partly involving opioid receptors, was ranging between 30% and 52%. Notably, BCM5 alone had less pronounced effect compared to BCM5 in conjunction with the opioid antagonist.

Another potential area of interest may be BCMs' ability to enhance satiety, based on their activity to bind to μ-receptors. Milk-opioid peptides, including BCMs, showed the ability to modify the endocrine activity of the pancreas by increasing insulin output (Kitts, 1994; Thiruvengadam et al., 2021). Moreover, as a result of physiological stress and anxiety, BCMs used as sleep inducers have been shown to

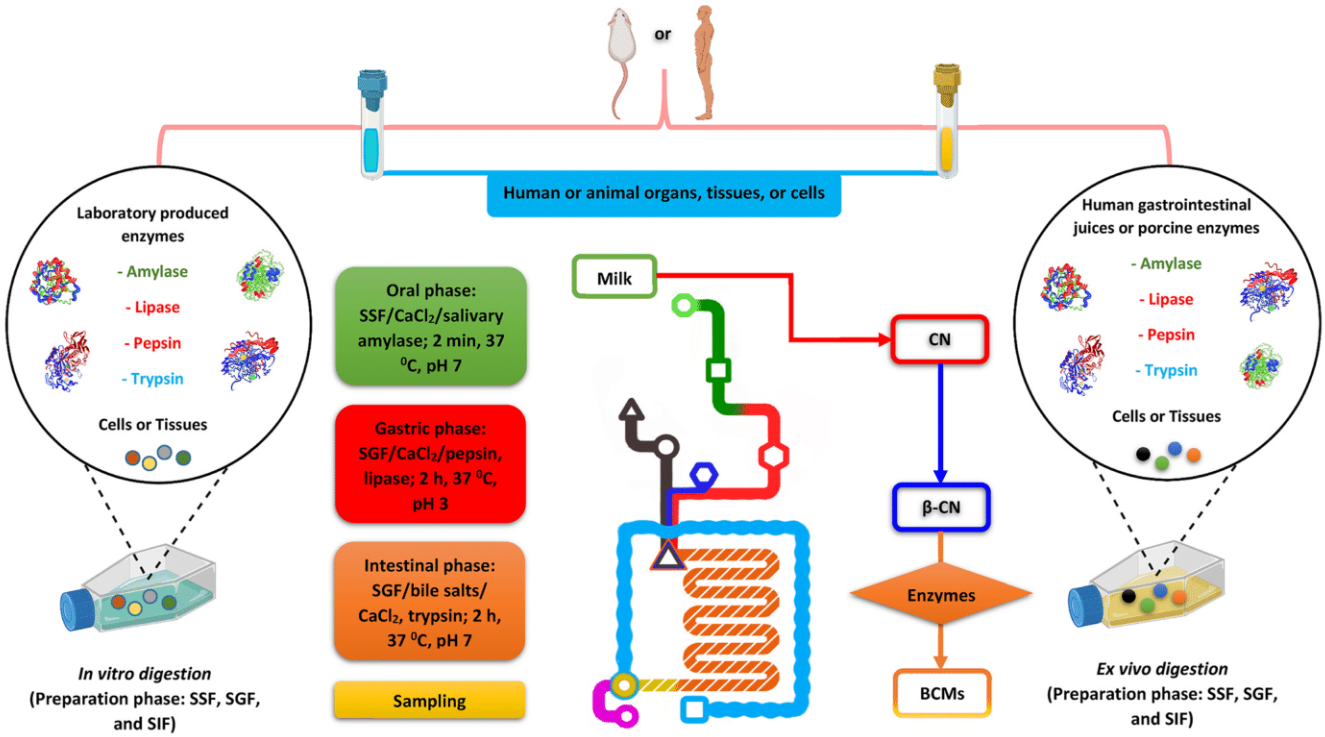

Fig. 3. Overview and flow diagram of the static and semi-dynamic *in vitro* and *ex vivo* digestion procedures proposed by COST INFOGEST. SSF (Simulated Salivary Fluid), SGF (Simulated Gastric Fluid), and SIF (Simulated Intestinal Fluid). Adapted in part from Asledottir et al. (2017); Asledottir et al. (2018); Brodkorb et al 2019; Minekus et al. (2014); Mulet-Cabero, Egger, et al. (2020).

promote a decrease in blood pressure in neonate rats suffering from insomnia, keeping them calm and more relaxed (Teschemacher et al., 1997; Thiruvengadam et al., 2021). In fact, after an intraperitoneal injection of BCMs in to 7-day old rats they exerted an analgesic effect on the nervous system, causing significant changes in sleep pattern, resulting in calmness (Taira, Hilakivi, Aalto, & Hilakivi, 1990). Specifically, the ratio of quiet state (defined as no body movement except for the occasional startle) to total recording time increased, and the percentage of movement during sleep decreased, demonstrating BCMs' behavioural effects and changes in sleep patterns in male rats (Taira et al., 1990). Overall, it appears that BCMs may have potential beneficial effects in the prevention and remedy of certain infections, illnesses, and diseases. Nevertheless, since the results presented are from a limited number of *in vitro* studies, more *in vitro* and *in vivo* work is warranted.

4. Strength and limitation of the present review - Are *in vitro* and *ex vivo* methods suitable for examining the effects of bovine β-casomorphins on human health?

The scientific data described in this review can be categorised as a combination of three related approaches. Definitive: where the reported results are shown to be repeatable in various investigatory settings (*in vitro* and *ex vivo* models*)*. Developing: where significant results have been obtained, but repeatability of the findings under a number of other laboratory conditions is required before the data can be fully substantiated (*in vitro* and *ex vivo models* reflecting the *in vivo* approach). Finally, implicit: where the results were based on the derivations, assumptions, and theories obtained from the analysis of synthetically manufactured BCMs (e.g., protective effects of BCM7). One essential finding of this comprehensive review is the lack of detailed information that can explain the conditions, which may influence the behaviour of β-CN in the gastrointestinal tract. On the contrary, the simplicity of *in vitro* and *ex vivo* models allows one to rapidly screen many samples, and provide some mechanistic understanding of the processes involved. Promising candidate formulations can then be tested using more sophisticated *in vitro* or *ex vivo* models, animal feeding studies, or human trials. In fact and in spite of their limitations, *in vitro* and *ex vivo* models are particularly suited for investigating the pathway of milk proteins and their peptides in dairy products, matters of bioaccessibility, and some aspects of bioavailability (Asledottir et al., 2017, 2019; Dalziel et al., 2014; Mulet-Cabero, Torcello-Gómez, et al., 2020; Nguyen et al., 2021). The simulated *in vitro* and *ex vivo* digestion approaches can be divided into three phases: preparation, digestion procedure (oral, gastric, and intestinal phase) and sample treatment with subsequent analysis. They are adopted and reproduced from Brodkorb et al. (2019), Mulet-Cabero, Egger, et al. (2020), Minekus et al. (2014) and they are summarised in Fig. 3. What seems to be conclusive is that after the digestion of β-CN using laboratory produced enzymes, porcine enzymes, or human gastrointestinal enzymes, leads to the release of BCM7, however, its presence alone is not indicative of its fate in the gastrointestinal system (Osborne et al., 2014). Hence, BCM7 existence in the gut may initiate inflammation and prolonged gastrointestinal transit time, which may lead to reduced activity of membrane-bound DPP-4. Thus, the enzyme may start interacting with other hormones, neuropeptides, and chemokine, which might potentially alter the human microbiome causing bacterial changes, prolonged lactose fermentation, and overall ability to cleave BCMs (Barnett et al., 2014; Olivares, Schüppel, et al., 2018; Pal et al., 2015). These physiological effects contribute to the common adverse effects similar to those induced by the lactose intolerance, including bloating, abdominal cramps, and diarrhoea (Catanzaro, Sciuto, & Marotta, 2021). Notably, the clinically proven results of the relation between BCMs and lactase activity have not been found neither within *in vitro/ex vivo* nor *in vivo* human and animal studies (Daniloski, Cunha, et al., 2021). Moreover, if ingested, BCM7 can still be hydrolysed into some active forms by brush border peptidases, into BCM5, tetrapeptides, tripeptides, dipeptides, or fragments before accessing the chosen organ or the human plasma (Asledottir et al., 2017). Contrarily, if BCMs are not hydrolysed (proline-rich sequence, Pro^{61} - Phe^{62} - Pro^{63}, renders the BCMs resistant to proteases), but translocated across the epithelium, they present a structural stability that could be the origin of

potential immunomodulatory activity of BCMs (Asledottir et al., 2019); their existence would trigger the activation of serum DPP-4 and consequently BCMs clearance from the blood (Fig. 4). For these reasons, BCMs' penetration in the internal human organs within healthy individuals, excluding those with "leaky gut" or other medical conditions, is highly unlikely (Jarmołowska et al., 2019; Mulvihill & Drucker, 2014).

The *in vitro* and *ex vivo* studies examined in this review, have observed the presence of BCMs in human and animal cells, tissues and organs, and their ability to bind to the μ-opioid receptors, which indicates that their potential biological involvement cannot be neglected. Currently, there is insufficient evidence to fully support either the positive or the negative attributes of BCMs. The present results in the literature from the aforementioned studies contain scarce information or not completely described results to support the evidence if BCMs are really important, disadvantageous, or have no significance to human health (Brooke-Taylor et al., 2017). Further research is required to establish the complete effects of BCMs on human health taking into consideration different population cohorts and dietary traits.

5. Concluding remarks

Scientific and industrial communities are engaged in an ongoing debate over the impact of BCMs, particularly BCM5 and BCM7 on human health. While the aim of the review was not only focused on answering the possible therapeutic and unfavourable effects of BCMs, its intent was also to address the pitfalls of using only *in vitro* and *ex* vivo approaches, and the influence and fate of BCMs in human and animal cells, tissues, and organs. In particular, the effects and the relative activity of BCMs are dependent on their binding affinities to the μ-opioid receptor and ability to translocate across the intestinal barrier in their active form. Subsequently, although *in vitro* and *ex vivo* digestion protocols are efficient, useful, and highly utilised within the research community, it should be remembered that these digestion protocols are limited to the intestinal process without simulating the enzymes of the brush border membrane and the final absorption of nutrients. It is also difficult to compare with *in vivo* conditions during the final stages of digestion. Thus, the origins of the reported negative effects and therapeutic impacts of BCMs on the human and animal cells, tissues, or organs remain unclear, while the mechanism for their release from their precursor is not well determined, and their identification remains a contested issue.

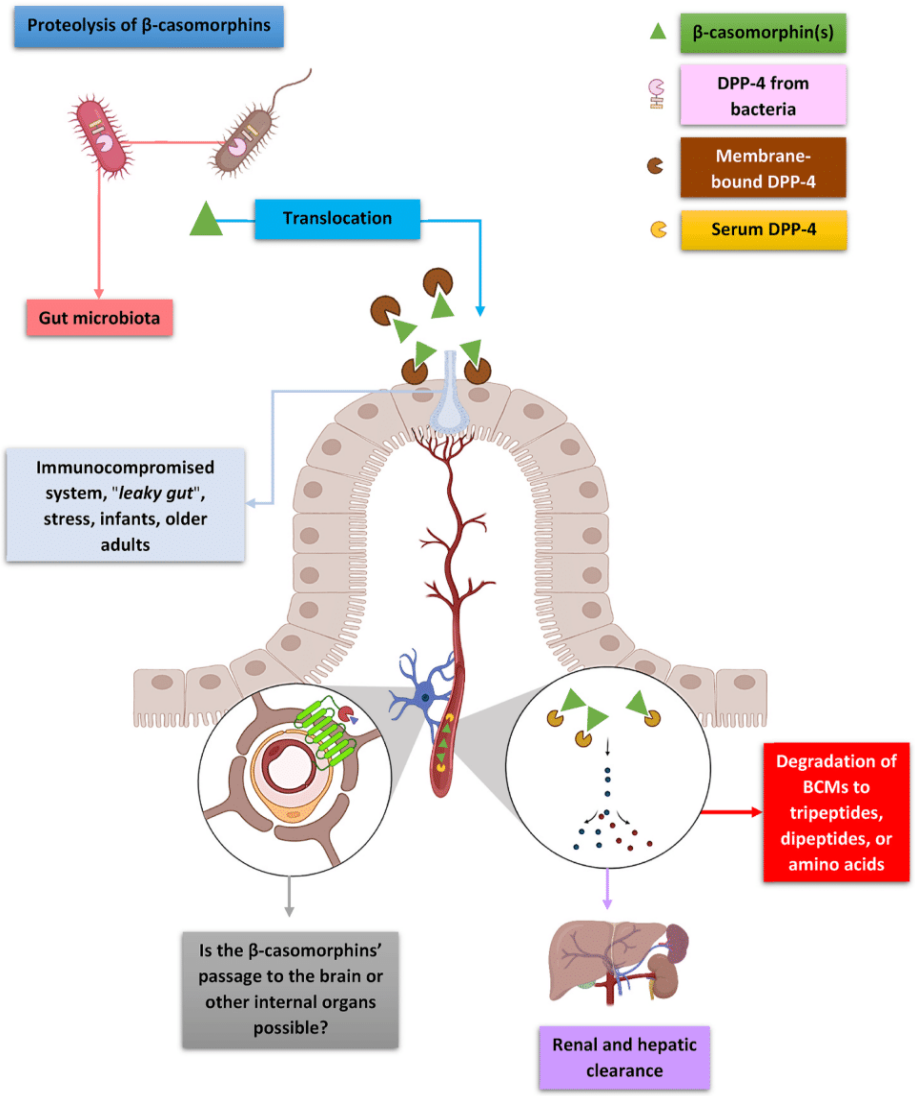

Fig. 4. A schematic model of possible scenarios in which gut microbiota-produced DPP-4, membrane-bound DPP-4 (enterocytes) and serum DPP-4 (endothelial cells) might influence human health during proteolysis of β-casomorphins.

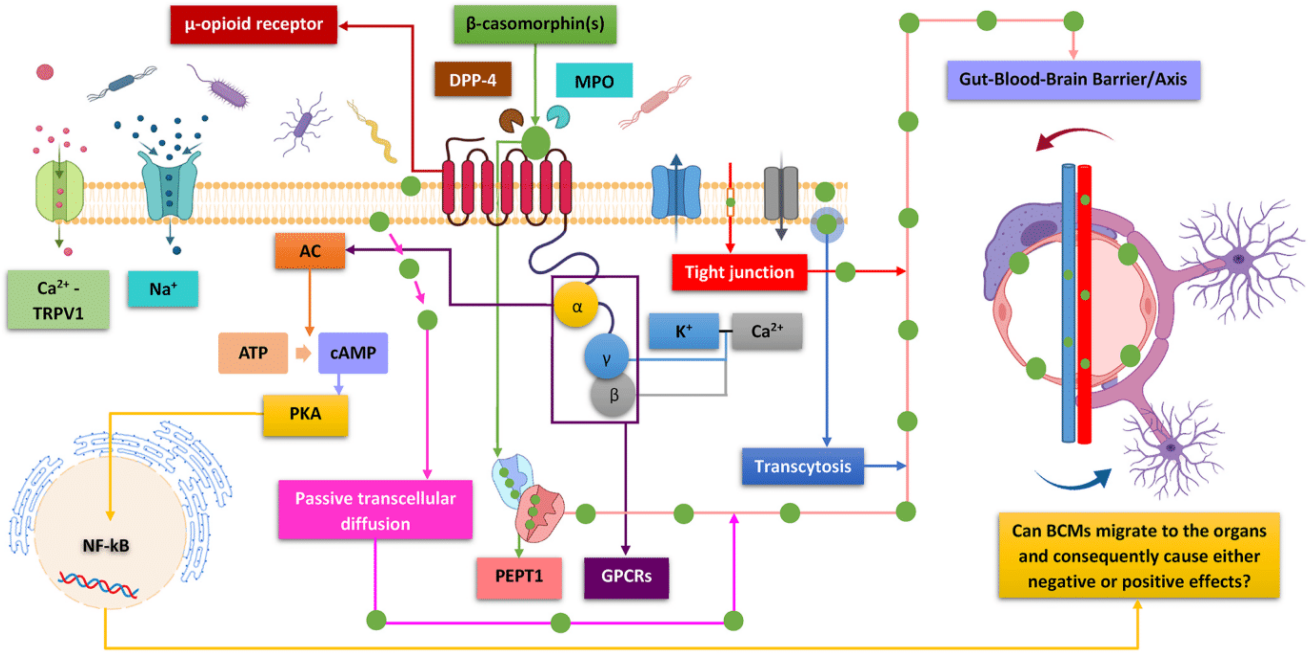

Fig. 5. Transport and bioactivity β-casomorphins. The blood-gut-brain axis and β-casomorphins (BCMs) liberation and transport. Transient receptor potential cation channel subfamily V member 1 (TRPV1); Dipeptydil peptidase - 4 (DPP-4); Myeloperoxidase (MPO); Potassium (K^+), Calcium (Ca^{2+}), Sodium (Na^{2+}) channels; Adenylyl cyclase (AC); Adenosine triphosphate (ATP); Cyclic adenosine monophosphate (cAMP); Nuclear Factor NF-κB (NF-kB); Protein kinase A (PKA); G-protein coupled receptors (GPCRs: α, β, and γ subunits); Peptide transporters (PEPT1). Adapted in part from Kodukula and Zeng (2018); Listos et al. (2019); Tyagi et al. (2020).

The context and final viewpoint of the BCM 'conundrum story' is that categorising and classifying BCMs still remains a significant challenge for both the research and industrial communities. Consequently, longer intervention studies (preferably *in vivo*) with relevant outcomes are essential to provide an adequate and comprehensive understanding on the health impact of BCMs and their release and degradation in milk and dairy products.

Author contributions

Davor Daniloski conceived the study, research question, and designed the review. Davor Daniloski and Todor Vasiljevic found the studies and the final inclusion criteria was checked by Noel A. McCarthy. Davor Daniloski wrote the original draft, conceptualised, reviewed, edited the manuscript and designed the figures and tables. Todor Vasiljevic and Noel A. McCarthy provided critical feedback and analysis, secured funding, reviewed and edited the manuscript and supervised the study. All authors have contributed to the manuscript and reviewed the final version.

Data availability statement

The data used, analysed and elaborated in this review have been stated in figures and tables within the manuscript.

Declaration of competing interest

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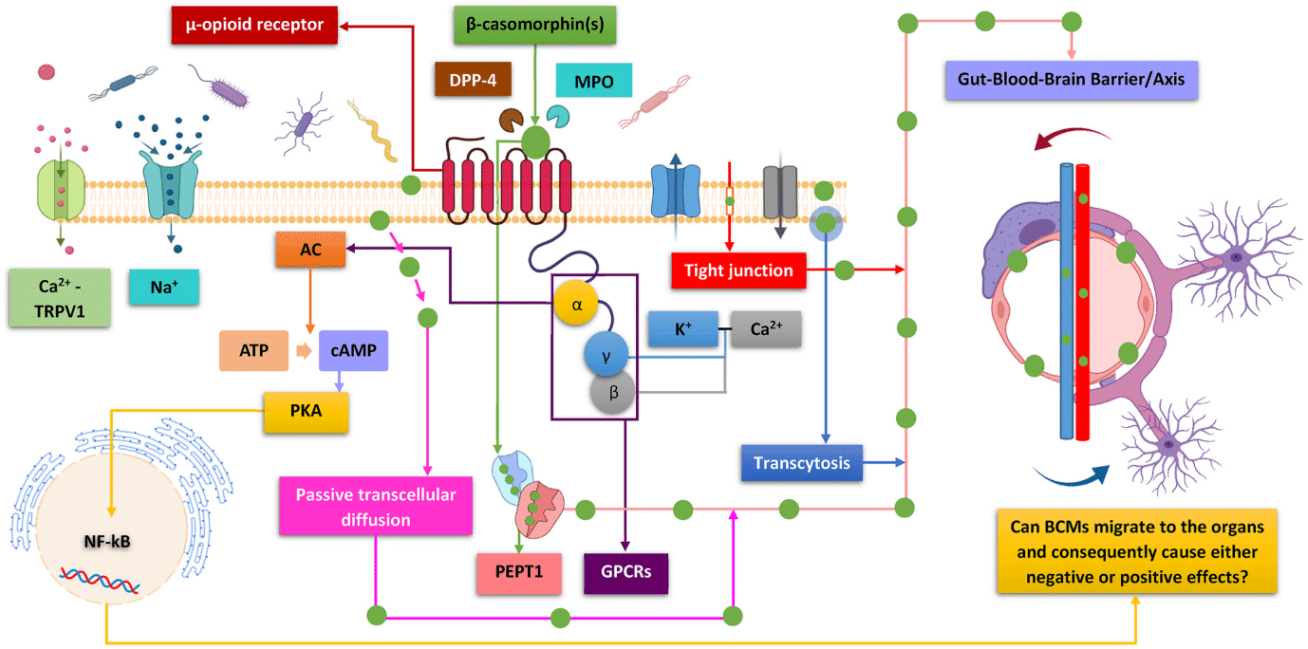

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Conclusions and future perspectives. Chapter 14.

14.1. Scientific and industrial impact of the research

Over the last century, the genetic selection in dairy cattle have significantly evolved, nowadays, the emphasis is on improving milk production, composition, and yield (Miglior et al., 2017). In this regard, numerous studies found that searching for a specific casein genotype, might positively influence milk composition and dairy production traits (Aleandri, Buttazzoni, Schneider, Caroli, & Davoli, 1990; Comin et al., 2008; Ikonen, Ojala, & Ruottinen, 1999; Marziali & Ng-Kwai-Hang, 1986; Ng-Kwai-Hang, Hayes, Moxley, & Monardes, 1986; Penasa et al., 2010; Ristanic et al., 2024).

From a scientific perspective, this thesis provided new insights into the effects of β-casein phenotypes on the structure and composition of milk and dairy products. By using advanced spectroscopic techniques, the thesis established a novel method that demonstrated how different β-casein variants affected the structure-functional properties of casein, milk, and dairy products.

On a commercial level, this research is valuable to the dairy industry as it provides knowledge on the possible impacts of changing national milk pools to the β-casein A2 variant. The study shows that A1/A1 and A1/A2 milks have significantly different heat coagulation properties compared to A2/A2 milk, which is less heat stable. This information might tailor decisions by dairy processors when producing sterilised milk or milk powders. These differences are also observed in acid- and rennet-induced gels, as well as in yoghurt and cheese made from different milk types, with A2/A2 milk showing the poorest gelation properties and more porous gel structure. The findings suggested that while A2/A2 milk may have disadvantages in terms of processability for cheese and yoghurt production, it may have advantages due to its proposed and alleged easier intestinal digestibility. This information is crucial for dairy processors and can help them make informed decisions about the types of milk they select for various dairy products, and, possible health implications.

14.2. General summary and conclusions

The overall research question addressed in this thesis was: What is the impact of a single amino acid mutation in the β-casein sequence on the casein micelle structure and techno-functional properties of dairy products? **Chapter 2** summarised the research conducted in recent years and highlighted the distinct structure-functionality differences between β-caseins A1 and A2. While isolated from the casein micelle but also purified, β-casein A2 tends to form smaller micelles compared to β-casein A1, it is evident that A2/A2 milk tend to have larger average

micelle sizes as opposed to those milks with β-casein A1. This information, hence, highlighted the urge for understanding the factors influencing these protein variations in the milk system, rather than examine them as standalone proteins.

The first research study, as described in **Chapter 3** summarises in detail the findings and key determinants of individual casein, but particularly the involvement of β-caseins A1 and A2 in the structuring of the casein micelle. Using one- and two-dimensional Fourier Transform Infrared (FTIR) and Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopies we were able to predict the structure of casein molecules at pH levels ranging from 6.7 to 2.3. In A1/A1 casein micelle, intramolecular β-sheets and α-helixes were predominant conformational motifs, giving a greater stability of the molecule compared to those micelles with β-casein A2. Conversely, A1/A2 and A2/A2 caseins showed dominance of β-turns, aggregated β-sheets, but especially PPII helixes, primarily influenced by β-casein and the presence of an additional proline in their structure (which was the only difference among the samples). Interestingly, at pH 5.7, all samples exhibited similar behaviour, raising questions about the behaviour of these phenotypes during cheese processing or the final phase of *in vitro* human digestion.

In Chapters 4 and 5 milk samples carrying either β-caseins A1/A1, A1/A2, or A2/A2 were exposed to cold, ambient, pasteurisation, and ultra-high temperature conditions to determine to what extent the β-casein phenotypes might rule the differences among the milk samples. In both chapters, mainly β- and κ-casein appeared to be continually impacted by temperature effects, which may potentially be attributed to a change in the mineral balance towards the micellar phase at higher heat treatment temperatures or dissociation of β-casein out of the casein micelle as a consequence of reduced hydrophobic attraction at 4 °C. Hence, the amino acid mutation and decreased κ-casein content in A2/A2 milk might lead to an increased micelle size, lower net negative charge, and decreased amount of minerals compared to the other milks with β-casein A1. Additionally, both, FTIR and NMR spectroscopies, showed a potential to depict conformational differences among the milk samples, thus giving an indication that both protocols might easily be adopted in the control of industrial processes in a very near future.

By realising the power that both FTIR and NMR might possess in identification of some conformational features in milks, in **Chapter 6**, milk samples containing β-casein A2 were shown to possess greater proportions of poly-proline II (PPII) helices (unstable protein secondary structure), with lower amounts of α-helix motifs (stable protein secondary structures) compared to milks containing β-casein A1. These differences could likely be due to

the presence of proline in the primary structure of β-casein A2 and I. Furthermore, the tendency of proline to create PPII helices might be an additional reason for distinguishing between these two families. Nevertheless, to fully develop a predictive model using FTIR for differentiating milk based on particular casein genotype, a number of factors (but not limited to) such as, breed, lactation stage, diet, season, environmental factors, milk protein phenotypes, must be taken into consideration, which were not included within this study.

Chapter 7 examined all three, structure, functionality, and stability of A1/A1, A1/A2, and A2/A2 sodium caseinate dispersions and emulsions. This study discovered that A2/A2 sodium caseinate showed a higher amount of PPII helices, while A1/A1 and A1/A2 sodium caseinates had more α-helices and β-sheets. Once the emulsions were produced, A2/A2 sodium caseinate experienced a structural reordering with a notable increase in α-helical content after adsorption to the emulsion interface. Additionally, both samples with β-casein A1 resulted in lower solubility and reduced emulsifying properties compared to A2/A2 sodium caseinate. These findings indicated that A2/A2 sodium caseinate possessed greater emulsification activity and stability in comparison to A1/A1 or A1/A2 sodium caseinates.

The objective of the study in **Chapter 8** was to investigate the influence of natural variation in β-casein composition in milk on acid-induced gelation of milk. The gels formed from A2/A2 skim milk exhibited notably reduced elastic modulus, water holding capacity, and gel permeability in comparison to gels with β-casein A1. Microscopic examination revealed a denser microstructure and smaller pore size in A1/A1 and A1/A2 acid-induced gels. The results showed that the micellar κ-casein in both milks with β-casein A1 was almost 2 times greater compared the same protein amount in A2/A2 milk. Also, extensive dissociation of calcium phosphate upon acidification, including losing the stable protein secondary structures, resulted in a weaker gel from A2/A2 milk.

Chapters between 9 and 12 provided detailed insights in the production of dairy products and simulated *in vitro* digestion of skim milk powders, yoghurts, and cheeses, all having the same composite genotype (as_1-as_2-κ-caseins-β-Lactoglobulin), but only different β-casein phenotype. It is worth mentioning that Chapter 9 is a systematic review, not an experimental study. Samples carrying the homozygous β-casein A2/A2 were presented with poorer rehydration properties, softer yoghurt, and rennet gels strength (longer time was needed for the milk gelation and coagulation), and more porous microstructure. Interestingly, even though, during cheese making all milk samples were standardised to the same protein to fat ratio (0.95,

w/w), A2/A2 cheese was harder and more cohesive compared to all other cheese types in the final maturation phase. It is worth noting, in all three dairy products mentioned above, during gastric digestion, coagulum formation occurred within the first 5 - 15 min (15 min were seen only in cheese samples due the presence of fats in the samples). However, the protein breakdown of A2/A2 samples within the gastric phase was slower. Additionally, the final gastric clot of A2/A2 digesta was characterised with a condensed protein network that was comprised of significantly higher levels of aggregated β-sheets as opposed to that of A1/A1 and A1/A2 digest. These three studies explained the importance of the products' dairy matrix on the gastric *in vitro* digestion of skim milk powders, yoghurts, and cheeses.

In **Chapter 13,** for the first time in the literature, the pathway of a β-casomorphin peptides liberated from dairy products with various β-casein phenotypes was described and was extensively elaborated. Although, β-casomorphin peptides, but especially β-casomorphin 7 might be liberated from dairy products carrying β-casein A2, albeit in a lower amount than from those with β-casein A1, the likelihood of their presence in the human blood after dairy digestion is improbable (excluding those with "*leaky gut*" or other medical conditions). Particularly, β-casomorphin 7 can still be broken down into active forms by brush border peptidases, forming β-casomorphin 5, tetrapeptides, tripeptides, dipeptides, or fragments before reaching the targeted organ or entering the human bloodstream. Conversely, if β-casomorphins are not completely hydrolysed but are transported across the intestinal epithelium, their presence would trigger the activation of serum dipeptidyl peptidase IV, leading to the clearance of these peptides from the blood. All these results are subsequently gathered in **Chapter 14** (this chapter) giving an overview and final indications of the thesis, but also future recommendations for farmers, the dairy industry, and the consumers wellbeing.

14.3. Transitioning to A2/A2 dairy herd: "Alert" or "Alternative"?

Milk production, fertility and calving performance of genotyped animals were found to be influenced by the genetic variants of β-casein. Over the last two decades, the frequency of A2/A2 Holstein cow genotype increased by 20 % (Scott, Haile-Mariam, MacLeod, Xiang, & Pryce, 2023). Namely, genotyping of females for the β-casein locus has been pursued by farmers who have wished to build an A2/A2 homozygous herd for commercial sales of A2/A2 milk (Newton, Axford, Ho, & Pryce, 2020). Nevertheless, recent data revealed that this could lead to an increased risk of inbreeding, because of the desire to quickly build an entire herd of

homozygous A2/A2 cows to fulfil milk sales contracts (Scott, Haile-Mariam, MacLeod, Xiang, & Pryce, 2023). It is well known that inbreeding can result in a loss of genetic diversity, decreased response to selection, reduced animal performance, and ultimately, decreased farm profitability (Leroy, 2014). Interestingly, while breeder associations have been promoting interest in A2/A2 milk production, some preliminary results indicated that cows carrying β-casein A1 exhibited higher reproductive performance than those carrying β-casein A2 (Sebastiani et al., 2022; Ardicli, Aldevir, Aksu, & Gumen, 2023). Additionally, as shown in the thesis, in general, milk containing the β-casein A2 variant was less heat stable and created weaker gels with implications for milk powder, yoghurt and cheese production efficiency (Daniloski et al., 2024a; Daniloski et al., 2024b; Daniloski et al., 2024c).

In contrast, subsequent evaluation of gastric digestion of milk and fermented dairy products has shown significant differences between conventional and A2/A2 milk, with slower gastric digestion and firmer gastric curd formation noted during simulated *in vitro* digestion of A2/A2 dairy products, which may have potential benefits for infant nutrition products in particular (Daniloski et al., 2024a; Daniloski et al., 2024b; Daniloski et al., 2024c). The results of the thesis have been discussed with a number of milk processors, and the dairy industry should remain cognisant to the implications of a transition of the dairy herd towards an A2/A2 dominant genotype.

14.4. What about the future?

While the focus of current research predominantly centres on the genetic variants of β-casein and their influence on milk and dairy product properties, but also *in vitro* gastric digestion pattern, future investigations should extend beyond β-casein as a contributing factor in the differences observed. Understanding the interactions and synergies among different caseins, including αs_1-, αs_2-, and κ-caseins could provide comprehensive knowledge on how these proteins influence the structure and functionality of dairy matrices. For instance, the interaction among α-, β-, and κ-caseins (including their genetic variants) is known to influence the stability and structure of the casein micelles (Day, Williams, Otter, & Augustin, 2015; Holt & Sawyer, 1993; Waugh, 1958), but how and why? Future studies could explore how variations in a single casein gene might affect the expression or function of other caseins, leading to changes in milk composition, product properties and their digestibility.

Research on casein genotypes holds promise in shedding light on the intricate architecture of the casein micelle. By elucidating the role of genetic variants in shaping the structural properties of casein micelles, future studies can contribute to a better understanding of micelle formation, stability, and functionality. This knowledge will not only enrich our fundamental understanding of dairy technology but also offer practical applications for optimising milk processing and dairy product development. Additionally, it would be interesting to further explore casein micelle structure as a function of casein genotype, using not only multidimensional NMR, but also small angle X-ray and small angle neutron scattering methods that were not employed in the current thesis.

In vitro digestion protocol is efficient and highly utilised within the research community and in the current thesis, however, it should be remembered that this digestion protocol possesses some limitations. Hence, it will be interesting in the future, if the impact of the dairy matrices from A1/A1, A1/A2, and A2/A2 dairy products can further be evaluated on both, gastric and intestinal levels by using exclusively *in vivo* human trials. Additionally, the insights gained from the comprehensive review in **Chapter 13** will significantly contribute to my future experimental and peptidomics research on β-casomorphins from A1/A1, A1/A2, and A2/A2 milk and dairy types. This research will focus on understanding the behaviour of these peptides during both *in vitro* and *in vivo* gastric and intestinal digestion processes.

In addition to traditional methods, the emergence of novel techniques such as precision fermentation, offers exciting prospects for modifying and harnessing the potential of β-caseins in food applications. By precisely engineering β-casein variants through fermentation, new ways of explaining the structure of the casein micelle and interactions between particular caseins can be explored (Raynes et al., 2024). These precision fermented β-caseins present a frontier for innovation in dairy technology, offering opportunities to enhance the quality, nutritional profile, and functionality of dairy products.

Table 1. An overview table of the differences between the β-casein A1 and A2 variants in many aspects found within the thesis, such as casein micelle structure, techno-functional properties, and *in vitro* gastric digestion of milk and dairy products.

Sample type	Technological trait	Outcome	Reference
Casein micelle: Structure and functionality			
Milk samples (n = 9) - Australian Holstein A1/A1 (n = 3) - Australian Holstein A1/A2 (n = 3) - Australian Holstein A2/A2 (n = 3)	Temperature: 37 °C pH: 6.7, 5.7 and 2.3	1. At 6.7 and 2.3, intramolecular β-sheets and α-helixes particularly found in A1/A1 and A1/A2 casein micelles (stable structure) 2. At 6.7 and 2.3, PPII helixes were mainly present in A2/A2 casein micelle (unordered structure) 3. At pH 5.7, all casein micelles, structurally and functionally behaved similarly	Daniloski, Markoska, McCarthy, and Vasiljevic (2023)
Chilled milk *vs* milk at room temperature			
Milk samples (n = 3) - Australian Holstein A1/A1 (n = 1) - Australian Holstein A1/A2 (n = 1) - Australian Holstein A2/A2 (n = 1)	Temperature: 4 and 20 °C	1. A2/A2 milk had a lower amount of κ-casein 2. A2/A2 milk contained a higher amount of phosphorus, but a lower content of calcium 3. A1/A1 and A1/A2 milks had similar behaviour	Daniloski, McCarthy, Markoska, Auldist, and Vasiljevic (2022a)

Heat treatment			
Milk samples (n = 15) - Australian Holstein A1/A1 (n = 5) - Australian Holstein A1/A2 (n = 5) - Australian Holstein A2/A2 (n = 5)	Heating treatment 1. 72 °C for 15 s 2. 121 °C for 2.6 min 3. 140 °C for 3 s	1. A1/A1 and A1/A2 milks were characterised by greater amounts of calcium and phosphorus 2. Histidine present in A1/A1 milk might govern the formation of dehydroalanine 3. Aggregated β-sheets increased in all three milks during the heat treatment	Daniloski, McCarthy, and Vasiljevic (2022b)
Heat stability			
Milk samples (n = 28) - Irish Holstein A1/A1 (n = 8) - Irish Holstein A1/A2 (n = 10) - Irish Holstein A2/A2 (n = 10)	Temperature: 140 °C pH range: 6.2 - 7.4	1. A2/A2 milk was less heat stable 2. Higher κ-casein content in A1/A1 and A1/A2 milks might positively influenced their bigger heat stability 3. Heat-induced formation of the β-lactoglobulin/κ-casein complex in the serum	Daniloski, Hailu, Brodkorb, Vasiljevic, and McCarthy (2024a)
Milk ingredients (caseinate)			
Milk samples (n = 3) - Australian Holstein A1/A1 (n = 1) - Australian Holstein A1/A2 (n = 1) - Australian Holstein A2/A2 (n = 1)	Structure, interfacial, and emulsifying properties of sodium caseinates	1. The β-casein A2 appeared to be able to reach the oil droplet surface more rapidly 2. Sodium caseinates carrying β-casein A2 were more efficient as emulsifying agent	Daniloski, McCarthy, Auldist, and Vasiljevic (2022c)

		3. A1/A1 and A1/A2 sodium caseinate emulsions had lower levels of α-helixes	
Milk powders			
Milk samples (n = 28) - Irish Holstein A1/A1 (n = 8) - Irish Holstein A1/A2 (n = 10) - Irish Holstein A2/A2 (n = 10)	Structure, functionality, and rehydration properties of skim milk powders	1. A1/A1 and A2/A2 powders had a cohesive flow behaviour (flow index lower than 4) 2. A1/A2 powder was classified as an easy-flowing powder (flow index higher than 4) 3. A1/A2 had larger powder particle size and more dimpled structure 4. Bigger levels of random coils in A2/A2 milk powder altered its solubility	Daniloski et al. (2024a)
Milk gelation and yoghurt production			
Milk samples (n = 52) - Australian Holstein A1/A1 (n = 5) - Australian Holstein A1/A2 (n = 15) - Australian Holstein A2/A2 (n = 32)	Acid-induced gelation	1. A2/A2 milk gel was more porous 2. Less κ-casein was present in A2/A2 milk 3. High levels of aggregated β-sheets were found in both acid gels with β-casein A1	Daniloski, McCarthy, Gazi, and Vasiljevic (2022d)
Milk samples (n = 28) - Irish Holstein A1/A1 (n = 8) - Irish Holstein A1/A2 (n = 10) - Irish Holstein A2/A2 (n = 10)	Yoghurt manufacturing Milk inoculated with:	1. A1/A1 and A1/A2 milks had higher gel strength, greater β-sheet motifs, and lower yoghurt gel porosity	Daniloski et al. (2024b)

	(YC-380 yoghurt culture, 500U · 2500L^{-1})	2. In A2/A2 yoghurt lower concentration of κ-casein and lower whey protein denaturation was observed	
Rennet coagulation and Cheddar cheese production			
Milk samples (n = 30) - Irish Holstein A1/A1 (n = 10) - Irish Holstein A1/A2 (n = 10) - Irish Holstein A2/A2 (n = 10)	Rennet-induced coagulation	1. A1/A1 and A1/A2 rennet gels were firmer, more cohesive, and needed shorter time to gel 2. Insignificant differences among the milk samples regarding their protein, fat, and mineral levels	Daniloski et al. (2024c)
Milk samples (n = 30) - Irish Holstein A1/A1 (n = 10) - Irish Holstein A1/A2 (n = 10) - Irish Holstein A2/A2 (n = 10)	Cheese manufacturing Milk inoculated with: - Starter cultures (R-604 and LH-B02) - Fermentation-produced bovine chymosin (CHY-MAX)	1. A2/A2 cheese was 1.1 times harder and more cohesive compared to A1/A1 and A1/A2 cheeses 2. Higher levels of aggregated β-sheets were found in A2/A2 cheese 3. During cheese ripening, both cheeses with β-casein A1 showed a more porous microstructure	Daniloski et al. (2024c)
Gastric *in vitro* digestion of milk			
Milk samples (n = 28) - Irish Holstein A1/A1 (n = 8)	INFOGEST: *In vitro* semi-dynamic digestion	1. Visible and higher protein degradation in A1/A1 and A1/A2 milks	Daniloski et al. (2024a)

- Irish Holstein A1/A2 (n = 10) - Irish Holstein A2/A2 (n = 10)		2. A2/A2 final digesta was less porous 3. A1/A1 and A1/A2 digesta had higher levels of random coils	
Gastric *in vitro* digestion of yoghurt			
Milk samples (n = 28) - Irish Holstein A1/A1 (n = 8) - Irish Holstein A1/A2 (n = 10) - Irish Holstein A2/A2 (n = 10)	INFOGEST: *In vitro* semi-dynamic digestion	1. Protein breakdown in A2/A2 yoghurt digesta was lower 2. A weakly cross-linked protein structure and altered secondary protein conformation with greater levels of random coils were observed in A1/A1 and A1/A2 digesta 3. A1/A1 and A1/A2 yoghurts had higher levels of denatured β-lactogloblin. Heat denaturation probably caused an opening of β-lactogloblin's globular structure, thus exposing it to the action of pepsin	Daniloski et al. (2024b)
Gastric *in vitro* digestion of Cheddar cheese			
Milk samples (n = 30) - Irish Holstein A1/A1 (n = 10) - Irish Holstein A1/A2 (n = 10) - Irish Holstein A2/A2 (n = 10)	INFOGEST: *In vitro* semi-dynamic digestion	1. Lower protein degradation in A2/A2 cheese during the gastric digestion 2. Potential binding of β-casein A2 with κ-casein probably lowered the interactions of κ-	Daniloski et al. (2024c)

		casein with other molecules (pepsin), which would lead to an increased quantity of κ-casein in the digesta 3. A1/A1 and A1/A2 digesta had lower levels of aggregated β-sheets	

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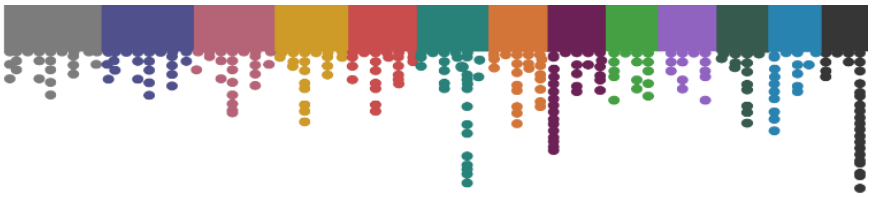
The end….

(Because every party needs a final slow dance!)

Annexes and supplementary material



Chapter 4. Conformational and physicochemical characteristics of bovine skim milk obtained from cows with different genetic variants of β-casein

https://www.sciencedirect.com/science/article/pii/S0268005X21006020#appsec1

Supplementary tables

Table 1. General characteristics of the Australian Holstein Friesian cows sampled and included in the study.

	Cow samples		
General characteristics (Average)	**A1/A1**	**A1/A2**	**A2/A2**
Milk yield (kg)	32.70	35.20	40.70
Liveweight (kg)	524.00	530.00	620.00
Condition score	4.80	4.10	3.90
Days in milk	45.00	80.00	43.00

Table 2A. The approximate composition of A1/A1 milk included in the study.

A1/A1 milk					
Overall approximate composition				Average	SD
Fats (%)	5.31	5.14	5.13	5.19	0.10
Density (kg/m^3)	30.57	29.71	29.67	29.98	0.51
Lactose (%)	5.09	4.95	4.94	4.99	0.08
Total solids (%)	9.28	9.02	9.00	9.10	0.16
Protein (%)	3.39	3.29	3.28	3.32	0.06
Water (%)	0.00	0.00	0.00	0.00	0.00
Temperature (^{0} C)	17.70	23.40	25.40	22.17	4.00
Freezing point (^{0} C)	- 0.61	- 0.59	- 0.59	-0.59	0.01
Salts (%)	0.76	0.74	0.73	0.74	0.02
pH	6.51	6.49	6.47	6.49	0.02

Table 2B. The approximate composition of A1/A2 milk included in the study.

A1/A2 milk					
Overall approximate composition				Average	SD
Fats (%)	3.98	3.88	3.90	3.92	0.05
Density (kg/m^3)	30.19	29.64	29.68	29.84	0.30
Lactose (%)	4.88	4.78	4.79	4.81	0.06
Total solids (%)	8.89	8.72	8.73	8.78	0.10
Protein (%)	3.25	3.18	3.19	3.21	0.04
Water (%)	0.00	0.00	0.00	0.00	0.00
Temperature (^{0} C)	19.80	24.10	26.00	23.30	3.18
Freezing point (^{0} C)	- 0.57	- 0.56	- 0.56	- 0.56	0.01
Salts (%)	0.73	0.71	0.71	0.72	0.01
pH	6.40	6.32	6.22	6.31	0.09

Table 2C. The approximate composition of A2/A2 milk included in the study.

A2/A2 milk					
Overall approximate composition				Average	SD
Fats (%)	3.75	3.72	3.87	3.78	0.08
Density (kg/m^3)	29.15	28.37	28.36	28.63	0.46
Lactose (%)	4.22	4.10	4.10	4.14	0.07
Total solids (%)	7.67	7.45	7.45	7.52	0.13
Protein (%)	3.21	3.32	3.37	3.30	0.08
Water (%)	0.00	0.00	0.00	0.00	0.00
Temperature (^{0} C)	22.70	25.20	26.40	24.77	1.89
Freezing point (^{0} C)	- 0.47	- 0.45	- 0.45	- 0.46	0.01
Salts (%)	0.83	0.65	0.78	0.75	0.09
pH	6.51	6.48	6.78	6.59	0.17

Table 3A. Levels of significance (p value) for all minerals depending on the milk type variants (GV) and temperature (Temp).

Parameters	Ca	K	Mg	Na	P	Ca^{2+}
Genetic variant	0.000 ***	0.000 ***	0.000 ***	0.000 ***	0.000 ***	0.002 **
Temperature	0.008 **	0.932	0.000 ***	0.000 ***	0.000 ***	0.681
GV.Tem	0.000 ***	0.000 ***	0.000 ***	0.000 ***	0.000 ***	0.561

*≤ 0.05 *; ≤ 0.01 **; ≤ 0.001 ****

Table 3B.

Levels of significance (p value) for the zeta potential and particle size depending on the milk type variants (GV) and temperature (Temp).

Parameters	Zeta potential	Particle size
Genetic variant	0.000 ***	0.000 ***
Temperature	0.000***	0.000***
GV.Tem	0.209	0.000***

*≤ 0.05 *; ≤ 0.01 **; ≤ 0.001 ****

Table 3C. Levels of significance (p value) for the total percentage areas of different secondary structures in amide I depending on the milk type variants (GV) and temperature (Temp).

Parameters	Side chain	Aggregated β-sheet	β-turn	Intramolecular β-sheet	α-helix
Genetic variant	0.024 *	0.022 *	0.000 ***	0.162	0.000 ***
Temperature	0.003 **	0.732	0.064	0.749	0.000 ***
GV.Tem	0.478	0.294	0.028 *	0.380	0.000 ***

*≤ 0.05 *; ≤ 0.01 **; ≤ 0.001 ****

Table 4A. Levels of significance (p value) for all minerals depending on the temperature (Temp) and the genetic variants (GV) of micellar casein.

Parameters	Ca	K	Mg	Na	P	Ca^{2+}
Genetic variant	0.000 ***	0.000 ***	0.000 ***	0.000 ***	0.000 ***	0.001 ***
Temperature	0.000 ***	0.000 ***	0.000 ***	0.000 ***	0.000 ***	0.234
GV.Tem	0.000 ***	0.000 ***	0.000 ***	0.000 ***	0.000 ***	0.188

*≤ 0.05 *; ≤ 0.01 **; ≤ 0.001 ****

Table 4B. Levels of significance (p value) for the zeta potential and particle size depending on the temperature (Temp) and the genetic variants (GV) of micellar casein.

Parameters	Zeta potential	Particle Size
Genetic variant	0.000 ***	0.008 **
Temperature	0.000 ***	0.000 ***
GV.Tem	0.358	0.010 *

*≤ 0.05 *; ≤ 0.01 **; ≤ 0.001 ****

Table 4C. Levels of significance (p value) for the total percentage areas of different secondary structures in amide I depending on the temperature (Temp) and the genetic variants (GV) of micellar casein.

Parameters	Side chain	Aggregated β-sheet	β-turn	Intramolecular β-sheet	α-helix
Genetic variant	0.597	0.545	0.334	0.002 **	0.000 ***
Temperature	0.465	0.880	0.085	0.055	0.202
GV.Tem	0.355	0.114	0.590	0.027 *	0.000 ***

*≤ 0.05 *; ≤ 0.01 **; ≤ 0.001 ****

Chapter 5. Impact of heating on the properties of A1/A1, A1/A2, and A2/A2 β-casein milk phenotypes

https://www.sciencedirect.com/science/article/pii/S0268005X22001242#appsec1

Supplementary tables

Table 1A. The approximate composition of A1/A1 milk included in the study

A1/A1 milk					
Overall gross composition				Average	Standard deviation
Fats (%)	5.28	5.31	5.12	5.24	0.10
Density (kg/m^3)	1036.53	1035.86	1034.88	1035.76	0.83
Lactose (%)	5.41	5.24	5.08	5.24	0.17
Total solids (%)	9.84	9.53	9.24	9.54	0.30
Protein (%)	3.61	3.50	3.39	3.50	0.11
Protein (Kjeldahl method %)	4.31	4.28	4.19	4.26	0.06
Temperature (°C)	21.10	22.40	22.25	21.92	0.71
Freezing point (°C)	- 0.62	- 0.59	- 0.57	- 0.59	0.02
Salts (%)	0.81	0.79	0.76	0.79	0.03

Table 1B. The approximate composition of A1/A2 milk included in the study

A1/A2 milk					
Overall gross composition				Average	Standard deviation
Fats (%)	3.94	3.91	3.89	3.91	0.03
Density (kg/m^3)	1033.22	1032.91	1033.03	1033.05	0.16
Lactose (%)	4.08	4.78	4.79	4.55	0.41
Total solids (%)	8.86	8.68	8.70	8.75	0.10
Protein (%)	3.25	3.10	3.19	3.18	0.08
Protein (Kjeldahl method %)	3.22	3.17	3.03	3.14	0.10
Temperature (°C)	22.4	21.5	22.8	22.23	0.67
Freezing point (°C)	- 0.55	- 0.53	- 0.53	- 0.54	0.01
Salts (%)	0.73	0.72	0.72	0.72	0.01

Table 1C. The approximate composition of A2/A2 milk included in the study

A2/A2 milk					
Overall gross composition				Average	Standard deviation
Fats (%)	3.69	3.72	3.85	3.75	0.09
Density (kg/m^3)	1031.36	1031.03	1031.23	1031.21	0.17
Lactose (%)	4.57	4.49	4.52	4.53	0.04
Total solids (%)	8.30	8.15	8.21	8.22	0.08
Protein (%)	3.05	2.99	3.01	3.02	0.03
Protein (Kjeldahl method %)	2.67	2.55	2.52	2.58	0.08
Temperature (°C)	21.7	21.9	22.8	22.13	0.59
Freezing point (°C)	- 0.51	- 0.40	- 0.50	- 0.50	0.00
Salts (%)	0.68	0.67	0.68	0.68	0.01

Table 2. Effect of β-casein phenotype and heat treatment on proteins in bovine milk

Temperature	Protein content (mg/mL)							
	Sample	κ-casein	αs_2- casein	αs_1-casein	β-casein A1	β-casein A2	β-Lactoglobulin	α-Lactalbumin
20 °C	A1	5.04 ± 0.06 ^{a}	2.39 ± 0.06 ^{ab}	13.04 ± 0.48 ^{a}	14.78 ± 0.12 ^{a}	n/d	6.82 ± 0.12 ^{a}	4.49 ± 0.05 ^{a}
	A1/A2	3.62 ± 0.03 ^{b}	2.28 ± 0.05 ^{bc}	9.88 ± 0.20 ^{bc}	6.24 ± 0.14 ^{d}	5.64 ± 0.06 ^{b}	2.94 ± 0.08 ^{bc}	2.41 ± 0.10 ^{c}
	A2	2.27 ± 0.05 ^{d}	1.98 ± 0.03 ^{de}	8.16 ± 0.05 ^{e}	n/d	8.84 ± 0.15 ^{a}	3.07 ± 0.06 ^{bc}	1.11 ± 0.05 ^{f}
72 °C	A1	3.61 ± 0.19 ^{b}	1.73 ± 0.07 ^{f}	9.47 ± 0.06 ^{cd}	11.65 ± 1.12 ^{bc}	n/d	4.29 ± 0.02 ^{b}	3.02 ± 0.03 ^{b}
	A1/A2	3.54 ± 0.22 ^{b}	2.52 ± 0.11 ^{a}	9.73 ± 0.09 ^{bcd}	6.20 ± 0.41 ^{d}	5.81 ± 0.11 ^{b}	2.61 ± 0.15 ^{bc}	1.47 ± 0.04 ^{e}
	A2	2.22 ± 0.06 ^{de}	2.18 ± 0.08 ^{bcd}	8.10 ± 0.11 ^{e}	n/d	8.59 ± 0.47 ^{a}	2.11 ± 0.09 ^{c}	0.92 ± 0.06 ^{fg}
121 °C	A1	3.58 ± 0.20 ^{b}	1.74 ± 0.05 ^{f}	9.76 ± 0.10 ^{bc}	12.45 ± 0.07 ^{b}	n/d	2.75 ± 0.10 ^{bc}	2.13 ± 0.04 ^{c}
	A1/A2	2.49 ± 0.02 ^{cd}	2.17 ± 0.14 ^{cd}	10.15 ± 0.09 ^{b}	6.16 ± 0.05 ^{d}	5.94 ± 0.06 ^{b}	1.80 ± 0.03 ^{c}	1.19 ± 0.05 ^{ef}
	A2	1.88 ± 0.05 ^{e}	1.75 ± 0.03 ^{f}	7.66 ± 0.14 ^{ef}	n/d	8.91 ± 0.22 ^{a}	1.96 ± 0.08 ^{c}	0.93 ± 0.31 ^{fg}
140 °C	A1	2.81 ± 0.17 ^{c}	1.01 ± 0.04 ^{g}	9.65 ± 0.07 ^{bcd}	11.31 ± 0.09 ^{c}	n/d	1.82 ± 0.08 ^{c}	1.81 ± 0.04 ^{d}
	A1/A2	2.38 ± 0.04 ^{d}	1.92 ± 0.05 ^{ef}	9.24 ± 0.13 ^{d}	6.11 ± 0.02 ^{d}	6.02 ± 0.05 ^{b}	1.53 ± 0.12 ^{c}	0.62 ± 0.01 ^{g}
	A2	1.44 ± 0.10 ^{f}	2.00 ± 0.07 ^{de}	7.42 ± 0.03 ^{f}	n/d	8.94 ± 0.06 ^{a}	1.36 ± 0.09 ^{c}	n/d

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$), n/d = not detectable.

Table 3. Effect of β-casein phenotype and heat treatment on proteins in serum

Temperature	Protein content (mg/mL)							
	Sample	κ-casein	αs_2- casein	αs_1-casein	β-casein A1	β-casein A2	β-Lactoglobulin	α-Lactalbumin
20 °C	A1	0.27 ± 0.03 ^{d}	0.41 ± 0.08 ^{a}	0.05 ± 0.01 ^{de}	0.97 ± 0.06 ^{c}	n/d	7.05 ± 0.05 ^{a}	1.01 ± 0.05 ^{de}
	A1/A2	0.39 ± 0.02 ^{cd}	0.10 ± 0.03 ^{cd}	0.04 ± 0.01 ^{de}	0.59 ± 0.02 ^{e}	0.92 ± 0.02 ^{f}	2.58 ± 0.07 ^{e}	1.16 ± 0.06 ^{c}
	A2	0.56 ± 0.05 ^{b}	0.10 ± 0.02 ^{cd}	0.29 ± 0.08 ^{c}	n/d	3.07 ± 0.07 ^{c}	4.19 ± 0.03 ^{c}	1.41 ± 0.07 ^{b}
72 °C	A1	0.47 ± 0.05 ^{bc}	0.22 ± 0.05 ^{b}	0.00 ± 0.00 $^{n/d}$	0.69 ± 0.03 de	n/d	5.42 ± 0.10 ^{b}	1.13 ± 0.05 ^{cd}
	A1/A2	0.42 ± 0.03 ^{c}	0.08 ± 0.01 ^{cd}	0.01 ± 0.01 ^{e}	0.29 ± 0.03 ^{f}	0.41 ± 0.04 ^{g}	1.71 ± 0.11 ^{f}	1.07 ± 0.03 ^{cd}
	A2	0.56 ± 0.07 ^{b}	0.11 ± 0.02 ^{c}	0.13 ± 0.02 ^{d}	n/d	1.85 ± 0.07 ^{d}	3.59 ± 0.14 ^{d}	1.66 ± 0.04 ^{a}
121 °C	A1	0.55 ± 0.07 ^{b}	0.34 ± 0.02 ^{a}	0.03 ± 0.01 ^{de}	1.57 ± 0.04 ^{b}	n/d	0.61 ± 0.03 ^{g}	0.51 ± 0.03 ^{f}
	A1/A2	0.48 ± 0.04 ^{bc}	0.09 ± 0.03 ^{cd}	0.09 ± 0.02 ^{de}	0.77 ± 0.05 ^{d}	1.08 ± 0.03 ^{e}	0.47 ± 0.09 ^{g}	0.92 ± 0.09 ^{e}
	A2	0.80 ± 0.02 ^{a}	0.17 ± 0.03 ^{bc}	0.43 ± 0.04 ^{b}	n/d	3.36 ± 0.05 ^{b}	1.88 ± 0.05 ^{f}	1.03 ± 0.02 ^{cde}
140 °C	A1	0.50 ± 0.02 ^{bc}	0.15 ± 0.01 ^{bc}	0.01 ± 0.00 ^{e}	2.51 ± 0.12 ^{a}	n/d	0.57 ± 0.04 ^{g}	0.08 ± 0.02 ^{h}
	A1/A2	0.41 ± 0.06 ^{c}	0.01 ± 0.01 ^{d}	0.26 ± 0.02 ^{c}	0.76 ± 0.06 ^{d}	1.05 ± 0.02 ^{ef}	0.23 ± 0.03 ^{h}	0.20 ± 0.02 ^{gh}
	A2	0.49 ± 0.03 ^{bc}	0.13 ± 0.01 ^{bc}	0.62 ± 0.08 ^{a}	n/d	4.45 ± 0.14 ^{a}	1.77 ± 0.07 ^{f}	0.34 ± 0.04 ^{g}

Mean values within a column that do not share a common superscript letter are significantly different ($p \leq 0.05$), n/d = not detectable.

Chapter 6. Authentication of β-casein milk phenotypes using FTIR spectroscopy

https://www.sciencedirect.com/science/article/pii/S0958694622000346#appsec1

Supplementary material

Table 1. The approximate average composition of A1/A1 milk (n = 5) included in the study.

Parameter	Overall approximate composition of samples					Average	Standard deviation
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5		
Fats (%)	3.67	3.98	3.78	3.77	3.75	3.79	0.11
Density (kg m^{-3})	1038.08	1037.75	1037.73	1037.77	1037.74	1037.81	0.15
Lactose (%)	5.54	5.49	5.49	5.51	5.57	5.52	0.03
Total solids (%)	10.07	9.98	9.98	9.92	9.94	9.98	0.06
Protein (%)	3.69	3.66	3.66	3.61	3.63	3.65	0.03
Temperature (°C)	20.70	18.20	18.20	19.80	19.75	19.33	1.10
Freezing point (°C)	–0.63	–0.62	–0.62	–0.64	–0.67	–0.64	0.02
Salts (%)	0.83	0.82	0.82	0.85	0.84	0.83	0.01

Table 2. The approximate average composition of A1/I milk (n = 5) included in the study.

Parameter	Overall approximate composition of samples					Average	SD
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5		
Fats (%)	3.72	3.71	3.72	3.73	3.72	3.72	0.01
Density (kg m^{-3})	1032.73	1033.06	1033.03	1033.03	1033.04	1032.98	0.14
Lactose (%)	4.72	4.77	4.76	4.76	4.76	4.75	0.02
Total solids (%)	8.58	8.66	8.65	8.65	8.65	8.64	0.03
Protein (%)	3.15	3.18	3.18	3.18	3.18	3.17	0.01
Temperature (°C)	21.10	22.00	21.80	20.80	18.50	20.84	1.40
Freezing point (°C)	–0.53	–0.53	–0.53	–0.53	–0.53	–0.53	0.00
Salts (%)	0.71	0.71	0.71	0.71	0.71	0.71	0.00

Table 3. The approximate average composition of A1/A2 milk (n = 5) included in the study.

Parameter	Overall approximate composition of samples					Average	SD
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5		
Fats (%)	3.45	3.77	3.69	3.72	3.49	3.62	0.14
Density (kg m^{-3})	1034.76	1034.71	1034.65	1034.58	1034.58	1034.66	0.08
Lactose (%)	5.02	5.01	5.00	5.01	5.01	5.01	0.01
Total solids (%)	9.12	9.10	9.08	9.11	9.11	9.10	0.02
Protein (%)	3.35	3.34	3.33	3.32	3.32	3.33	0.01
Temperature (°C)	20.90	20.00	21.67	21.14	21.14	20.97	0.61
Freezing point (°C)	–0.56	–0.56	–0.56	–0.57	–0.57	–0.57	0.00
Salts (%)	0.75	0.75	0.75	0.77	0.77	0.76	0.01

Table 4. The approximate composition of I/I milk (n = 5) included in the study.

Parameter	Overall approximate composition of samples					Average	SD
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5		
Fats (%)	3.88	3.84	3.89	3.87	3.88	3.87	0.02
Density (kg m^{-3})	1035.75	1036.49	1036.69	1036.42	1036.39	1036.35	0.35
Lactose (%)	5.17	5.28	5.26	5.27	5.26	5.25	0.04
Total solids (%)	9.39	9.59	9.56	9.57	9.56	9.53	0.08
Protein (%)	3.45	3.52	3.51	3.51	3.51	3.50	0.03
Temperature (°C)	21.10	20.80	19.70	19.80	18.70	20.02	0.96
Freezing point (°C)	–0.58	–0.60	–0.59	–0.59	–0.59	–0.59	0.01
Salts (%)	0.78	0.79	0.79	0.79	0.79	0.79	0.00

Table 5. The approximate composition of A2/I milk (n = 5) included in the study.

Parameter	Overall approximate composition of samples					Average	SD
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5		
Fats (%)	3.81	3.78	3.82	3.79	3.83	3.81	0.02
Density (kg m^{-3})	1035.19	1035.06	1035.03	1035.22	1035.29	1035.09	0.09
Lactose (%)	5.09	5.07	5.07	5.08	5.09	5.08	0.01
Total solids (%)	9.25	9.21	9.20	9.25	9.26	9.22	0.03
Protein (%)	3.39	3.38	3.38	3.40	3.41	3.38	0.01
Temperature (°C)	20.60	19.50	19.60	19.80	19.90	19.90	0.61
Freezing point (°C)	–0.57	–0.57	–0.57	–0.58	–0.59	–0.57	0.00
Salts (%)	0.76	0.76	0.76	0.76	0.77	0.76	0.00

Table 6. The approximate composition of A2/A2 milk (n = 5) included in the study.

Parameter	Overall approximate composition of samples					Average	SD
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5		
Fats (%)	3.55	3.61	3.59	3.61	3.71	3.61	0.06
Density (kg m^{-3})	1036.17	1037.34	1037.28	1036.21	1036.34	1036.67	0.59
Lactose (%)	5.23	5.39	5.39	5.33	5.34	5.34	0.07
Total solids (%)	9.50	9.78	9.80	9.81	9.82	9.74	0.14
Protein (%)	3.49	3.59	3.60	3.49	3.52	3.54	0.05
Temperature (°C)	22.10	21.20	19.80	22.10	22.20	21.48	1.02
Freezing point (°C)	–0.59	–0.61	–0.61	–0.59	–0.60	–0.60	0.01
Salts (%)	0.78	0.81	0.81	0.79	0.82	0.80	0.02

Table 7. Levels of significance (*p*-value) for the total percentage areas of different secondary structures in amide I depending on the milk phenotype (PT). [a]

Phenotype	Parameters					
	Side chain	Intramolecular β-sheet	Random coil	α-helix	β-turn	Aggregated β-sheet
β-casein	0.123	0.008 **	0.000 ***	0.000 ***	0.000 ***	0.044 *

[a] *p*-values indicated by: *, ≤ 0.05; **, ≤ 0.01; ***, ≤ 0.001.

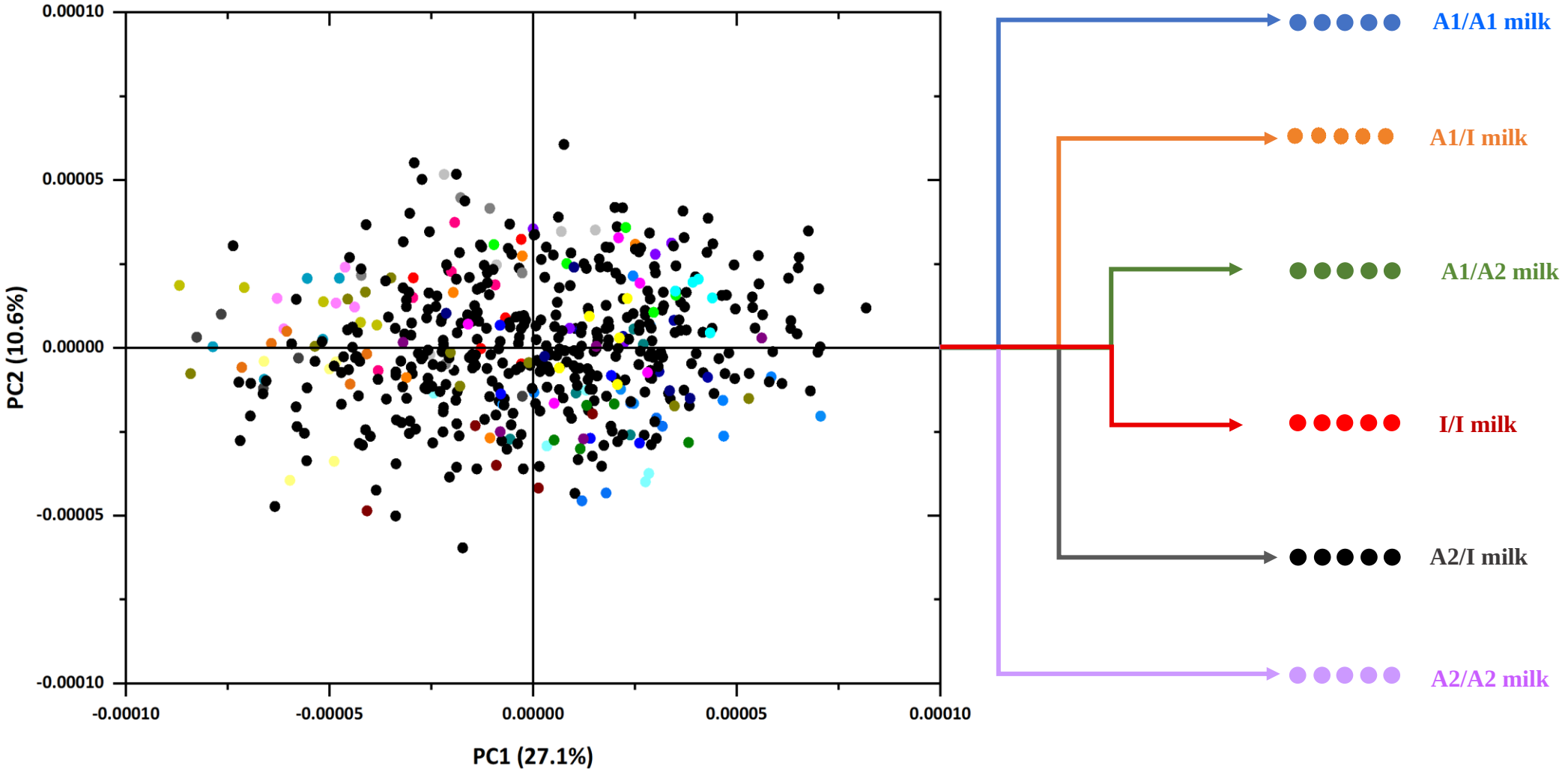


Figure 1. Scatter plot of the PCA scores of FTIR spectra of milk samples and the samples chosen for further analysis.

Health-related outcomes of genetic polymorphism of bovine β - casein variants: A Systematic Review of randomised controlled trials

https://www.sciencedirect.com/science/article/pii/S0924224421001825#appsec1

Supplementary tables

Table 1. The effect of bovine milk, β-CN, and BCM7 on gastrointestinal tract

Study	Exposure	Intervention (Dosage)	Results
Chabance et al. (1998)	Exposure to skim bovine milk (milk powder 147 g/L) with a protein content of 4.2 g/100 g for a period of 14 days.	**Gastrointestinal intubation technique** 1. Ingestion of 500 ml test (4 h) meal (skim milk) in 5 - 10 min. 2. Every 20 min, 5 ml of gastric contents waere aspirated with a manual syringe. 3. The stomach was washed with 200 ml of 150 mM NaCI solution. 4. No additional food and fluids. Passage of milk peptides into the blood circulation was performed on 3 days. Blood samples were collected at 5; 20; 40; 60 min; and 8 h, after the ingestion of 500 ml of water (Vittel and skim milk).	**Identification of β-CN peptides liberated in the human stomach after skimmed milk ingestion** 1. 20 min: f1-12; f33-44; f107-114; f29-41; f30-41; f106-120. 2. 1 h: f6-17; f29-40; f164-175. 3. 4 h (β-CN peptide sequences): f164-175. **Identification of β-CN peptides liberated in the human duodenum after skimmed milk ingestion** 1. 20 min (β-CN peptide sequences): f7-18; f114-119; f84-92; f83-93. 2. 40 min (β-CN peptide sequences): f7-16; f145-156; f1-12; f155-165; f1-12. 3. 4 h (β-CN peptide sequences): f69-80.

Boutrou et al. (2013)	Milk proteins from raw milk (CNs separated from WPs). The experiment was conducted in a period of 9 days.	1. Protein intake of 1.4 g · kg^{-1} · d^{-1}. The daily protein intake was 30 g CN, 27.5 g maltodextrine (Roquette), and 2.5 g orange flavour. 2. At the beginning of the standardization, each subject was given 7 shakers that contained the supplement powder for self-administration after dissolution in 500 ml H_2O.	**Identification of β-CN peptides liberated in the jejunum effluent after CN ingestion** 1. 30 min (β-CN peptide sequences): f60-66 (3.60 ± 0.35 mg). 2. 30 min (β-CN peptide sequences): f108-113 (40 mg). 3. 2 h (β-CN peptide sequences): f60-66 (4 mg). 4. 6 h (β-CN peptide sequences): f57-; f58-; f59-; f60-66; f108-113.
Barnett, McNabb, Roy, Woodford, and Clarke (2014)	Skim milk-based diets containing β-CN of either the A1 or A2 genetic variants. The experiment lasted 7 days.	1. The rats were fed skim milk-based diets containing β-CN of either the A1 or A2 type for 36 h or 84 h, together with water. Food and water were provided ad libitum. 2. All rats were orally gavage 24 h before the end of the feeding periods with an inert tracer (TiO_2). Half of the rats in each group were also injected with naloxone at this time.	GITT (gastrointestinal transit time): In rats fed the A1 β-CN diet, TiO_2 recovery was significantly lower in the saline-treated group than in the naloxone-treated group at 8 h ($p = 0.01$) and 11 h ($p = 0.049$), but not at 14 h ($p = 0.17$). On the contrary, in rats fed the A2 β-CN diet, the cumulative recovery of TiO_2 was not significantly different between the naloxone-treated and saline-treated groups at 8 h ($p = 0.55$), 11 h ($p = 0.84$), or 14 h ($p = 0.38$). MPO (myeloperoxidase): Its activity was 65 % higher in the A1S group than in the A2S group (0.52 vs 0.32 units/3min/mg protein, $p = 0.04$). MPO activity was also 64 % higher in the A1S group than in the A1N group (0.52 vs 0.32 units/3 min/mg protein, $p = 0.04$).

		3. For injection, 6 mg of naloxone (N) was dissolved in 20 ml of normal saline (S) and administered at a dose of 333 ml per 100 g of body weight, giving a final dose of 1 mg/kg body weight. Food intake was not recorded during the study.	HIS (histological injury score): The HIS inflammation scores were 55 % higher in the A1S group than in the A2S group; however, this difference was not statistically significant (17.45 vs 11.33; p = 0.36). DPP-4: Its activity in jejunum was 40 % higher in the A1S group than in the A2S group (38.3 vs 27.3 pkatal/µg protein; p = 0.002). DPP-4 activity was 37 % higher in the A1N group than in the A2S group (p = 0.001).
Haq, Kapila, Sharma, Saliganti, and Kapila (2014)	Separation of β-CN from whole fat bovine milk; Karan Fries cattle with A1/A1, A1/A2, or A2/A2 genotype. The experiment was conducted over period of 30 days.	1. Control group: basal diet (18.4 % starch, 65 % Bengal gram, 2.55 % oil, 2.05 % mineral mixture, 1 % vitamin mixture and 11 % cellulose) 2. Experimental Group 1: β-CN (A1/A1 genetic variant). 3. Experimental Group 2: β-CN (A1/A2 genetic variant). 4. Experimental Group 3: β-CN (A2/A2 genetic variant). All three experimental groups were fed with 85 mg β-CN/animal/day suspended in 200 µl phosphate-buffered saline (PBS).	MPO: Comparison of A1/A1 β-CN and A1/A2 β-CN vs A2/A2 β-CN variants. All three genetic protein variants increased the activity of MPO in murine intestine ($p<0.01$) by 179.06 % and 31.68 %. CD4 T Cells (IL-4): These results also indicated an increase ($p<0.01$) in IL-4 levels on feeding A1/A1 β-CN (272.13 %) and A1/A2 β-CN (282.29 %), compared to A2/A2 β-CN consumption. IgE: Feeding A1/A1 and A1/A2 β-CN significantly increased ($p<0.001$) total IgE levels by 50.38 % and 46.46 %, respectively, compared to A2/A2 consumption. IgG: Intestinal fluid showed an elevated levels of IgG by consumption of A1/A1 β-CN (91.08 %; $p<0.001$) and A1/A2 β-CN (33.54 %; $p<0.01$), compared to A2/A2 β-CN. IgA: No changes between the genetic variants. Leu in GIT: Feeding on animals with A1/A1 β-CN and A1/A2 β-CN increased the number of total leukocytes

			by 153 % and 135.73 %, respectively, compared to A2/A2 β-CN consumption.
			TLR-2: A1/A1 β-CN consumption hugely increased ($p<0.001$) TLR-2 expression by 141.39 %, 220.71 % and 349 % compared to A1/A2 β-CN, A2/A2 β-CN and control group mice, respectively.
Ho, Woodford, Kukuljan, and Pal (2014)	Whole milk based diet containing A1 β-CN or A2 β-CN. The experiment lasted 8 weeks (including the washout period).	1. 2 weeks (Initially): Group 1 - A1 and Group 2 - A2: were consuming 750 ml/day of their allocated milk which contained ≈ 7.5 g of either A1 β-CN or A2 β-CN. 2. After that 4 Weeks: Washout with rice milk (both groups - no dairy products). 3. Finally another 2-week period: Group 1 - A2 and Group 2 - A1: were consuming 750 ml/day of their allocated milk which contained ≈ 7.5 g of either A1 β-CN or A2 β-CN.	**BSS stool consisteany values (mean ± s.e.m.; MD)** All participants during the 2-week period: A1 - 3.87 ± 0.02; A2 - 3.56 ± 0.02; 0.31.
			Self-described as milk tolerant during the 2-week period: A1 - 3.82 ± 0.02; A2 - 3.47 ± 0.02; 0.35.
			Self-described as milk intolerant during the 2-week period: A1 - 4.02 ± 0.04; A2 - 3.87 ± 0.05; 0.15.
			Digestive discomfort & abdominal pain: Although all mean values were numerically higher on the A1 diet, none were statistically significant.

			Faecal calprotectin: There were no overall differences in faecal calprotectin between the A1 (41.6 μg/g) and A2 diets (35.8 μg/g), MD (15 vs 14 μg/g), no information on SD.
Jianqin et al. (2015)	Bovine milk containing either A1/A2 β-CN (conventional milk) with a ratio of 58:42 or only A2 β-casein (industry milk). The duration of the examination was 14 days.	1. 2 weeks (Initially): Group 1 = A1 β-CN & Group 2 = A2 β-CN: were consuming 250 ml/day of their allocated milk after meals. 2. After that 2 Weeks: Washout period - no dairy products. 3. Finally another 2-week period: Group 1* = A2 β-CN & Group 2* = A1 β-CN: were consuming 250 ml/day of their allocated milk after meals. SBTT - small bowel transit time CTT - colonic transit time WGTT - whole gastrointestinal transit time	SBTT (hours): G1 - 3.62 ± 1.46 / SBTT (hours): G1* - 4.02 ± 1.45. / SBTT (hours): G2 - 3.79 ± 1.89 / SBTT (hours): G2* - 3.90 ± 1.85 CTT (hours): G1 - 35.41 ± 8.68 / CTT (hours): G1* - 28.23 ± 5.50 / CTT (hours): G2 - 35.31 ± 6.92 / CTT (hours): G2*- 29.62 ± 7.41 WGTT (hours): G1 - 39.95 ± 8.45 / WGTT (hours): G1 - 33.41 ± 5.68 / WGTT (hours): G1 - 40.14 ± 6.81 / WGTT (hours): G1 - 34.36 ± 6.90 **Gastrointestinal inflammation (between intervention)** As participants moved from milk containing A1/A2 β-CN to milk containing only A2 β-CN, 36.4 % reported change in major inflammation of the intestine and 22.7 % in stomach inflammation. In comparison, as participants moved from milk containing only A2 β-CN to milk containing A1/A2 β-CN, 11.1 % demonstrated progress in small intestine and stomach inflammation. **Stool frequency - baseline (no./wk)** / **Stool frequency - after 2 wk (no./wk)**

			- G1: 7.68 ± 1.98 - G1*: 7.95 ± 2.3 - G2: 7.83 ± 1.59 - G2*: 7.57 ± 1.95	- G1: 11.05 ± 4.21 - G1*: 7.91 ± 1.15 - G2: 10.43 ± 3.46 - G2*: 7.87 ± 1.91
			Stool consistency baseline (BSS) - G1: 4.05 ± 0.65 - G1*: 4.08 ± 0.46 - G2: 4.07 ± 0.51 - G2*: 4.09 ± 0.67	**Stool consistency after 2 wk (BSS)** - G1: 4.42 ± 0.74 - G1*: 4.05 ± 0.25 - G2: 4.35 ± 1.11 - G2*: 4.08 ± 0.61
			Diarrhoea (measured as adverse event) during the first 2 weeks: A1/A2 β-CN milk: 8 events in 5 participants.	Diarrhoea (measured as adverse event) during the first 2 weeks: A2 β-CN milk: 3 events in 3 participants.
Crowley, Williams, Roberts, Dunstan, and Jones (2013)	Bovine milk A1 β-CN vs bovine milk A2 β-CN. The experiment had been conducted over a period of 6 weeks (including the washout period).	1. 2 weeks (Initially): Group 1 = A1 β-CN & Group 2 = A2 β-CN: were consuming 400 ml/day of their allocated milk. 2. After that 2 Weeks: Washout with CMP and soy protein free	The resolution of chronic functional constipation (CFC) has been observed by 14 participants (out of 22; 64 %) who consumed A1 β-CN milk. The resolution of CFC has been observed by 16 participants (out of 25; 64 %) who consumed A2 β-CN milk.	

		milk (both groups - no dairy products 400 ml/day). 3. Finally another 2-week period: Group 1* = A2 β-CN & Group 2* = A1 β-CN: were consuming 400 ml/day of their allocated milk.	Bowel motions per fortnight (SD) has been observed by 10.5 participants (out of 22; 5.75 %) who consumed A1 β-CN milk. Bowel motions per fortnight (SD) has been observed by 10.56 participants (out of 25; 5.24 %) who consumed A2 β-CN milk.
He, Sun, Jiang, and Yang (2017)	Bovine milk containing either A1/A2 β-CN (conventional milk) with a ratio of 58:42 or only A2 β-CN (industry milk). The duration of the examination was 8 days.	Day 1: Group 1 = A1/A2 β-CN and Group 2 = A2 β-CN: were consuming 300 ml/day of their allocated milk. 1. 1 h after milk consumption: breakfast (congee and a steamed bun - Beijing; comprised fried chicken, congee and bread in Shanghai and Guangzhou). 2. 3 h after milk consumption: urine sample and VAS questionnaire. 3. 12 h after consumption: VAS questionnaire. Day 2 - Day 7: Group 1 and Group 2 - Washout (Dairy - free diet).	1. GIT symptoms: All six gastrointestinal symptom scores at 1 h and 3 h were significantly lower after consuming A2 β-CN vs conventional milk (A1/A2 β-CN) (all P<0.0001). At 12 h, significant differences remained for bloating, abdominal pain, stool frequency, and stool consistency (all P<0.0001). 2. Urine samples: Symptom scores were consistently lower with A2 β-CN in both lactose absorbers (urinary galactose ≥ 0.27 mmol/l) and lactose malabsorbers (urinary galactose < 0.27 mmol/l). 3. Age: the age group did not have a significant impact on gastrointestinal symptoms when evaluated using GEE analysis or Kruskal - Wallis test.

		Day 8: Group 1* = A2 β-CN and Group 2* = A1/A2 β-CN: were consuming 300 ml/day of their allocated milk. 1. 1 h after milk consumption: breakfast (congee and a steamed bun - Beijing; comprised fried chicken, congee and bread in Shanghai and Guangzhou). 2. 3 h after milk consumption: urine sample and VAS questionnaire. 3. 12 h after consumption: VAS questionnaire.		
Sheng, Li, Ni, and Yelland (2019)	Bovine milk containing either A1/A2 β-CN (conventional milk) or only A2 β-CN (industry milk). The duration of the intervention was 19 days. The examination was based on visual analogy scales (VAS) questionnaire to assess baseline gastrointestinal symptoms.	1. Phase 1 (days 1 - 5): Group 1 = A1/A2 β-CN and Group 2 = A2 β-CN: were consuming x 2; 150 ml/day (300 ml/day) of their allocated milk. 2. Washout: 9 days (days 6 - 14). No info on a diet. 3. Phase 3 (days 15 - 19): Group 1* = A2 β-CN and Group 2* = A1/A2 β-CN: were consuming x 2; 150 ml/day (300 ml/day) of their allocated milk.	GIT symptoms (bloating, abdominal pain, flatulence, and heavy or full stomach): There were no significant differences in baseline total VAS scores between the 2 sequence groups in both phases (phase 1: P = 0.915; phase 2: P = 0.801).	
			Markers of gut inflammation: CM (PostI. - Phase 1) - 0.77 (0.08) ng/ml, IL-4 ($p<0.05$) - 11.2 (2.0) g/l, IgG ($p<0.0001$)	**Markers of gut inflammation: A2 β-CN (PostI. - Phase 1)** - 0.73 (0.08) ng/ml, IL-4 ($p<0.05$) - 9.2 (1.6) g/l, IgG ($p<0.0001$)

			- 7.15 (1.36) g/l, IgG1 (p<0.05) - 3.15 (2.05 – 5.49) ng/ml, BCM7 (p<0.01) - 1.44 nmol/ml (0.37 -3.88), GSH (p<0.001) - 90.5 (11.5 - 153) IU/ml, IgE (p<0.05)	- 6.04 (1.25) g/l, IgG1 (p<0.05) - 2.54 (1.95 – 4.38) ng/ml, BCM7 (p<0.01) - 2.22 nmol/ml (0.68 - 3.88), GSH (p<0.001) - 52.2 (16.3 - 160) IU/ml, IgE (p<0.05)
			Analysis of stool frequency: consumption of conventional milk was associated with a significantly higher stool frequency and significantly higher BSS scores compared with the consumption of milk containing A2 β-CN.	
Milan et al. (2020)	UHT processed cow's milk (conventional milk, A1/A2 β-CN) or only A2 β-CN (industry milk). The duration of the study was 12 hours, divided in three visits (three different days).	Three Groups of participants 1. Group 1: LI = self-reported intolerant, diagnosed lactose intolerant (n = 10) 2. Group 2: NLDI = self-reported non-lactose dairy intolerant, but diagnosed lactose tolerant (n = 20)	**Bowel movement (Number of participants' Symptoms)** - Abdominal pain (LI): A2M – 6; CON – 12; LF-CON – 2 (p<0.001). - Abdominal pain (NLDI): A2M – 8; CON – 13; LF-CON – 9 (p<0.001). - Abdominal pain (DT): A2M – 5; CON – 3; LF-CON – 0 (p<0.001).	

		3. Group 3: DT = diagnosed lactose tolerant or dairy tolerant (n = 10) All three groups of participants consumed 750 ml conventional milk (CON; containing A1/A2 β-CN and lactose – Visit 1), A2 Milk (A2M; exclusively containing A2 β-CN with lactose – Visit 2), or lactose-free conventional milk (LF-CON; A1/A2 β-CN without lactose – Visit 3).	- Abdominal fullness (LI): A2M – 3; CON – 6; LF-CON – 0 ($p<0.001$). - Abdominal fullness (NLDI): A2M – 12; CON – 12; LF -CON – 4 ($p<0.001$). - Abdominal fullness (DT): A2M – 6; CON – 1; LF-CON – 0 ($p<0.001$).
			- Abdominal bloating (LI): A2M – 8; CON – 8; LF-CON – 5 ($p<0.001$). - Abdominal bloating (NLDI): A2M – 9; CON – 8; LF-CON – 5 ($p<0.001$). - Abdominal bloating (DT): A2M – 7; CON – 2; LF-CON – 0 ($p<0.001$).
			- Abdominal distension (LI): A2M – 4; CON – 7; LF-CON – 4 ($p<0.001$). - Abdominal distension (NLDI): A2M – 7; CON – 3; LF-CON – 1 ($p<0.001$). - Abdominal distension (DT): A2M – 5; CON – 2; LF-CON – 0 ($p<0.001$).
			- Loose BMs (diarrhoea; BSS score >6) (LI): A2M – 1; CON – 7; LF-CON – 0 ($p<0.001$). - Loose BMs (diarrhoea; BSS score >6) (NLDI): A2M – 0; CON – 4; LF-CON – 4 ($p<0.001$).

			- Loose BMs (diarrhoea; BSS score >6) (DT): A2M – 5; CON – 2; LF-CON – 2 ($p<0.001$). **Stool (Symptoms)** Over 12 h, MD score did not differ between milks or tolerance groups (No comparable).
Guantario et al. (2020)	Conventional bovine milk, A1/A2 β-CN and A2/A2 β-CN. The experiment lasted for 4 weeks.	Three Groups of participants (males and females). All diets were same; groups 2 and 3 were only differently supplemented: 1. Group 1 (n = 8 mice): Control Diet (CTRL), containing a casein-like amino acid mix (CTRL). 2. Group 2 (n = 9): Diet supplemented with A2/A2 β-CN lyophilised milk. 3. Group 3 (n = 7): Diet supplemented with A1/A2 β-CN lyophilised milk. Vh - villus height Cd - crypt depth	**Cytokine Secretion** Both milk-supplemented diets induced a significant increase in NK cell percentage as compared to CTRL ($p \leq 0.001$), whereas T lymphocytes were increased in A1/A2 mice, as compared to both CTRL and A2/A2. A2/A2 milk supplementation could significantly modify the gut immune phenotype of old mice as compared to A1/A2 milk and CTRL group. **Gut Enzymatic Activities** The difference in DPP-4 activity among the three groups has not been shown. **Histomorphological Evaluation (mean ± SD)** 1. Vh (D): CTRL = 10.0 ± 1.76; A1/A2 = 8.33 ± 0.58; A2/A2 = 9.57 ± 0.63. 2. Cd (D): CTRL = 1.18 ± 0.15; A1/A2 = 1.09 ± 0.11; A2/A2 = 0.99 ± 0.14. 3. Vh/Cd (D): 8.51 ± 1.20; A1/A2 = 7.72 ± 1.09; A2/A2 = 9.86 ± 1.71.

			Immunohistochemical staining (IHC) 1. $CD8^+$ IHC (D): CTRL = 1.79 ± 0.79; A1/A2 = 2.07 ± 1.48; A2/A2 = 3.11 ± 1.47. 2. $CD45^+$ IHC (D): CTRL = 3.05 ± 1.12; A1/A2 = 3.20 ± 1.11; A2/A2 = 4.11 ± 0.53.
			IgGs No significant differences in IgG serum levels were observed between CTRL, A1/A2, and A2/A2 group.
			Short-Chain Fatty Acids 1. Isobutyrate (F): CTRL = 25.1 ± 10.5; A1/A2 = 77.4 ± 62.0; A2/A2 = 80.04 ± 32.6. 2. SCFAs (F): CTRL = 60.1 ± 20.9; A1/A2 = 127 ± 79.2; A2/A2 = 131 ± 42.6.
			Enterobacteriaceae and *Enterococcaceae* were scored in the CTRL group, but not in the A1/A1 and A2/A2. These bacteria's families were previously correlated with ageing.

Table 2. The effect of bovine milk, β-CN, and BCM7 on cardiovascular diseases

Study	Exposure	Intervention (Dosage)	Results (Mean)	
Chin-Dusting et al. (2006)	Exposure to bovine milk (milk powder) contains either A1 β-CN or A2 β-CN (10 g/d) throughout the 12-week study.	15 Participants with high risk of CVD, consumed 25 g/d of the dairy shake (A1 β-CN or A2 β-CN) in 200 ml water or fruit juice. More specifically, all participants were advised to not consume dairy products (excluding cheese). 1. Group 1: All participants consumed A1 β-CN for six weeks. 2. Group 2: All participants consumed A2 β-CN for other six weeks. Control visits were made at week 0, 6, 12, 18 and 24.	TGs – A1 (BL): 1.4 mmol/l	TGs – A2 (BL): 1.4 mmol/l
			TGs – A1 (6w): 1.2 mmol/l	TGs – A2 (6w): 1.5 mmol/l
			TGs – A1 (12w): 1.4 mmol/l	TGs – A2 (12w): 1.3 mmol/l
			TC – A1 (BL): 6.3 mmol/l	TC – A2 (BL): 6.3 mmol/l
			TC – A1 (6w): 3.6 mmol/l	TC – A2 (6w): 3.7 mmol/l
			TC – A1 (12w): 5.6 mmol/l	TC – A2 (12w): 5.7 mmol/l
			LDL – A1 (BL): 3.7 mmol/l	LDL – A2 (BL): 3.7 mmol/L
			LDL – A1 (6w): 3.6 mmol/l	LDL – A2 (6w): 3.7 mmol/l
			LDL – A1 (12w): 3.3 mmol/l	LDL – A2 (12w): 3.4 mmol/l
			HDL – A1 (BL): 1.8 mmol/l	HDL – A2 (BL): 1.8 mmol/l
			HDL – A1 (6w): 1.8 mmol/l	HDL – A2 (6w): 1.8 mmol/l
			HDL – A1 (12w): 1.6 mmol/l	HDL – A2 (12w): 1.7 mmol/l

			SBP – A1 (BL): 127 mmHg	SBP – A2 (BL): 127 mmHg
			SBP – A1 (6w): 131 mmHg	SBP – A2 (6w): 127 mmHg
			SBP – A1 (12w): 131 mmHg	SBP – A2 (12w): 131 mmHg
			DBP – A1 (BL): 77 mmHg	DBP – A2 (BL): 77 mmHg
			DBP – A1 (6w): 76 mmHg	DBP – A2 (6w): 73 mmHg
			DBP – A1 (12w): 77 mmHg	DBP – A2 (12w): 75 mmHg
Venn, Skeaff, Brown, Mann, and Green (2006)	Exposure to low-fat cow's commercial milk contain either A1/A2 or A2 β-CN. Exposure to full-fat cow's cheese A1/A2 or A2 β-CN. The study lasted two 4.5 week periods without washout.	Participants consumed 500 ml/d A1 β-CN or A2 β-CN milk and 28 g/d A1 β-CN or A2 β-CN cheese. More specifically, all participants were advised to not consume dairy products. 1. Group 1: All participants consumed A1 β-CN for 4.5 weeks. 2. Group 2: All participants consumed A2 β-CN for 4.5 weeks.	TGs – A1/A2 (BL): 1.48 mmol/l	TGs – A2 (BL): 1.48 mmol/l
			TGs – A1/A2 (4.5w): 1.33 mmol/l	TGs – A2 (4.5w): 1.34 mmol/l
			TC – A1/A2 (BL): 5.92 mmol/l	TC – A2 (BL): 5.92 mmol/l
			TC – A1/A2 (4.5w): 5.60 mmol/l	TC – A2 (4.5w): 5.63 mmol/l
			LDL – A1/A2 (BL): 3.97 mmol/l	LDL – A2 (BL): 3.97 mmol/L
			LDL – A1/A2 (4.5w): 3.73 mmol/l	LDL – A2 (4.5w): 3.75 mmol/l
			HDL – A1/A2 (BL): 1.28 mmol/l	HDL – A2 (BL): 1.28 mmol/l
			HDL – A1/A2 (4.5w): 1.26 mmol/l	HDL – A2 (4.5w): 1.27 mmol/l
Kamiński, Cieslinska, and	Exposure to raw bovine milk contain either		TC – A1/A2 (1w): 87.33 mg/dl	TC – A2 (1w): 88 mg/dl

Fiedorowicz (2012)	A1/A1 β-CN or A2/A2 β-CN over a period of 6 weeks.	All subjects were fed with the normal diet with addition of A1/A1 or A2/A2 β-CN milk. 1. Group 1: All participants consumed A1/A1 β-CN for 6 weeks. 2. Group 2: All participants consumed A2/A2 β-CN for other 6 weeks.	TC – A1/A2 (2w): 89.67 mg/dl	TC – A2 (2w): 90.33 mg/dl
			TC – A1/A2 (3w): 87.67 mg/dl	TC – A2 (3w): 87 mg/dl
			TC – A1/A2 (4w): 92 mg/dl	TC – A2 (4w): 87 mg/dl
			TC – A1/A2 (5w): 94 mg/dl	TC – A2 (5w): 92.67 mg/dl
			TC – A1/A2 (6w): 109 mg/dl	TC – A2 (6w): 106.67 mg/dl
			HDL – A1/A2 (1w): 40 mg/dl	HDL – A2 (1w): 43.67 mg/dl
			HDL – A1/A2 (2w): 42 mg/dl	HDL – A2 (2w): 47.33 mg/dl
			HDL – A1/A2 (3w): 43.67 mg/dl	HDL – A2 (3w): 46 mg/dl
			HDL – A1/A2 (4w): 48 mg/dl	HDL – A2 (4w): 45.67 mg/dl
			HDL – A1/A2 (5w): 50 mg/dl	HDL – A2 (5w): 49.33 mg/dl
			HDL – A1/A2 (6w): 55.33 mg/dl	HDL – A2 (6w): 54 mg/dl

Table 3. The effect of bovine milk, β-CN, and BCM7 on diabetes mellitus

Study	Exposure	Intervention (Dosage)	Results
Chin-Dusting et al. (2006)	Exposure to bovine milk (milk powder) contain either A1 β-CN or A2 β-CN (10 g/d) throughout the 24 - week study.	Participants consumed 25 g/d of the dairy shake (A1 β-CN or A2 β-CN) prepared in 200 ml water or fruit juice. More specifically, all participant were advised to not consume dairy products (excluding cheese). 1. Group 1: All participants consumed A1 β-CN for 12 weeks. 2. Group 2: All participants consumed A2 β-CN for another 12 weeks. Control visits were made at week: 0, 6, 12, 18 and 24.	**Plasma insulin concentration, A1 (mean)** Baseline: 11.8 mU/l. After 6 weeks: 10.1 mU/l. After 12 weeks: 8.8 mU/l.
			Plasma insulin concentration, A2 (mean) Baseline: 11.8 mU/l. After 6 weeks: 7.8 mU/l. After 12 weeks: 9 mU/l.
Chia et al. (2018)	Five generations have been exposed to bovine milk diet (milk powder) containing either A1 β-CN or A2 β-CN (60.53 g/100 g) for a period of 30 weeks.	4 generations, out of F0 generation have been included in the study: F1, F2, F3, and F4 and were fed either A1 β-CN or A2 β-CN supplemented diets.	**Incidence of Type 1 Diabetes (T1D)** No difference in diabetes incidence was observed between the two cohorts from F0 to F2 generations (F1: A1 18.4 % vs. A2 21.6 %; F2: A1 18.2 % vs. A2 13.2 %). In F3 generation, the incidence of diabetes increased twice in the group which had been fed with A1 β-CN (40 %) in comparison with the group A2 β-CN (20.7 %).

		1. Group 1 (F0 – F4): Half of the participants consumed A1 β-CN for 30 weeks. 2. Group 2 (F0 – F4): Half of the participants consumed A2 β-CN for 30 weeks.	F4 generation: Fasting BGLs were notably higher in NOD mice fed with A1 β-CN (7.0 ± 0.4 mM) vs A2 β-CN (5.5 ± 0.5 mM, $p<0.05$).
			F0 - F4 generations: Splenic $CD4^+CD25^+FoxP3^+$, $CD4^+CD25^-FoxP3^+$, macrophage, $CD4^+$, $CD8^+$, B-cell number had not been changed in NOD mice which consumed A1 β-CN or A2 β-CN. Only significant decrease in Treg from $CD4^+CD25^-FoxP3^+$ was observed in subjects consuming A1 β-CN diet.
			The presence of BCM7 peptide in both types of diet and all generations was not detected.
			Compared to A2 β-CN fed NOD mice, A1 β-CN supplementation increased the level of several bacterial species, such as *Streptococcus pyogenes* & *Streptococcus suis* (bacteria related with incidence of T1D).
			Both A1 β-CN and A2 β-CN supplemented diet did not have an effect on the gastrointestinal integrity of the mice.

Table 4. The effect of bovine milk, β-CN, and BCM7 on neurological disorders

Study	Exposure	Intervention (Dosage)	Results
González-Domenech et al. (2020)	Normal diet (ND); and Gluten-free and Casein-free diet (GFCF) were examined in the study over a period of 1 year. No washout between treatments.	1. Group 1: All participants were consuming a ND for 6 months. 2. Group 2: All participants were consuming a GFCF diet for 6 months. 3. Group 1: All participants were consuming a GFCF for additional 6 months. 4. Group 2: All participants were consuming a ND diet for additional 6 months.	**BCM7 concentration in patients' urine (mean ± SEM)** 1. Before the GFCF diet: 3.63 ± 4.4 ng/ml (n = 17). 2. After the GFCF diet: 2.30 ± 3.0 ng/ml (n = 10). No significant decrease of BCM7 in urine after the examination period.
Jianqin et al. (2015)	Cow's milk containing either A1/A2 β-CN (conventional milk) with a ratio of 58:42 or only A2 β-casein (industry milk). The duration of the examination was 14 days.	1. 2 weeks (Initially): Group 1 = A1 - β-CN & Group 2 = A2 - β-CN: were consuming 250 ml/day of their allocated milk after meals. 2. After that 2 Weeks: Washout period – no dairy products. 3. Finally another 2-week period: Group 1* = A2 - β-CN & Group 2* = A1 - β-CN: were consuming 250 ml/day of their allocated milk after meals.	**Subtle Cognitive Impairment Test** The computer-based test for speed and effectiveness showed that participants who consumed milk containing A1/A2 β-CN genetic variant demonstrated slightly longer processing times and higher error levels comparable to participants who consumed only milk consisting of A2 β-CN genetic variant.

Table 5. The effect of bovine milk, β-CN, and BCM7 on athletes' performances and other health-related outcomes

Study	Exposure	Intervention (Dosage)	Results
Yadav et al. (2020)	Fat-free bovine milk contain either A1/A1, A1/A2, or A2/A2 β-CN over a period of 30 weeks (the treatment has been maintained for 5 days a week in addition to the standard pellet diet - a quantity equivalent to a one glass of milk for humans).	Male Balb/c mice were randomly divided into 4 experimental groups: 1. Control: RO water (water purified by reverse osmosis). 2. A1/A1: RO water + 10 ml/kg body weight A1/A1 milk (oral gavage). 3. A1/A2: RO water + 10 ml/kg body weight A1/A2 milk (oral gavage). 4. A2/A2: RO water + 10 ml/kg body weight A2/A2 milk (oral gavage).	**Airway hyperresponsiveness (Flexi Vent)** 1. Penh: A1/A1 > A2/A2 2. Airway resistance (AR): A1/A1 > Control and A2/A2 3. Penh + AR: Intermediate response = A1/A2 **Th2 cytokines levels in mice lungs** 1. Control: - IL-4 in BAL (≈ 16.0 pg/ml); - IL-5 in BAL (≈ 12.0 pg/ml); - IL-5 in serum (≈ 5.71 pg/ml); - INF_y in BAL (≈ 13.7 pg/ml); - INF_y in serum (≈ 32.0 pg/ml). 2. A1/A1: - IL-4 in BAL (≈ 54.0 pg/ml); - IL-5 in BAL (≈ 33.1 pg/ml); - IL-5 in serum (≈ 32.0 pg/ml); - INF_y in BAL (≈ 14.3 pg/ml); - INF_y in serum (≈ 33.1 pg/ml). 1. A1/A2: - IL-4 in BAL (≈ 38.9 pg/ml); - IL-5 in BAL (≈ 24.0 pg/ml); - IL-5 in serum (≈ 11.4 pg/ml); - INF_y in BAL (≈ 16.0 pg/ml);

			- INF_y in serum (≈ 32.0 pg/ml). 1. A2/A2: - IL-4 in BAL (≈ 18.9 pg/ml); - IL-5 in BAL (≈16.0 pg/ml); - IL-5 in serum (≈ 10.3 pg/ml); - INF_y in BAL (≈ 17.1 pg/ml); - INF_y in serum (≈30.3 pg/ml).
			IgG and IgE levels in mice lungs 1. Control: - IgE in BAL (≈ 12.4ng/ml); - IgG in BAL (≈ 2739 ng/ml); - IgE in serum (≈ 2143 ng/ml); - IgG in serum (≈ 928571 ng/ml). 2. A1/A1: - IgE in BAL (≈ 14.8 ng/ml); - IgG in BAL (≈ 8095 ng/ml); - IgE in serum (≈ 4929 ng/ml); - IgG in serum (≈ 1160714 ng/ml). 3. A1/A2: - IgE in BAL (≈ 13.3 ng/ml); - IgG in BAL (≈ 4881 ng/ml); - IgE in serum (≈ 3214 ng/ml); - IgG in serum (≈ 982143 ng/ml). 4. A2/A2: - IgE in BAL (≈ 12.90 ng/ml); - IgG in BAL (≈ 4643 ng/ml); - IgE in serum (≈1714 ng/ml);

			- IgG in serum (≈ 1160714 ng/ml).
			Cellular infiltration of lymphocytes and eosinophils in mice 1. Control: - Tot. BAL C x 10^5 (≈ 0.87); - Tot. Blood C x 10^6 (≈ 12.8); - Eosi. BAL C x 10^5 (≈ 0.01); - Eosi. Blood C x 10^6 (≈ 0.05); - Lym. BAL C x 10^5 (≈0.05); - Lym. Blood C x 10^6 (≈ 8.60). 2. A1/A1: - Tot. BAL C x 10^5 (≈ 1.87); - Tot. Blood C x 10^6 (≈ 17.1); - Eosi. BAL C x 10^5 (≈ 0.04); - Eosi. Blood C x 10^6 (≈ 0.08); - Lym. BAL C x 10^5 (≈ 0.08); - Lym. Blood C x 10^6 (≈ 10.8). 3. A1/A2: - Tot. BAL C x 10^5 (≈ 1.6); - Tot. Blood C x 10^6 (≈ 14.4); - Eosi. BAL C x 10^5 (≈ 0.03); - Eosi. Blood C x 10^6 (≈ 0.07); - Lym. BAL C x 10^5 (≈ 0.06); - Lym. Blood C x 10^6 (≈ 8.80).

			4. A2/A2: - Tot. BAL C x 10^5 (≈ 0.70); - Tot. Blood C x 10^6 (≈ 12.3); - Eosi. BAL C x 10^5 (≈ 0.01); - Eosi. Blood C x 10^6 (≈ 0.05); - Lym. BAL C x 10^5 (≈ 0.05); - Lym. Blood C x 10^6 (≈ 8.40).
Kirk et al. (2017)	Diet with semi-skimmed bovine milk contain either A1/A2 β-CN - regular milk (RM) or A2 β-CN for a period of 5 days. Placebo group (PL) (50 g maltodextrin mixed with water) has been invited.	Participants randomly separated in three groups consumed 500 ml/d A1/A2 β-CN RM or A2 β-CN milk. More specifically, all participant were advised to not consume additional dairy products. 1. Group 1 (RM): 7 team sport players consumed RM (A1/A2) β-CN for 4 days. 2. Group 2: 7 team sport players consumed A2 β-CN for 4 days. 3. Group 3 (PL): 7 team sport players consumed maltodextrin mixed with water for 4 days. Control visits were made at 0, 24, 48, 72 h.	**20 - m Sprint** 1. 0 and 24 h: No differences between groups have been found. 2. 48 h: Sprint time recovered quicker in A2 β-CN and RM consumers in contrast with the PL consumers, representing 3.3 ± 0.1, 3.3 ± 0.3, and 3.6 ± 0.3, respectively. Moreover, the repeated sprint bout decreased decrements by 5.1 % (A2 β-CN) and 5.2 % (RM) immediately after the 20 - m sprint time ($p<0.05$). **Countermovement jump height (CMJ):** 48 h: CMJ recovered quicker in A2 β-CN and RM consumers in contrast with the PL consumers, representing 33.4 ± 6.6, 33.1 ± 7.1, and 29.2 ± 3.6, respectively. Moreover, the repeated sprint bout decreased decrements by 7.2 % (A2 β-CN) and 6.3 % (RM) immediately after the CMJ height ($p<0.05$). **Maximal Voluntary Isometric Contraction (MVIC)**: There were not shown any differences between the time and groups ($p>0.05$).

			Visual Analogue Scale (Muscle Soreness): There were not shown any differences between the time and groups ($p>0.05$).
Deth, Clarke, Ni, and Trivedi (2015)	Bovine milk containing either A1/A2 β-CN (conventional milk) with a ratio of 58:42 or only A2 β-CN (industry milk). The duration of the examination was 14 days.	1. 2 weeks (Initially): Group 1 = A1 β-CN and Group 2 = A2 β-CN: were consuming 250 ml/day of their allocated milk after meals. 2. After the 2 Weeks: Washout period – no dairy products. 3. Finally another 2-week period: Group 1 = A2 β-CN and Group 2 = A1 β-CN: were consuming 250 ml/day of their allocated milk after meals.	**Plasma Glutathione (GSH - mean ± SE):** 1. A1/A2 β-CN: 1.99 ± 0.50 nmol/ml 2. A2 β-CN: 4.01 ± 0.61 nmol/ml The change of the GSH concentrations was higher in the first period [Sequence = A2 to A1/A2 (4.07 nmol/ml) in comparison with the second period sequence = A1/A2 to A2 (2.70 nmol/ml) for 1.37 nmol/ml]. **BCM7 concentrations** 1. A1/A2 β-CN: 0.87 - 0.98 ng/ml 2. A2 β-CN milk: 0.71–0.73 ng/ml

Explanation of the terms used within the thesis

1. **Proteoform** refers to the different forms of proteins produced from the genome with a variety of sequence variations, splice isoforms, and myriad posttranslational modifications that are critical elements in all biological systems (Smith et al., 2013). A case in point is β-casein A2-5P, a proteoform that refers to a genetic variant β-casein A2.
2. **Genetic variant** of a protein refers to a version of the protein that is encoded by a different allele (variant) of the gene that codes for that protein. These variants can result in differences in the amino acid sequence of the protein, which can affect its structure, function, and properties (Caroli, Chessa, & Erhardt, 2009). In particular, β-casein A1 and β-casein A2 are genetic variants.
3. **Phenotype** is the observable trait that is the result of the interaction between its genetic makeup (genotype) and the environment. Identifying phenotypes of proteins is a central challenge of modern genetics in the post-genome era and this is still a discussion at the protein level up until now (Hu et al., 2011). For instance, β-casein A1/A1, β-casein A1/A2 and β-casein A2/A2 are phenotypes.
4. **Haplotype** is at the gene level rather than the protein level. Haplotype refers to groups of genetic variants that co-occur on single chromosomes (Snyder, Adey, Kitzman, & Shendure, 2015). Namely, at the casein locus, CSN1S1 [B] - CSN1S2 [A] - CSN2 [A2] - CSN3 [A] is a haplotype and it represents a cow whose genomic DNA encodes for αs_1-casein B, αs_2-casein A, β-casein A2, and κ-casein A.
5. **Genotype** refers to the entire collection of genes of an organism, not limited to a single chromosome or locus on a chromosome. And this is also at the gene level, rather than a protein level (Churchill, 1974; Johannsen, 1911; Mahner & Kary, 1997).

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Conferences presentations, attended schools, and PhD mobility

1. 8th International Conference on Food Digestion, Portugal, 2024
2. University of Wisconsin - Madison, Center for Dairy Research: Research Fellow, USA, 2023
3. IDF World Dairy Summit, USA, 2023
4. IFT First: Annual Event and Expo, USA, 2023
5. 2nd Dairy Science Summer School: Your role in future green dairy systems, University College Cork, Ireland, 2023
6. 3rd Annual Dairy Science and Technology Symposium, delivering with dairy: From primary production to primary purpose, University College Cork, Ireland, 2023
7. Nordic Rheology Conference: Soft Matter Science meets Food, Aarhus University, Denmark, 2023
8. Rheology Course: Soft Matter Science meets Food, Aarhus University, Denmark, 2023
9. 50th Food Science and Technology Annual Conference, University College Cork and The Institute of Food Science and Technology of Ireland, Ireland, 2022
10. 7th Symposium on Science and Technology of Fermented Milk, International Dairy Federation, Belgium, 2022
11. Teagasc, The Agriculture and Food Development Authority: Walsh Scholar, Ireland, 2022 - 2023
12. Summer School: Dairy Nutrition and Health, Wageningen University, The Netherlands, 2022
13. Summer School: Dairy Protein Biochemistry, Wageningen University, The Netherlands, 2022
14. FrieslandCampina: PhD Visiting Research Fellow, The Netherlands, 2022
15. International Conference on Food Analysis, Melbourne, Australia, 2021
16. Winter School: Food Proteins, Wageningen University, The Netherlands and Aarhus University, Denmark, 2020

Awards and grants obtained during the PhD studies

1. Finalist for the Early Career Award, 8th International Conference on Food Digestion, Portugal, 2024
2. Winner of The Gold Medal Award as part of the Walsh Scholar of the Year Competition, Teagasc, Ireland, 2023
3. Winner of The Best Presentation as part of the Walsh Scholar of the Year Competition, Teagasc, Ireland, 2023
4. Walsh Scholar of the Year in The Food Programme, Teagasc, Ireland, 2023
5. Winner of The Early Career Scientist Award Professor Pavel Jelen, International Dairy Federation, Chicago, USA, 2023
6. International Training Award, Teagasc, The Agriculture and Food Development Authority, Ireland, 2023
7. Best Student Oral Presentation Second Prize, 50th Food Science and Technology Annual Conference, University College Cork and The Institute of Food Science and Technology of Ireland, Ireland, 2022
8. Winner of The Oral Presentation Award, 7th Symposium on Science and Technology of Fermented Milk, International Dairy Federation, Belgium, 2022
9. COVID-19 2022 RTP Allowance Programme Scholarship, Victoria University, Australia, 2022
10. Crystal Prize Best Paper 2020 Scientific Session - PhD Students and Young Scientists, University of Ruse, Bulgaria, 2020
11. Walsh Scholarship Programme, Government and Teagasc, Ireland, 2020 - 2024
12. International Postgraduate Research Scholarship, Government and Victoria University, Australia, 2020 - 2024