EFFECT OF CARBON EMISSION REDUCING DIET ON BOVINE MILK PROPERTIES

A thesis submitted for the degree of Doctor of Philosophy

by

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I dedicate this thesis to my parents,

Mr. Maddun Abbas & Mrs. Ratna Dewi

Who have instilling in me the desire to work hard

and learn new things

Abstract

This study was aimed to investigate the effect of methane emission reducing diet on composition and processing properties of bovine milk. Treatment diets included supplementation of cottonseed oil (800 g/d, CSO), *A. mearnsii*- condensed tannin (400 g/d, TAN) or their combination (CPT) to a control diet, consisting of 6.0 kg dry matter concentrates and ad libitum hay. In contrast to the TAN diet, the CSO and CPT diets decreased milk fat content and the proportion of saturated fatty acids but increasing proportions of monounsaturated and polyunsaturated fatty acids. The TAN diet had no effect on feed intake, milk yield and milk protein content, but it depressed the yields of protein and lactose. All supplemented diets did not affect protein content or composition, nitrogen content, or casein to total protein ratio of the resulting milk. All treatment diets did not alter gelation time (GT), storage modulus (G′), loss modulus (G″), loss tangent (tan δ), permeability, water holding capacity and hardness of acid- and rennet-induced gels. These diets had a marginal effect on fat and protein recoveries, cheese yield, composition, textural properties and organic acid production of the cheeses. All cheeses showed comparable primary and secondary proteolysis compared to that of control. Degrees of primary and secondary proteolysis kept increasing throughout ripening time. Cheeses manufactured from milk of cows fed a diet containing cottonseed oil had higher concentration of medium- and long-chain fatty acids (FA) but lower in short-chain FA. In contrast, supplementation of condensed tannin in the diet resulted in cheese with comparable FA composition to that of control. Throughout ripening time, the

concentration of short-, medium-, and long-chains FAs tend to remain stable. A further study is required to establish the effect of diet supplementation on lipolysis of cheeses. Furthermore, dietary treatments can be used to produce Cheddar cheese with a new property without compromising consumer preferences for appearance, firmness, flavour, aroma, and overall acceptance. Results support that these supplemented diets could be used to mitigate methane emission without altering milk processing properties such as milk coagulation and Cheddar cheese making properties.

Certificate

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CERTIFICATE

This is to certify that the thesis entitled "Effect of carbon emission reducing diet on bovine milk properties" submitted by **Aprianita** in partial fulfilment of the requirement for the award of the Doctor of Philosophy in Food Technology at Victoria University is a record of bonafide research work carried out by her under my personal guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Werribee, Australia
Date 07-10-2015

Prof. Todor Vasiljevic
(Principal supervisor)

Student Declaration

"I, **Aprianita**, declare that the PhD thesis entitled "EFFECT OF CARBON EMISSION REDUCING DIET ON BOVINE MILK PROPERTIES" 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".

Signature Date: 20-05-2015

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List of Abbreviations

Abbreviations key:

GHG	Greenhouse gases
CH_4	Methane
CO_2	Carbon dioxide
H_2	Hydrogen
Ca	Calcium
P	Phosphor
CFCs	Chlorofluoro-carbons
N_2O	Nitrous oxide
NH_3	Ammonia
NaCl	Sodium chloride
LA	Linoleic acid (C18:2 n-6)
ALA	Linolenic acid (C18:3 n-3)
ARA (AA)	Arachidonic acid (C20:4 n-6)
EPA	Ecosapentaeinoic acid (C20:5 n-3)
DHA	Docosahexaenoic acid (C22:6 n-3)
FA	Fatty acid
FFAs	Free fatty acids
VFAs	Volatile fatty acids
MUFA	Monounsaturated fatty acid
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acid
SCFA	Short-chain saturated fatty acid
LCFA	Long chain saturated fatty acid
VA	Vaccenic acid

List of Abbreviations

CLA	Conjugated linoleic acid
AA	Amino acid
CT	Condensed tannins
HT	Hydrolysable tannins
MFD	Milk fat depression
GDL	Glucono-δ -lactone
LAB	Lactic acid bacteria
NSLAB	Non-starter lactic acid bacteria
MNFS	Moisture non-fat solids
FDM	Fat dry matter
S/M	Salt to moisture
DPI	Department of Primary Industry
PRP	Proline-rich protein
CN	Casein
β-Lg	β-lactoglobulin
α-Lac	α-Lactalbumin
BSA	Bovine serum albumin
IG	Immunoglobulins
ATP	Adenosine-triphosphate
GTP	Guanosine-triphosphate
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
MP	Microbial protein
MCP	Microbial crude protein
HG/LR	High grain/low roughage
VFI	Voluntary feed intake
DM	Dry matter
NDF	Non-dietary fibre
GMP	Glycomicropeptide
HCl	Hydrochloric acid

List of Abbreviations

CCP	Colloidal calcium phosphate
IP	Isoelectric point
SAOR	Small amplitude oscillatory rheology
G′	Storage modulus
G″	Loss modulus
tan δ	Tangent δ
GT	Gelation time
WHC	Water holding capacity
CE	Capillary electrophoresis
RP-HPLC	Reverse-Phase High Performance Liquid Chromatography
WSN	Water soluble nitrogen
PTA-SN	Phosphtungstic acid-soluble nitrogen
TCA-SN	Triflouroacetic acid-soluble nitrogen
LPL	Lipoprotein lipase
TPA	Texture profile analysis
CON	Control
CSO	Cottonseed oil
CPT	Cottonseed oil plus tannins
TAN	Tannins
$CaCl_2$	Calcium chloride
SSC	Somatic cell count
MFGM	Milk fat globule membrane
PR	Protein recovery
FR	Fat recovery
Ya	Actual cheese yield
Yp	Predicted yield
Yma	Moisture-adjusted yield
GLM	General linier model
L	Lightness
a	Greenness

b	Yellowness
EMP	Embden-Meyerhof-Parnas

1. Introduction

1.1 BACKGROUND

Global warming is one of the most serious threats to the global environment ever faced in human history. This phenomenon is related to the increase in concentration of greenhouse gases (GHG) in the atmosphere (Howden and Reyenga, 1999). Annually, the accumulation of these gases increases by 0.3 to 0.9% mainly due to anthropogenic effects on the carbon and nitrogen cycles (Boadi et al., 2004). Besides extreme climate changes in the future, other expected consequences of this global warming include coastal flooding, spreading disease, and mass extinctions (Johnson and Johnson, 1995; Moss et al., 2000; Boadi et al., 2004). Therefore, it is generally accepted that the concentration of GHG in the atmosphere should not be allowed to continue to rise.

Of all the GHG, carbon dioxide (CO_2) receives the most attention since it contributes up to 53% of the total GHG contribution to the overall earth warming. However, other gasses are also of importance; these include methane (CH_4), tropozone, chlorofluoro-carbons (CFCs), and nitrous oxide (N_2O), which contribute 17%, 13%, 12%, and 5%, respectively, to the climate change (Johnson and Johnson, 1995). Methane is the second largest contributor to the global warning. This gas is more than 20 times as effective as CO_2 at trapping heat in the atmosphere and has a fast accumulation rate (Boadi et al., 2004). Currently ~70% of methane production arises from anthropogenic sources and the remainder from natural sources with agriculture considered being responsible for about two thirds of the anthropogenic sources (Johnson and Johnson, 1995; Boadi et al., 2004). Globally, domestic ruminant livestock including dairy cattle is considered to be the largest sources of

methane released into the atmosphere (Johnson and Johnson, 1995; Moss et al., 2000; Boadi et al., 2004). A similar trend occurs in Australia where agriculture sector contributes 16% of the GHG emission in this country and become the second largest emission source after the energy sector. The majority of agricultural emissions in Australia are associated with animal agriculture (enteric fermentation), which contributes 65% to the total of agricultural emissions (Howden and Reyenga, 1999).

Besides its potential to cause environmental problems, methane emission from dairy cows is also associated with loss of feed energy to support animal productivity (Boadi et al., 2004). Therefore, an ideal strategy to reduce enteric methane emission should be able to reduce methane emission without compromising animal productivity (Hegarty, 2009). In general, strategies to mitigate enteric methane emission include biotechnology, additives and feeding (Moss et al., 2000; Boadi et al., 2004). Among these strategies, feeding strategy especially through lipid supplementation is more preferable since, besides ability to reduce methane emission (Beauchemin and McGinn, 2008; Grainger et al., 2010), lipids are often added to animal diet to increase energy density to support milk production (Chilliard et al., 2001; Jenkins and McGuire, 2006; Hegarty, 2009). In addition, supplementation of lipids high in polyunsaturated fatty acids (PUFA) into animal diet can be directed to improve health quality of milk and other dairy products manufactured from the milk since this supplementation associated with reduction in the amount of saturated fatty acids (SFA) and increase in the amount of PUFA in milk (Bauman and Griinari, 2001). Besides lipid supplementation, dietary plant secondary metabolites such as

condensed tannins (CT) have been reported that could reduce methane emission through their effect on metanogenic microbes (Ushida and Jouany, 1996). The presence of condensed tannins in animal diet has attracted many attentions due to their effect on feed intake and animal performance (Patra and Saxena, 2010).

While reduction of methane release appears an on-going research focus, a little attention has been devoted to the effects of these strategies from the whole farming system including the effect of these strategies on the quality and yield of primary products of farming including milk (Beauchemin and McGinn, 2008). In other side, production or economic benefits are the main factors which determine adoptability of mitigation practices by the livestock producers (Hristov et al., 2013). Thus, when assessing methane mitigation practises, we should also consider adoptability of these practices by the farmers who unlikely to adopt mitigating strategy which has no positive economic benefits for them (Beauchemin and McGinn, 2008).

In general, milk composition can be affected by several factors such as physiological stages, genetics and dietary requirements of animals (Lindmark-Masson, 2008; Bionaz et al., 2012). Among these factors, animal diet could result in a quicker response (Emery, 1988). According to Walker et al. (2004), animal diet could influence chemical composition of milk by direct transfer of plant compounds or by modifying the cow's metabolism. Compared to other milk chemical components such as protein and lactose, milk fat is more sensitive to any changes in animal diet (Bauman and Griinari, 2001). Feeding lipids high in polyunsaturated FA may alter rumen biohydrogenation and induce milk fat depression (MFD), which

associated with reduction in milk fat content, decrease in the amount of short and medium (C4:0-C14:0) FA, and increase in the proportions of monounsaturated and polyunsaturated FA (Bauman and Griinari, 2001). Thus this supplementation could be directed to improve the health quality of milk and other dairy products manufactured from the milk. Compared to the effect of supplemented lipids on milk fat content, effect of lipid on milk protein content (Medeiros et al., 2010; He and Armentano, 2011) and composition (Zhang et al., 2006) is less pronounced and the reported effects vary among studies. Supplemented fat could affect milk protein content by affecting the availability of amino acids (Dunkley et al., 1977) or metabolised energy (de Veth et al., 2006; Weerasinghe et al., 2012) required for protein synthesis. Adding fat into animal diet also could increase its energy density to support milk production (Chilliard et al., 2001; Jenkins and McGuire, 2006; Weiss et al., 2011).

Condensed tannins (CT) are plant secondary metabolites belonging to a phenolic group which are found in many forage legumes, trees and shrubs (Barry and McNabb, 1999). Once being chewed, these compounds are able to form pH dependent-complexes with protein, cellulose, starch, and minerals in the forages (Reed, 1995; Makkar, 2003). Their ability to form these complexes then might influence the effect of CT on composition of the milk (Khiaosa-Ard et al., 2009). Condensed tannin could affect the availability of amino acid for milk protein synthesis by binding protein in forages thus protecting degradation of this protein in rumen (Barry and McNabb, 1999; Woodward et al., 1999; Min et al., 2003). In addition, their binding with fibre or cellulose may influence production of volatile

fatty acids which are required for synthesis of milk lipids (Dahlberg et al., 1988; Khiaosa-Ard et al., 2009). Condensed tannins may also affect composition of milk fatty acids through their effect on the activity of rumen microflora (Piredda et al., 2002; Cabbidu et al., 2005).

Alteration in milk composition may subsequently affect milk technological characteristics such as coagulation (Johnson et al., 2001). Milk coagulation is an important process during transformation of milk into a variety of dairy products such as yoghurt and cheeses (Hallen, 2008). Milk coagulation occurs after destabilisation of casein micelles which then leads to formation of an aggregated protein network (Hallen, 2008). This process can be induced by addition of acids, rennet, or their combination (Horne and Banks, 2004). Milk coagulation induced by adding acidifying agents such as glucono-δ-lactone (GDL) is essential for the manufacture of yoghurt and fresh acid coagulated cheeses such as cream cheese, cottage cheese, and quarg (Phadungath, 2005). The rennet-induced coagulation is the basic step during cheese manufacturing (Lee and Lucey, 2004). Besides rheological characteristics such as gelation time and curd firmness, other coagulation parameters such as permeability and water holding capacity are also important in determining manufacturing process and the quality of dairy products. Milk coagulation is mainly affected by milk physicochemical properties, which include pH, and content of protein, casein, fat, and minerals in milk (Phadungath, 2005). Therefore animal diet has a potential to alter milk composition and milk coagulation properties. Diet may affect milk coagulation properties partly through its effect on content and composition of protein in milk (Masucci et al., 2006; de Renobales et al., 2012)..

Most of milk production in Australia is transformed into cheese with the Cheddar cheese as the most popular and the most produced in this country (Dairy Australia, 2013). In 2012/2013, total cheese production in Australia was 338,300 tonnes. Thus, approximately one third of the total milk production during that period was manufactured into cheeses (Dairy Australia, 2013). Cheese is a dairy product which protein and fat of the milk are concentrated (Van Boekel, 1994). Therefore, any changes in milk composition, especially fat and protein content may influence yield and quality of the resulting cheese.

Many biochemical processes occur in cheese during ripening period, and these include proteolysis, lipolysis, and glycolysis, all of which may affect the overall quality of the cheese (McSweeney and Sousa, 2000). Proteolysis is the most pronounce process during cheese ripening, in which caseins are degraded into smaller peptides and amino acids by proteinases, peptidases, coagulant, starter bacteria, and non-starter bacteria (McSweeney and Sousa, 2000). The extent of proteolysis during cheese ripening can be affected by the activity of the above microorganisms, cheese pH, and content of fat in cheese (Fenelon et al., 2000). Proteolysis also may affect cheese texture through degradation of protein matrix during ripening period, which softens cheese texture (Fenelon et al., 2000). In addition, proteolysis plays an important role during development of cheese flavour through production of small peptides and free amino acids (Fenelon et al., 2000; Upadhyay et al., 2004).

Lipolysis, which involves hydrolysis of lipids in cheese, may directly contribute to the cheese flavour by producing free fatty acids (FFA) (Fox, 1993) or

indirectly by producing substrates for synthesis of volatile compounds (Marilley and Casey, 2004) Another biochemical process during cheese ripening is Glycolysis, in which starter lactic acid bacteria (LAB) and non-starter lactic acid bacteria (NSLAB) metabolise lactose residue in cheese curd (McSweeney and Fox, 1993). Glycolysis could affect cheese texture due to development of crystals (McSweeney and Fox, 2004). In addition, metabolism of L-lactate into CO_2 and acetate by LAB and NSLAB could produce CO_2 and acetate which then may affect cheese flavour by causing off-flavour perception (Aston and Dulley, 1982).

Textural property of Cheddar cheese is an important factor that may affect overall quality of the cheese and consumer acceptance (Lucey et al., 2003). Cheese texture can be affected by cheese pH, degree of proteolysis, and cheese chemical composition especially content of fat, protein, moisture, moisture non-fat solids (MNFS), and fatty acid profile (Baer et al., 1996; Lucey et al., 2005). Changes in cheese hardness mainly occur during the early ripening period, which might be related to the extensive proteolysis, which disrupts protein matrix (Lucey et al., 2003). Moreover, cheese hardness may also be affected by concentration of C16:0 and cis-9-C18:1 which are the major milk saturated and unsaturated FA that have high and low melting points, respectively (Coppa et al., 2011).

Similar to cheese texture characteristic, sensory properties of cheese are also critical in terms of consumer acceptance (Soryal et al., 2005). Animal diet may affect cheese sensory properties through several mechanisms i.e. direct transfer of feed components, modification of fat and protein in milk, transfer of endogenous enzymes, which may affect proteolysis and lipolysis during cheese ripening, and

modification of microbial ecosystem (Coulon et al., 2004). In addition, cheese sensory properties can be affected by manufacturing process, chemical composition of the cheesemilk and biochemical process during cheese ripening such as proteolysis, glycolysis, and lipolysis (Fox, 1993; Fox and McSweeney, 1998). Fatty acid composition in cheese might also affect cheese sensory properties (Urbach, 1990).

The current study was part of collaborating research between Victoria University and Department of Primary Industry (DPI)-Ellinbank, Victoria, in investigating the effects of fat and condensed tannin supplemented to cow's diet on enteric methane emission and consequently milk properties. In this project, cottonseed oil was used as a fat supplement; while condensed tannins extracted from *Acaccia mearnsii* were used as dietary condensed tannins which separately or in combination were added into cow's diet. Feeding treatment and measurement of methane production were under control of DPI, while analysis of the effect of these modified diets on yield, composition, and technological properties of the resulting milk was performed at Victoria University and become the core of this study.

1.2 RESEARCH OBJECTIVES

The main objective of this study was thus to ascertain the effects of specific methane mitigating diet fed to dairy cows on the physico-chemical and technological properties of resulting milk.

The specific aims of this project were:

(1) To investigate the effects of supplementing a dairy cow diet with cottonseed oil, condensed tannin, or combination of cottonseed oil and condensed tannin on milk fat and protein composition.

(2) To confirm that supplementing the cow diet with fat, tannin, or a combination of these would not affect the coagulation properties of the milk.

(3) To establish the effects of feeding fat and tannin-supplemented cow's diet on yield, composition, proteolysis, and textural properties of the respective cheddar cheese.

(4) To investigate the effects of cottonseed oil- and condensed tannin-supplemented diets on colour, organic acids, fatty acid profile, and sensory properties of the resulting cheddar cheese.

1.3 OUTLINE OF THESIS

With respect to the content of this thesis, the background and objectives of this thesis are stated in Chapter 1. Chapter 2.0 contains a review of current state of knowledge related to the effect of dietary fat and condensed tannins on chemical composition and coagulation properties of bovine milk. Effect as the supplemented diets on Cheddar cheese properties was also included. Chapter 3.0 examines the influence of supplemented cottonseed oil and condensed tannin on milk composition. Chapter 4.0 investigates the effects of the supplemented diets on milk coagulation properties. The effects of the diets on yield, composition, proteolysis, and textural

properties of the respective cheddar cheese are discussed in Chapter 5.0. Chapter 6.0 reports on the influence of the diets on colour, organic acids, fatty acid profile, and sensory properties of the resulting cheddar cheese. A conclusion of study base on results obtained from Chapter 3 to Chapter 6 as well as a future research direction are summarised in Chapter 7. Finally, List of references is presented in Chapter 8.

2 Literature review

2.1 METHANE EMISSION FROM RUMINANTS

2.1.1 Methane production in the rumen

Ruminants (cattle and sheep) are the main contributors to methane (CH_4) production in the agricultural sector (Johnson and Johnson, 1995; Howden and Reyenga, 1999; Moss et al., 2000). In the ruminant digestive system, CH_4 is mainly (80-90%) produced in the rumen (Howden and Reyenga, 1999; Martin et al., 2008a). The rumen provides a very stable environment for growth of bacteria that digest carbohydrates, organic acids, proteins and fats, as well as bacteria that produce ammonia, methane or synthesize vitamins (Moss et al., 2000). Once fodders have been chewed, bacteria digest carbohydrates released from the fodders through an oxidative process under anaerobic condition in the rumen. This process produces volatile fatty acids (VFAs), which become the main source of energy for the animal. The main VFAs produced are acetate, butyrate, and propionate. Besides the VFAs, the enteric fermentation also produces hydrogen (H_2) as an end product. The abundance and type of VFAs production determines the amount of H_2 produced. In general, production of acetate would result in twice the amount of H_2 compared to H_2 produced during production of butyrate. In contrast, the production of propionate uses up H_2 (Beauchemin and McGinn, 2008; Morgavi et al., 2010). Rumen methanogens, microorganisms that produce methane in rumen, use H_2 and carbon dioxide (CO_2) to produce methane (Figure 2.1). Thus production of methane by rumen methanogens is the main pathway for removing H_2 generated by anaerobic microbes. The generated methane is then released from animals into the atmosphere mainly through animal breathing (Howden and Reyenga, 1999; Martin et al., 2008a).

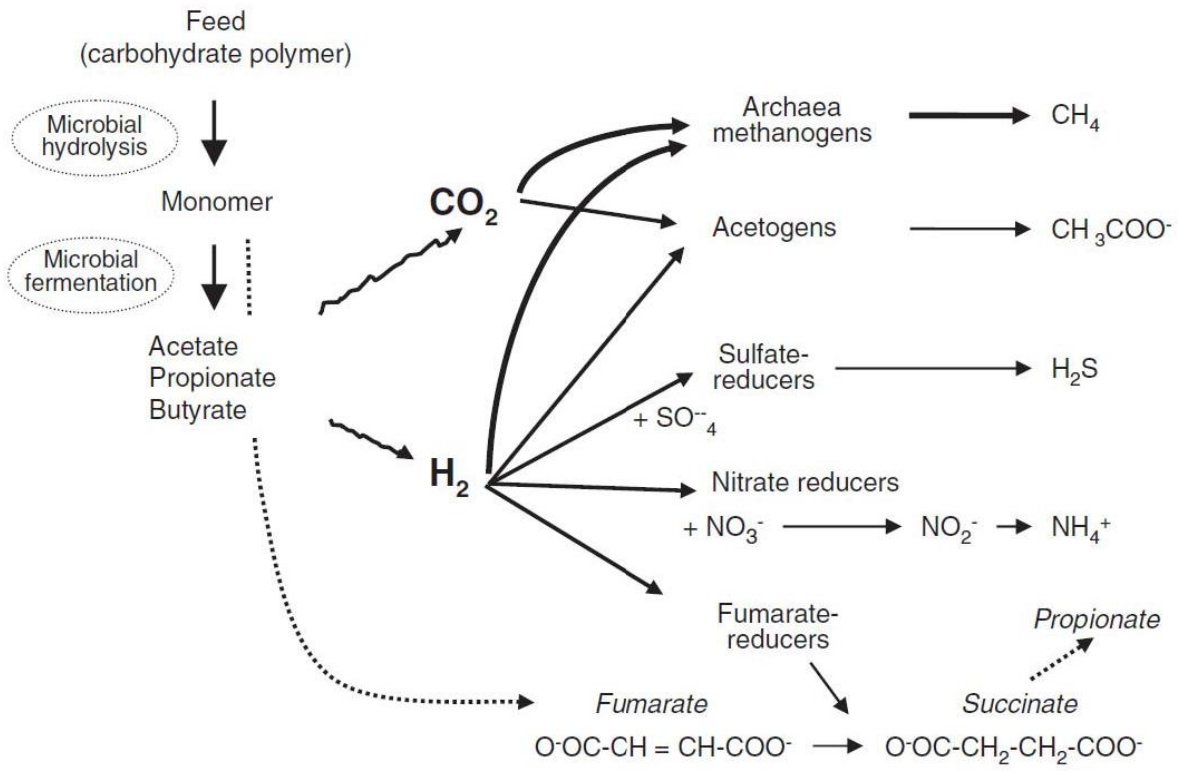


Figure 2.1. Schematic microbial fermentation of feed polysaccharides and H_2 reduction pathways in the rumen
Source: Morgavi et al. (2010)

2.1.2 Strategies to reduce enteric methane emission

Basically, there are three mitigation strategies to reduce methane emission from ruminants: biotechnology, additives, and feeding (Moss et al., 2000; Boadi et al., 2004; Martin et al., 2008b). These strategies are directed to reduce methane emission either by reducing production of hydrogen, transforming hydrogen into other products such as propionate, and inhibiting methane formation (Beauchemin and McGinn, 2008).

Biotechnology

Biotechnological strategy includes vaccination, use of probiotics and defaunation (Moss et al., 2000). Inhibition of methanogens by immunizing ruminants against their own methanogens could reduce methane emission by ~8% (Moss et al., 2000). Probiotic treatment involves the addition of exogenous microorganism, which can influence rumen fermentation (Moss et al., 2000; Boadi et al., 2004; Martin et al., 2008a).

Additives

Additive strategy to reduce methane production from ruminants includes the addition of antibiotics or chemicals to inhibit metanogenesis (Moss et al., 2000). However, the use of antibiotics and chemicals are prohibited due to their toxic effects on ruminants after prolonged feeding (Sahakian et al., 2010). Alternatively, plant extracts such as essential oils and saponnins could be used as additives through their inhibitory effect on metanogenesis and protozoa (Moss et al., 2000; Boadi et al., 2004; Martin et al., 2008a). However, despite their potency in reducing enteric methane emission, biotechnology and additive strategies are currently under investigation and need more development. Thus, these strategies are not expected to be commercially available in the near future (Beauchemin and McGinn, 2008).

Feeding

Feeding strategy is another option to reduce production of enteric methane through modification of animal diet (Beauchemin and McGinn, 2008). The strategy could reduce methane production in the ruminants by lowering the amount of

methane formed per kilogram of feed. This can be achieved by feeding high grain diet, choosing types of grain, supplementing diets with fats and oils, feeding legume forages such as alfalfa and clover, and feeding corn silage or small grain silage. Feeding strategy also can be performed by adding plant secondary compounds (i.e. condensed tannins, saponins and essential oils), yeast and organic acid to animal diet (Beauchemin and McGinn, 2008). However, despite their reducing effect on enteric methane emission, the amount of methane produced during processing or transporting the grain and forage as well as the cost associated with this strategy should be considered (Moss et al., 2000).

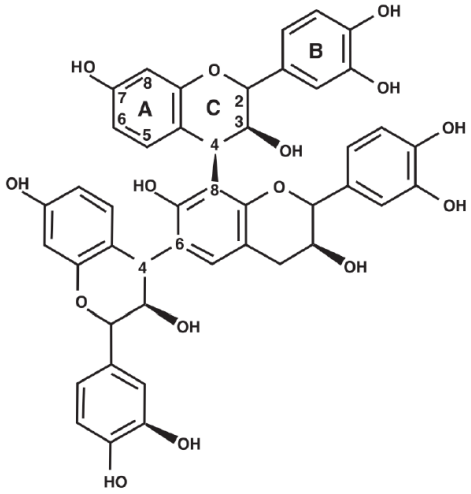
Feeding strategy through lipids supplementation is preferable since, in addition to their ability to reduce methane emission (Grainger et al., 2009), they also increases energy density to support milk production (Chilliard et al., 2001; Hegarty, 2009; Weiss et al., 2011). Cottonseed oil is a by-product extracted from the kernels of cotton plants such as *Gossypium hirsutum* and *G. herbaceum* (Balasubramanya and Shalkh, 2007). Linoleic acid forms the highest percentage (50.76%) of fatty acids in cottonseed oil, followed by palmitic, oleic, stearic, and myristic with 24.70, 20.87, 2.20, and 0.79%, respectively (Balasubramanya and Shalkh, 2007). Feeding cottonseed oil to dairy cows could provide high energy source to the animal as well as influence feed intake and milk composition (Mohamed et al., 1988; Zheng et al., 2005). Recently, it has been reported that inclusion of whole cottonseed (Grainger et al., 2010) in dairy cow's diet reduced methane emission in the animals. Effect of lipid supplementation on enteric methane emission is influenced by types and concentration of the supplemented lipids.

Tannins are phenolic plant secondary metabolites, which are widely distributed in forage legumes, trees, and shrubs (Min et al., 2003). This plant compound is also present in many animal fodders especially the species of *Acacia, Dichrostachys, Dorycnium, Hedysarum, Leucaena, Lotus, Onobrychis, Populus, Rumex* and *Salix* (Mueller-Harvey, 2006). Concentration of tannins in plants is influenced by plant maturity, temperature, grazing and soil fertility (McMahon et al., 2000). Based on their chemical structure, tannins are categorized as hydrolysable tannins (HT) and condensed tannins (CT). Hydrolysable tannins are mainly found in fruit pods and plant galls as plant protection mechanism against insects and herbivores (McLeod, 1974). This type of tannins contains a carbohydrate moiety as the central core with phenolic groups esterified on the hydroxyl group of the carbohydrate (Figure 2.2; Patra and Saxene, 2010). On the other hand, condensed tannins, which do not have a carbohydrate core, are complexes of oligomers and polymers of flavonoid units linked via carbon-carbon bonds (Figure 2.2; Naumann et al., 2013).

Due to presence of multiple phenolic hydroxyl groups, tannins are able to form complexes mainly with proteins and to a lesser extent with metal ions, amino acids, and polysaccharides (Makkar, 2003). The ability of tannins to form complexes with these compounds would govern the effect of tannins on metabolisms of protein, carbohydrate, and lipids in ruminants and consequently chemical composition of the resulting milk (Khiaosa-Ard et al., 2009). Effect of dietary condensed tannin in reducing methane emission from ruminants has been reported previously (Ushida and Jouany, 1996). Feeding ruminant with fodder tree especially from *Acacia*

species, as a part of methane mitigating practice is important not only from methane mitigating point of view, but also for development of a sustainable farming system since this species is drought tolerant and grows well in arid areas which make it a valuable source of feed (Krebs et al., 2003).

A



B

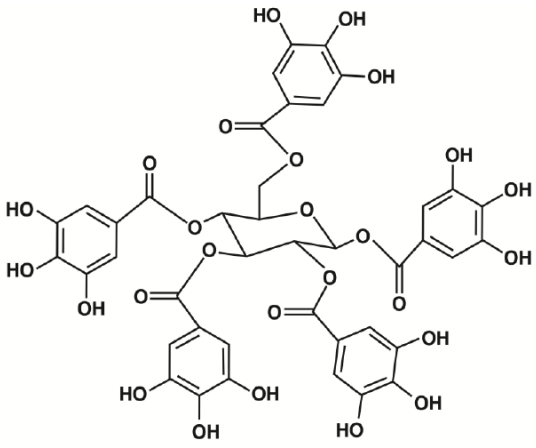


Figure 2.2 Monomeric units of condensed (A) and hydrolysable (B) tannins
Source: Naumann et al (2013)

2.1.3 Effect of lipids and condensed tannins supplementations on enteric methane emission

Effect of lipids supplementation on enteric methane emission

Dietary lipids could decrease enteric methane emission by lowering the activity of ruminal methanogens, decreasing protozoal numbers, and for lipids rich in unsaturated fatty acids (FA), through biohydrogenation of FA (Johnson and Johnson, 1995). The inhibition of methanogenesis by lipid supplementation is possibly due to the toxicity of long chain FAs to methanogenic bacteria (Henderson, 1973). However, there appears to be considerable variation in the effects of supplemental fats on CH_4 production and only a limited number of studies have been conducted using dairy cows (Johnson and Johnson, 1995). Reductions in CH_4 through lipid supplementation have been substantial in some cases: 26% reduction with extruded flaxseed, 49% reduction with flaxseed oil (Martin et al., 2008), and a 27% reduction with a mixture of sunflower and fish oil (Woodward et al., 2006). However, other dairy studies have shown no effects of fat addition on reduction of methane emission (Johnson et al., 2002).

Effect of condensed tannins supplementation on enteric methane emission

Even though the exact role of added condensed tannins in mitigating CH_4 emission from cows is still unclear, it was proposed that they could reduce CH_4 emission indirectly by reducing fibre digestion, which then decrease the amount of hydrogen (H_2) required by methanogens for CH_4 production (Carulla et al., 2005; Animut et al., 2008). Alternatively, supplemented condensed tannins could directly

affect methanogenic bacteria by reducing the amount of ciliate protozoa, which harbours methanogenic microbe in the rumen fluid (Ushida and Jouany, 1996). Tannins may also indirectly affect methanogenic microbes by influencing the number and type of phages (viruses) that infect methanogenic microbes. This would negatively affect methanogen or again reduce the number of ciliate protozoa (Boadi et al., 2004).

Despite the extensive body of research into dietary manipulation to reduce enteric methane emission, not enough attention has been paid to the effects of these modified diets on the resulting milk composition and its suitability for manufacturing purposes. An ideal methane mitigating strategy should result in a persistent decrease in methane emissions without detrimental effects on animal production (Beauchemin and McGinn, 2008). Cow's diet might influence milk production and milk composition such as fat content, fatty acid (FA) profile, protein content and protein composition by direct transfer of plant compounds or by modifying cow metabolism (Walker et al., 2004). The remaining part of this review would discuss effects of fat and condensed tannin supplemented-diets on production, composition, coagulation, and manufacturing properties of bovine milk.

2.2 EFFECT OF FAT AND CONDENSED TANNINS SUPPLEMENTATIONS ON ANIMAL FEED INTAKE, YIELD AND COMPOSITION OF BOVINE MILK

2.2.1 Effect of supplementations on animal feed intake and milk yield

2.2.1.1 Effect of dietary fat on feed intake and milk yield

According to Allen (2000), supplemented fat may reduce feed intake by decreasing palatability and reducing fibre digestion, which then causes prolonged rumen fill. In addition, supplemented fat may increase the level of cholecystokinin, a hormone that mediate dry matter intake (DMI), in blood plasma (Bradford et al., 2008), and can inhibit reticuloruminal motility (Matzinger et al., 2000). Reduction in fibre digestion associated with fat supplements rich in PUFA may alter microbial population and rumen environment (Doreau and Chilliard, 1997).

Effect of supplemented fat on feed intake is influenced by a type and amount of fat in diet (Dschaak et al., 2011) as well as levels of saturation (Harvatine and Allen, 2006). In general, rumen-protected fat has less pronounced effect on feed intake since this fat is released in the rumen slowly, thus prevents the rapid effect on the rumen environment. As a result, rumen-protected fat has lower effect on fibre digestion. In contrast, supplementation of free oil has been reported to depress feed intake, which might be due to freely available fat, and can affect directly the rumen microorganisms (Dschaak et al., 2011). A study using supplemented fat at different saturation levels i.e. saturated, intermediate, and unsaturated showed that feed intake decreased linearly with the increasing levels of unsaturated fat (Harvatine and Allen,

2006). According to Allen (2000), high concentration of unsaturated fat in diet is associated with reduction in fibre digestion, and subsequently may affect feed intake.

Effect of dietary fat on milk yield may be associated with the effect of dietary fat on feed intake and increase in energy density of diet (Allen, 2000; Harvatine and Allen, 2006). Limitation in dietary energy is one of the main reasons for suppression of milk yield (Allen, 2000). Without sufficient energy, cows would produce less milk, well below their potential milk production (Harvatine and Allen, 2006). According to Newbold (1994), reducing effect of dietary fat on *de novo* synthesis FA may provide alternative source of energy to increase milk production. However, reports on the effects of dietary lipids on feed intake and milk production of ruminants vary. It appears that the effects are affected by a source and a level of fat in diet as well as stage of lactation (Newbold, 1994; Medeiros et al., 2010). Therefore, results from individual lipid supplement cannot be generalized for all lipid supplements (Dschaak et al., 2011).

2.2.1.2 **Effect of condensed tannin supplementation on feed intake and milk yield**

The presence of condensed tannins in animal diets may reduce feed intake by reducing palatability, upsetting mouth and throat tissues, or exerting astringency sensation in animal mouth by forming complexes with salivary glycoproteins (Landau et al., 2000). Condensed tannins may react with the outer layer of the gut giving a signal that would control feed intake in the ruminants (Provenza et al., 1990). Reduction in feed intake is part of animal's defence mechanism against the

toxicity effect of tannins. Some animals such as mouse, rat and deer have an adaptation mechanism towards a high concentration of tannins in diets by producing proline-rich protein (PRP) in their saliva. This type of protein protects the animals by binding to the tannins and preventing interaction of the tannins with other proteins. This salivary protein also could minimise absorption of tannins and reduce toxicity of tannins (Austin et al., 1989). However, this kind of an adoptive defence mechanism is absent in ruminants such as sheep, cattle, and goat (Austin et al., 1989; McArthur et al., 1995).

Effect of dietary condensed tannins on feed intake is influenced by their concentration in diet (Waghorn et al., 1997; Barry and McNabb, 1999). Condensed tannins present at high concentration (10% of dry matter) in diets tend to depress feed intake (Waghorn et al., 1997; Barry and McNabb, 1999). However, reports on the effects of supplemented tannins on feed intake are inconsistent and appeared to be affected by factors such as animal species, physiological state of the animals and diet composition (McSweeny et al., 2001).

Effect of dietary condensed tannins on ruminant milk production varies among studies (Woodward et al., 1999; Turner et al., 2005). For example, dairy cows fed *Lotus corniculatus* had higher milk production compared to control (Woodward et al., 1999). In contrast, Wang et al (1996) reported that dietary condensed tannin showed no effect on milk production when early-lactating ewes were fed *L. corniculatus*. However, the same study reported an increase in milk production with mid- and late-lactation ewes. The author reported that the increase in milk

production was not accompanied with an increase in voluntary feed intake (Wang et al., 1996).

2.2.2 Effect of cottonseed oil and condensed tannin supplements on milk composition

In addition to their effect on animal feed intake and milk production, dietary fat and plant secondary metabolites, such as condensed tannins, may influence metabolism of lipids, carbohydrate, and nitrogen in the rumen (Figure 2.3; Lourenco et al., 2010), and further may result in alteration of milk composition (Dschaak et al., 2011). Prior to a discussion on the effect of fat and condensed tannins supplementation on milk composition, a brief summary of bovine milk composition is presented in this review.

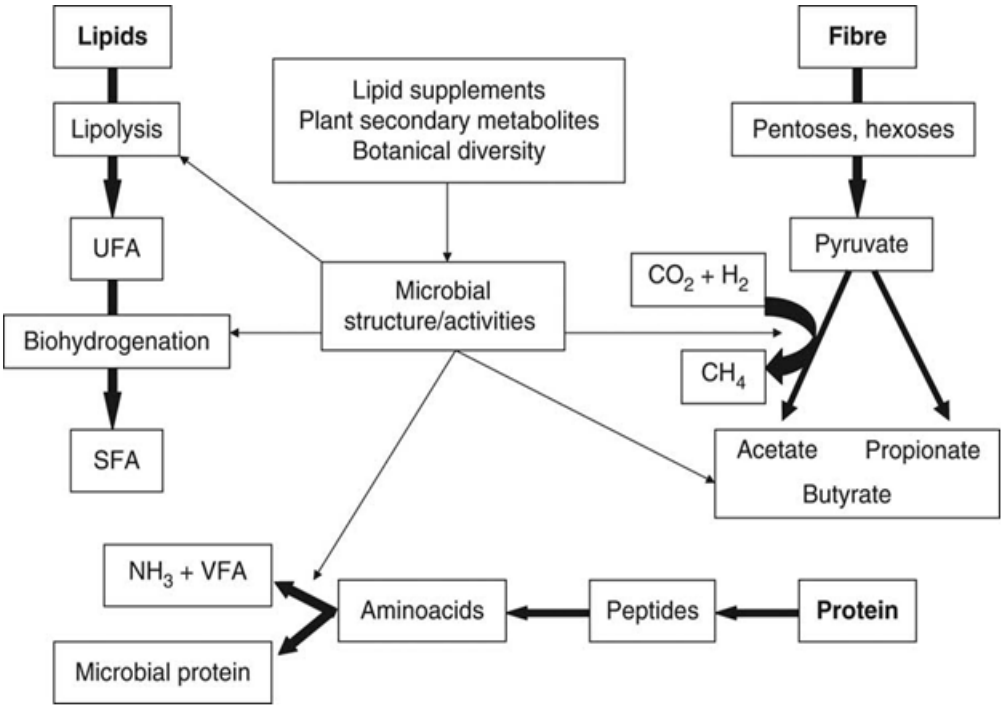


Figure 2.3 Intervention to manipulate fermentation and metabolism in the rumen
Source: Lourenco et al (2010)

2.2.2.1 **Composition of bovine milk**

The principal components of bovine milk are presented in Table 2.1. In general, bovine milk consist of water (~87%), lactose (~4.6%), fat (~4.4%), proteins (~3.4%), mineral (~0.7%), and vitamins (~0.1%) (Hennessy, 2011). This composition is influenced by several factors such as environment, animal genetic, diet, stages of lactation, and season (Fox and McSweeney, 1998). Milk composition is important not only because of its nutritional value, but also its role in technological properties of milk and dairy products. In addition, milk composition determines milk pricing in the dairy industry (Heck, 2009).

Table 2.1 The principal component of bovine milk
Source: Hennessy (2011)

Milk components	Percentage (%)
Water	86.6
Fat	4.4
Lactose	4.6
Casein	2.7
Whey protein	0.6
Non Protein Nitrogen	0.1
Minerals	0.7
Salts	0.17
Vitamin and enzymes	0.13

Milk Proteins

Content and composition of milk proteins have been receiving increasing interest as they affect nutritional values and technological properties of the milk (Heck et al., 2008). Nowadays in many countries, the value of milk proteins exceeds those of milk fat (Bauman and Griinari, 2001). Based on their chemical composition and physical properties, bovine milk proteins can be categorized into two major types i.e. caseins and whey proteins. Caseins contain phosphorous and would precipitate at pH 4.6. Whey proteins contain a large amount of sulphur-containing amino acids and remain in milk solution at pH 4.6 (Fox and McSweeney, 1998). Besides these two major milk proteins, others proteins such as lactoferrin, lactoperoxidase, lysozyme, β-2 microglobulin and other enzymes are also present in milk. The composition of proteins in bovine milk is presented in Table 2.2.

Table 2.2. Composition of protein in bovine milk
Source: Bionaz et al. (2012)

Milk proteins	Concentration (g. kg^{-1})
Total casein	**26**
α_{s1}-casein	10.7
α_{s2}-casein	2.8
β-casein	8.6
κ-casein	3.1
γ-casein	0.8
Total whey protein	**6.3**
β-lactoglobulin	3.2
α-lactalbumin	1.2
Serum albumin	0.4
Proteose peptone	0.8
Immunoglobulin	**0.80**
$IgG_{1,2}$	0.65
IgA	0.14
IgM	0.05
Lactoferrin	0.10
Lysozyme	126 x 10^{-6}

Caseins

Caseins are the major class of proteins in bovine milk comprising ~80% of the total milk protein. The casein fraction consists of several genetic variants i.e. α-caseins (α_{s1}-CN and α_{s2}-CN), β-casein (β-CN), κ-casein (κ-CN) and γ-casein (γ-CN) with specific amino acid compositions, genetic variations and functional properties for each type (Wedholm et al., 2006). The main characteristic of these proteins is the absence of a secondary structure, which provides them with a greater heat stability.

The lack of this secondary structure might be associated with the high amount of proline residues in the casein molecules (Paulson and Dejmek, 1990).

The α_{s1}-casein of bovine milk is the major structural component of the casein micelle and plays an important role during curd formation (Walstra and Jenness, 1984). This type of casein has eight or nine phosphorylation sites, which form two phosphorylation centres (de Kruif and Holt, 2003) and three hydrophobic regions (Swaisgood, 2003). The α_{s2}-casein is the most phosphorylated casein with eleven phosphorylated serine residues, with lesser amount containing 10, 12, or 13 phosphate groups (Swaisgood, 2003). Bovine β-casein has four or five phosphorylated serine residues. The N-terminus of this protein is very hydrophilic, followed by a relatively random hydropathy distribution in the rest of protein, and a hydrophobic C-terminus (Swaisgood, 2003).

The bovine κ-casein is the only glycosylated member of the casein family; about two-third of bovine casein molecules are glycosylated at one of six threonyl residues (Pisano et al., 1994). The presence of a glycan moiety in the C-terminal of κ-casein enhances its ability to stabilize the micelle by electrostatic repulsion, and may increase the resistance of the protein to proteolytic enzymes and high temperature (Minkiewiez et al., 1993; Dziuba and Minkiewiez, 1996). The level of glycosylation does not affect the micelle structure but it does affect the susceptibility of κ-casein to hydrolysis by chymosin, with susceptibility decreasing as the level of glycosylation increases (Zbikowska et al., 1992).

Whey proteins

Whey proteins comprise 20% of total bovine milk protein (Fox and McSweeney, 1998). The major components of whey protein family in bovine milk are β-Lactoglobulin (β-Lg) and α-Lactalbumin (α-Lac). Blood serum albumin, immunoglobulins, lactoferrin, transferrin, and many minor protein and enzymes are present in smaller amount (Fox and McSweeney, 1998). β-Lactoglobulin contains two disulphide bonds (between cys 66 and cys 160; and between cys119 and cys 119 and cys 121) which cause the amino acids chain to form a compact spherical shape. These disulphide bonds can be broken which then leads to loss of a compact structure. This process called denaturing process is important during yoghurt manufacturing (Fox and McSweeney, 1998). α-Lactalbumin plays an important role during lactose synthesis by acting as a substrate modifier, thus increasing specificity and affinity of galactosyltransferase for glucose during lactose synthesis in mammary gland (Johnke and Petersen, 2012).

In comparison to β-Lactoglobulins and α-Lactalbumin, minor whey proteins such as bovine serum albumin (BSA) and immunoglobulins (IGs) are present at much lower concentration in milk. Serum albumin binds to fatty acids and other small molecules; however the function of serum albumin is still unknown. Immunoglobulins are part of the mammary immune system and present at low amount in milk (Fox and McSweeney, 1998). Other proteins such as lactoferrin, lactoperoxidase, lysozyme, and β2-microglobulin are also found in bovine milk in small amounts. Lactoferrin has antibacterial properties and may also be an immunomodulator. The amount of this protein in bovine milk is low during

lactation, but is increased during mastitis and involution. Lactoperoxidase breaks down hydrogen peroxide as well as has antibacterial properties. Lysozyme cleaves carbohydrate polymers of bacterial wall. The function of β2-microglobulin in milk is still unknown. Besides minor proteins mentioned above, other enzymes such as proteases, protease activator, nucleases, glycosidase, etc. are also present in milk (Fox and McSweeney, 1998).

Synthesis of milk protein

Major milk protein components such as caseins (α-, β-, κ-, and γ- caseins), and whey proteins (β-lactoglobulin and α-lactalbumin) are synthesized in the mammary glands (Fox and McSweeney, 1998). Synthesis of milk protein from nitrogen sources requires the presence of rumen microorganisms, a large supply of energy from the carbohydrate fermentation, amino acid availability, and protein synthesis machinery (Bionaz et al., 2012). Most of feed proteins entering the rumen are degraded into ammonia and amino acids. Ammonia produced from this degradation is used by rumen microorganism for their growth. The availability of energy generated from carbohydrate metabolism would determine the extent of ammonia used for microbial protein synthesis. As cows eat more feed, the bacteria contain more protein and pass from the rumen to the abomasum more rapidly. Some of forages proteins may be resistant to ruminal degradation and pass undegraded to the small intestine. A portion of bacterial proteins is broken down within the rumen, but majority flow to the abomasum attached to the feed particles. Approximately, 60% of the amino acids absorbed through the small intestine are from bacterial proteins, and the remaining 40% is from ruminally undegraded dietary proteins

(Wattiaux, 1998). The strong acids secreted by abomasum stop all microbial activity and digestive enzymes start breaking down the proteins into amino acids (AA). Most of the amino acids absorbed by the mammary glands are used to synthesis milk protein (Figure 2.4; Wattiaux, 1998).

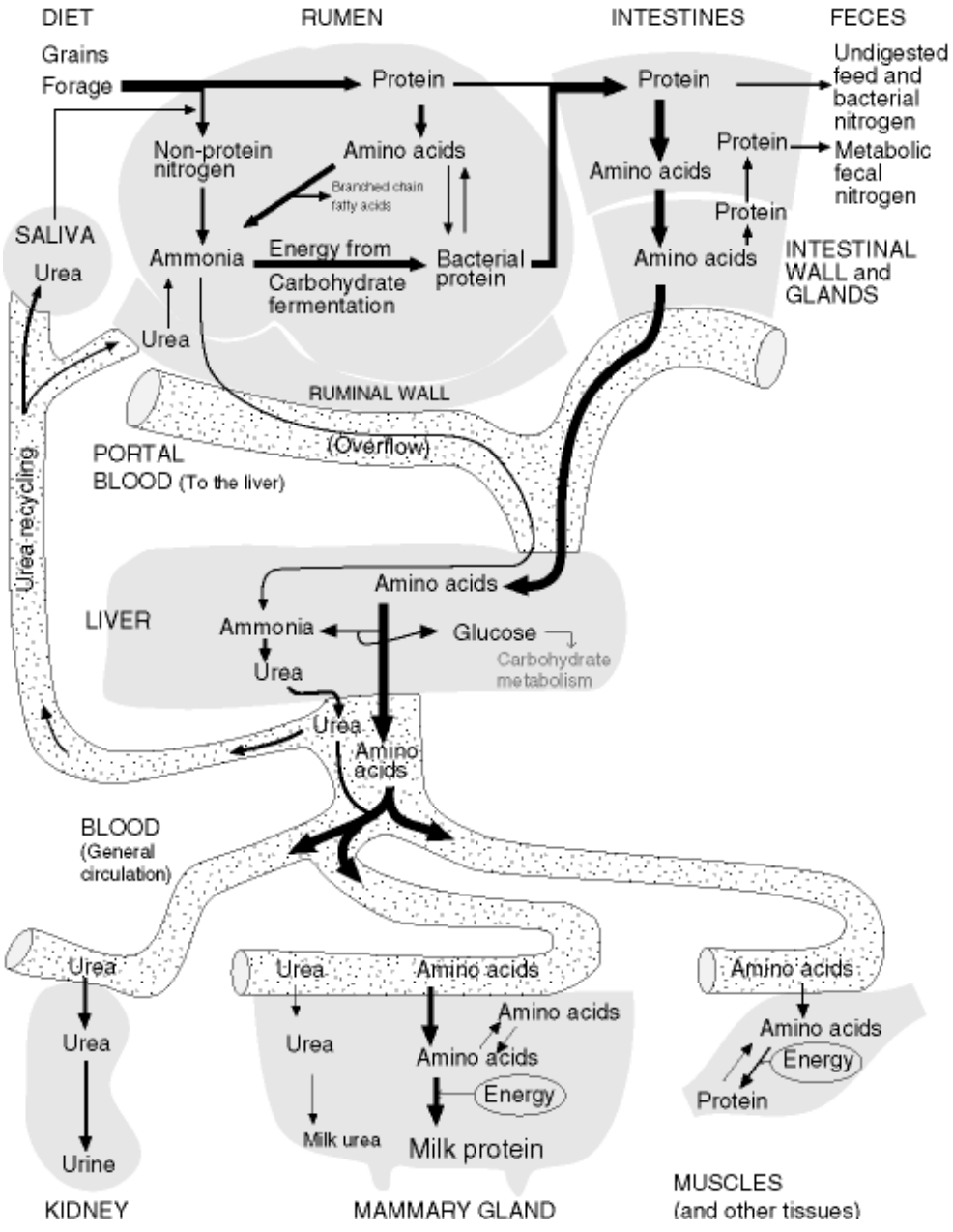


Figure 2.4 Synthesis of milk protein in ruminants
Source: Wattiaux (1998)

Effect of animal diet on milk protein synthesis

The amount and composition of milk protein are influenced by factors such as animal genetics, availability of energy, amino acids, parity, stages of lactation, breed, feed intake, and animal diet (van Knagsel et al., 2005; Bionaz et al., 2012). Amongst these factors, genetics of animals plays the most important role. However, even though effect of animal diet on milk protein content is relatively small, changes in animal diet may exert quicker response (Emery, 1988). Animal diets provide metabolized energy and amino acids, which are highly required during protein synthesis in the mammary glands (Van Knagsel et al., 2005). Metabolized energy generated from carbohydrates fermentation provides energetic precursors to generate intracellular energy transfer molecules such as adenosine-triphosphate (ATP), guanosine-triphosphate (GTP), nicotinamide adenine dinucleotide (NADH), and nicotinamide adenine dinucleotide phosphate (NADPH). This energy may also increase insulin secretion which positively affects signalling network during synthesis of milk protein (Hanigan et al., 2002).

Diets rich in more readily fermentable carbohydrates and high degradation of rumen organic matter may increase supply of energy for microbial protein synthesis in the rumen. Thus, greater supply of microbial protein would cause higher milk protein yield (Zhu et al., 2013). However, when fermentable energy is lacking or when crude proteins in the diet are highly degradable, not all ammonia produced in rumen is incorporated into microbial proteins. The excess ammonia is absorbed across the rumen wall and is transported to the liver. The liver converts the ammonia to urea which is released in the blood. Urea in blood can follow two routes: (1) it

returns to rumen through the saliva or through the rumen wall and converted back to ammonia to serves as nitrogen source for bacterial growth or (2) It is excreted into the urine by the kidney (Wattiaux, 1998).

Animal diet may also provide amino acids for milk protein synthesis in mammary glands. The presence of specific amino acids such as methionine and lysine can be a limiting factor during milk protein synthesis (Bequette et al., 1998). Besides being utilised for protein synthesis, these amino acids may be oxidized to produce energy through the Krebs cycle (Mepham, 1982; Bequette et al., 1998). Despite the importance of amino acid availability, metabolized energy has more significant role for milk protein synthesis as indicated by the increase of milk protein yield following abomasal starch infusion to cows, while infusion of casein could not increase the yield (Rius et al., 2010).

Milk fat content and fatty acid composition

Milk fat has been attracting great deal of attention due to its importance as the main energy component in milk and source of essential fatty acids (FA). In addition, milk fat may influence flavour and rheology of dairy products (Fox and McSweeney, 1998). Typically, bovine milk contains approximately 3.5% of fat; however, this amount may vary depending on type of animal diet, stage of lactation, breed, season, animal health and age, and interval between milkings (Fox and McSweeney, 1998). Approximately, 97.5% of total lipids in bovine milk fat consists of triacylglycerols; while other milk lipids such as diacylglycerol, cholesterol, phospholipids, and free fatty acids (FFA) are present in lower amounts (~2%, <0.5%, <1%, and ~0.1%, respectively) (Jensen et al., 1991). Milk fat triacylglycerols

are synthesized from many fatty acids, with fatty acids between C4:0 and C18:0 as the main components (Jensen et al., 1991).

Bovine milk fats contain more than ~400 fatty acids; however most of them are present in trace amount (Christie, 1995). The main fatty acids in bovine milk are presented in Table 2.3. Typically, fatty acid profile of bovine milk contain high amount of short- and medium chain fatty acids but low amount of polyunsaturated fatty acids (Fox and McSweeney, 1998). Saturated fatty acids (SFA) comprise 69.4% of total milk fatty acids, while the rest 30.6% are unsaturated fatty acids (UFA). From a quantitative point of view, palmitic acid (C16:0), which makes up 30% of total FAs, is the most important followed by stearic acid (C18:0), which accounts for 12% of total FAs. Myristic acid (C14:0), butyric acid (C4:0) and caprionic acid (C6:0) make up 11%, 4.4 and 2.4%, respectively (Table 2.2; Lindmark-Mansson, 2008).

Monounsaturated fatty acids (MUFA) account for ~30% of total FAs in milk with oleic acid (C18:1) constituting the biggest portion of MUFAs with 22.8%. Approximately, ~2.3% of the total fatty acids is comprised of polyunsaturated fatty acids (PUFAs). Linoleic acid (C18:2) and α-linolenic (C18:3) acid account for 1.6 and 0.7% of the total fatty acids respectively are the main components of PUFAs. The unsaturated fatty acids appear as cis or trans isomers. The concentration of trans-isomers in bovine milk is about 2.7% of the fatty acids with one or more trans-double bonds (Precht and Molkentin, 1995). Vaccenic acid (VA) (C18:1, 11t), constitutes ~2.7% total FA content, and it is the main trans 18:1 isomer. Milk fat also contains conjugated linoleic acid (CLA), with many different isomers such as

rummenic acid (RA) (cis-9, trans-11 CLA), and constitutes 75-90% of total CLA (Precht and Molkentin, 1995). The CLA in milk fat is generated through two pathways - endogenous synthesis in mammary glands and biohydrogenation of PUFA by rumen bacteria (Bauman and Lock, 2006). Majority of CLA in milk fat is endogenously synthesized in the mammary glands through the action of mammary Δ^9-desaturase on vaccenic acid (VA). Biohydrogenation of PUFA in rumen contributes to a lesser extent, the total amount of CLA in milk fat (Bauman and Lock, 2006).

Table 2.3 Example of fatty acid composition expressed as per cent by weight of total fatty acids
Source: Lindmark-Mansson (2008)

Shorthand designation[1]	Fatty acids		Amount (%)
	Systematic name	Trivial name	
C4:0	Butanoic acid	Butyric acid	4.4
C6:0	Hexanoic acid	Caproic	2.4
C8:0	Octanoic acid	Caprylic	1.4
C10:0	Decanoic acid	Capric	2.7
C12:0	Dodecanoic acid	Lauric	3.3
C14:0	Tetradecanoic acid	Myristic	10.9
C15:0	Pentadecanoic acid		0.9
C16:0	Hexadecanoic acid	Palmitic	30.6
C17:0	Heptadecanoic acid	Margaric	0.4
C18:0	Octadecanoic acid	Stearic	12.2
C20:0	Eicosanoic acid	Arachidic	0.2
SFA[1] total			69.4
10:1	Δ9-Tetradecenoic	Decenoic acid	0.3
14:1	Δ9-Hexadecenoic	Myristoleic acid	0.8
16:1	Δ10-Heptadecenoic	Palmitoleic acid	1.0
17:1	Δ9-Octadecenoic		0.1
18:1		Oleic acid	22.8
MUFA[2], cis, total			25.0
C18:2	Δ9,12-Octadecadienoic	Linoleic acid	1.6
C18:3	Δ9,12,15-Octadecatrienoic	Linolenic acid	0.7
PUFA[3], cis, total			2.3
16:1t			0.4
18:1t	sum of C18:1 trans isomers	all trans 4 to 16Octadecenoic	2.1
18:2t	sum of C18:2 trans isomers	all trans 9,12Octadecadienoic	0.2
Trans FA total			2.7
CLA[4]	Δ9,11-Octadecadienoic	Rumenic acid	0.4

[1]Shorthand designation: SFA= saturated fatty acid; MUFA= monounsaturated fatty acid; PUFA= polyunsaturated fatty acid; CLA= conjugated linoleic acid.

Synthesis of milk fat

In ruminants, fatty acids in milk are derived from *de novo* synthesis in mammary glands, blood lipids via diet or other organs, or from both sources, using acetate and β-hydroxybutyrate as substrates (Figure 2.5; Fox and McSweeney, 1998). These precursors are produced during feed fermentation by microorganisms in the rumen, which are then transported to the blood. Some β-hydroxybutyrate are reduced to butyrate and directly incorporated into milk fat. This is the reason for a higher concentration of butyric acid in milk, which stays at a fairly constant level in comparison to other fatty acids (Fox and McSweeney, 1998). Other fatty acids are produced through malonyl CoA pathway using acetyl CoA, as a precursor. Acetyl CoA is derived from acetate and oxidation of β-hydroxybutyrate. In cytoplasm, acetyl CoA is converted to malonyl CoA. This malonyl CoA pathway produces 100% of C10, C12, C14, and 50% of C16:0 fatty acids. Others fatty acids such as C4:0, C6:0, and C8:0 are synthesised from acetate and β-hydroxybutyrate through pathways not involving malonyl CoA (Fox and McSweeney, 1998).

Some fatty acids such as C18:0, C18:1, C18:2 and 50% of C16:0 are derived from blood lipids (Fox and McSweeney, 1998). These lipids are hydrolysed by lipoprotein lipase present in the alveolar blood capillaries resulting in monoglycerols, free fatty acids, and some glycerols. These are then transported across the basal membrane and re-incorporated into triacylglycerol inside the mammary cell (Fox and McSweeney, 1998). Palmitic acid is lengthened by successive addition of acetyl CoA in liver mitochondria. Approximately 30% of oleic (C18:1) and palmitoleic (C16:1) acids are produced by desaturating stearic and

palmitic acids in the mammary glands, respectively by microsomal enzymes in the secretory cells (Fox and McSweeney, 1998). Linoleic (C18:2) and linolenic (C18:3) acids cannot be synthesised by mammals; thus they must be supplied in the diet. These fatty acids may be elongated or/and further desaturated by similar mechanism from stearic acid to oleic acids (Fox and McSweeney, 1998). Meanwhile, fatty acid with odd number of carbons such as pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) are synthesized in the rumen by bacterial flora (German and Dillard, 2006).

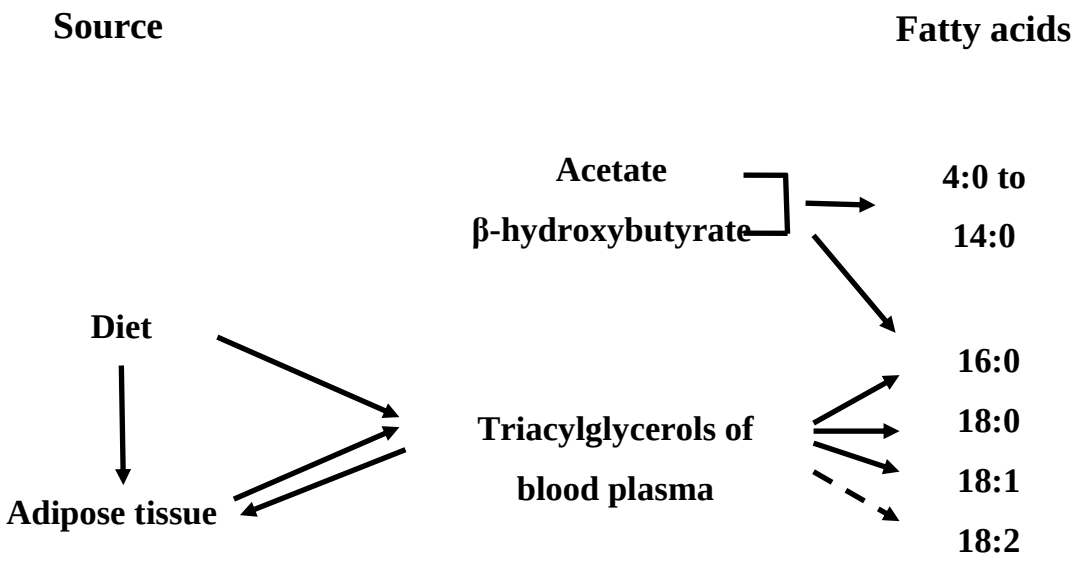


Figure 2.5 Sources of fatty acids in bovine milk
Source: Hawke and Taylor 1995)

2.2.2.2 **Effect of cottonseed oil on milk composition**

Effect of lipid supplementation on protein content of bovine milk

Reports on the effects of dietary lipids on yield and content of proteins in bovine milk varying ranging from decreasing (Wu and Huber, 1994; Dhiman et al., 2000), increasing (Medeiros et al., 2010), or no effect (Rego et al., 2005; 2009). There are some proposed theories, which explain mechanisms of how dietary lipids could reduce content of milk proteins. These theories include glucose deficiency (Smith et al., 1978), insulin resistance (Palmquist and Moser, 1981), increased energetic efficiency (DePeters and Cant, 1992) and somatotropin deficiency (Casper and Schingoethe, 1989). Based on glucose deficiency theory, lipids supplements may affect milk protein content by reducing the amount of glucose precursors (Smith et al., 1978). According to Palmquist and Moser (1981) fat supplements may cause insulin resistance, which then retards the uptake of amino acids for milk protein synthesis. However, insulin resistance theory is not supported by the fact that effect of fat supplements on plasma insulin is inconsistent (Mohamed et al., 1988).

According to DePeters and Cant (1992), fat supplements may increase energy efficiency for milk production through their effect on decreasing *de novo* fatty acid (FA) synthesis. Reduction in *de novo* FA synthesis would increase the availability of glucose for lactose synthesis, which then would increases milk production. Subsequently, increase in milk production would reduce mammary blood flow thus reducing amino acid availability for milk protein synthesis. However, this theory has not been supported by the available data (Cant et al., 1993). Moreover somatotropin deficiency theory explains that fat supplements may reduce milk fat synthesis by

reducing somatotropin from the pituitary gland, which then reduces amino acids uptake from blood for the synthesis of milk protein (Casper and Schingoethe, 1989). However, the data from subsequent studies did not support this assumption (Austin et al., 1991).

Another possible explanation for reducing effect of fat supplementation on milk protein synthesis is associated with the effect of fat on reducing the availability of amino acids by depressing carbohydrate digestibility or reducing microbial growth in the rumen (Dunkley et al., 1977). Inhibition of ruminal bacterial growth is important because microbial crude protein (MCP) derived from ruminal fermentation is the main component of microbial protein (MP) reaching the small intestine in lactating dairy cows, and qualitatively it has an essential amino acid (AA) profile that closely matches essential AA requirements for milk protein synthesis (NRC, 2007).

On the other hand, increase of protein content in ruminant milk due to fat supplementation appears to be related to increase of metabolized energy available (Hoover and Stokes, 1991). This increase is mainly due to reduction of milk fat synthesis in mammary glands and other peripheral organs such as adipose (de Veth et al., 2006; Weerasinghe et al., 2012). Increase in yield of milk proteins has been reported when diets were supplemented with conjugated linoleic acid (CLA) (Medeiros et al., 2010). Meanwhile, lack of an effect of supplemented fat on milk protein yield has been reported with sunflower oil (He and Armentano, 2011), and canola or flaxseed (Mutsvangwa et al., 2012). No change in protein content was observed in milk of cow fed diet supplemented with rapeseed, sunflower or linseed

(Rego et al., 2005). This lack of an effect could be related to unaltered flow of nitrogen for the milk protein synthesis (Mutsvangwa et al., 2012). Individual milk protein fractions appeared to be affected to a varying extent (Khorasani et al., 1991; Zhang et al., 2006). Inclusion of flaxseed to cow diet had minimal impact on protein fractions of the milk (Zhang et al., 2006), while reduction in casein concentration was observed in milk of cow fed canola fat (Khorasani et al., 1991).

Effect of fat supplementation on milk fat content and fatty acid composition

Basically, the amount and composition of fatty acids in milk are influenced by animal-related factors and feed-related factors (Lindmark-Mansson, 2008). Factors of animal origin include animal genetics, stage of lactation, mastitis, and ruminal fermentation. On the other hand, feed-related factors include fibre and energy intake, dietary fat, seasonal and regional effects (Lindmark-Mansson, 2008). Compared to animal origin-factors, effects of feed-related factors on milk fat content and fatty acid composition are more pronounced (Bauman and Griinari, 2001).

One phenomenon in milk fat related with animal feed is milk fat depression (MFD), a situation where certain diets affect yield of milk fat and alter milk fatty acid composition (Bauman and Griinari, 2001). Reduction in *de novo* fatty acids synthesis in mammary glands and increase in the amount of trans-C18:1 fatty acid are the main features when cows develop MFD (Bauman and Griinari, 2001). These changes shift the composition of milk fatty acid towards decreased proportion of short and medium chain fatty acids and increased proportion of long chain fatty acids (Bauman and Griinari, 2001). Despite of its effect on fat and fatty acids in milk, usually MFD has no impact on the yields of protein or lactose (Bauman and Griinari,

2001). Basically, MFD can be triggered by two types of diets; firstly diets that provide high amount of readily fermentable carbohydrate but low in fibrous components. The most common diet of this type is the high grain/low roughage (HG/LR) diet. Secondly, diet that contains high amount of unsaturated fat, which is obtained by adding oils (plant or fish oils), full-fat seeds, or meal containing the polyunsaturated fatty acids (Davis and Brown, 1970).

There are two categories of postulated theories, which explain the physiology behind MFD. Firstly, theories that consider reduction of milk fat synthesis in mammary glands which are due to inadequate supply of precursors for lipid synthesis i.e. acetate and β-hydroxybutyrate. This category includes acetate deficiency, β-hydroxybutyrate deficiency, and glucogenic-insulin theories. Acetate and β-hydroxybutyrate are important substrates for fat synthesis in mammary glands. Reduction in concentration of these two precursors is usually observed when cows are fed HG/LR diet (Van Soest, 1963; Davis and Brown, 1970; Sutton, 1985). However, infusion of acetate to cows fed normal diets or diet causing MFD only resulted in a slight to modest increase in milk fat yield (Davis and Brown, 1970). Thus, according to the authors, this theory cannot explain reduction in milk fat.

The β-hydroxybutyrate contributes to 8% of milk fatty acid carbon of *de novo* synthesis fatty acids (Palmquist et al., 1969). According to Van Soest and Allen (1959), increase of propionate in HG/LR diets may limit the supply of β-hydroxybutyrate by reducing the synthesis of hepatic synthesis of ketones, a source of β-hydroxybutyrate. However, other study indicated that the increase of propionate did not affect the turnover of β-hydroxybutyrate even though milk fat yield decrease

(Palmquist et al., 1969). This fact together with the low contribution of β-hydroxybutyrate as carbon source for fat synthesis has rendered β-hydroxybutyrate deficiency as the basis to explain diet-induced MFD (Bauman and Griinari, 2001).

The use of glucogenic-insulin theory to explain diet-induced MFD was firstly proposed by McClaymot and Vallance (1962). Insulin has stimulatory effect on lypogenesis and inhibitory effect on lyposis in adipose tissues. However, mammary glands are not responsive to insulin circulation. Release of insulin from pancreas is stimulated by propionate and glucose. Diet high in readily digestible carbohydrate but low in fibre (HG/LR diets) increases propionate production and gluconeogenesis rates in heparin (Annison et al., 1974). The increase of insulin enhances the uptake of lypogenic precursors and decreases the release of fatty acids by adipose tissues. This results in an increase in the use of acetate, β-hydroxybutyrate, and diet-derived long chain fatty acids in adipose tissues. As consequence, the availability of lipogenic precursor for milk fat synthesis in mammary glands becomes limited (Bauman and Griinari, 2001). However, other research using HG/LR diets showed that increase in insulin and reduction in acetate: propionate ratio was not companied by any significant effect on milk fat yield (Palmquist et al., 1969). When the increase of lypogenic enzyme activities is accompanied by a reduction in milk fat yield, it appears that the increase of this enzyme activity is more related to the effect of energy balance characteristic of cows fed HG/LR diets rather than to MFD (Benson et al., 1972). Thus, there is little support for glucogenic-insulin theory to explain diet-induced MFD (Bauman and Griinari, 2001).

Other theories consider reduction in milk fat content due to inhibition of one or more steps of milk fat synthesis in mammary glands. Vitamin B12/methyl-malonate and trans fatty acids theories are included in this category (Bauman and Griinari, 2001). The use of trans fatty acid theory as the basis of diet-induced MFD was firstly proposed by Davis and Brown (1970) and was elaborated by Pennington and Davies (1975). Trans C18:1 fatty acid, a major intermediate during biohydrogenation of unsaturated fatty acids by rumen bacteria (Figure 2.6), is proposed to inhibit milk fat synthesis in mammary glands (Bauman and Griinari, 2003). Effect of trans C18:1 fatty acid in decreasing the yield of milk fat is observed not only with a diet containing high polyunsaturated fat, but also in a HG/LR diet. The high amount of grain and low amount of roughage in diet may cause rumen fluid to be more acidic, thus alter microbial population in rumen since some bacteria are sensitive to acidic condition. This change then may increase the amount of trans FAs, which have a potential to reduce milk fat synthesis. However, abomasal infusion of some trans C18:1isomers such as trans-9 C18:1 (Rindsig and Schultz, 1974), or a mixture of trans-11 and trans-12 C18:1 (Bauman and Griinari, 2001) did not affect milk fat yield. Therefore, trans fatty acid theory, which is based on total trans-C18:1 can not to be used as the basis of a diet-induced MFD (Bauman and Griinari, 2001).

Another theory to explain the MFD phenomena is the biohydrogenation theory, which states that under certain condition, rumen biohydrogenation pathways may be altered and produces unique fatty acids intermediates able to inhibit *de novo* synthesis fatty acids (Figure 2.6; Bauman and Griinari, 2001). Studies showed that

reduction in the yield of milk fat is more associated with an increase of trans-10 C18:1 (Griinari et al., 1997; Newbold et al., 1998). CLA isomer i.e trans-10 cis-12 CLA, also has been reported to reduce milk fat synthesis, but cis-9, trans-11 CLA has no effect (Baumgard et al., 2000).

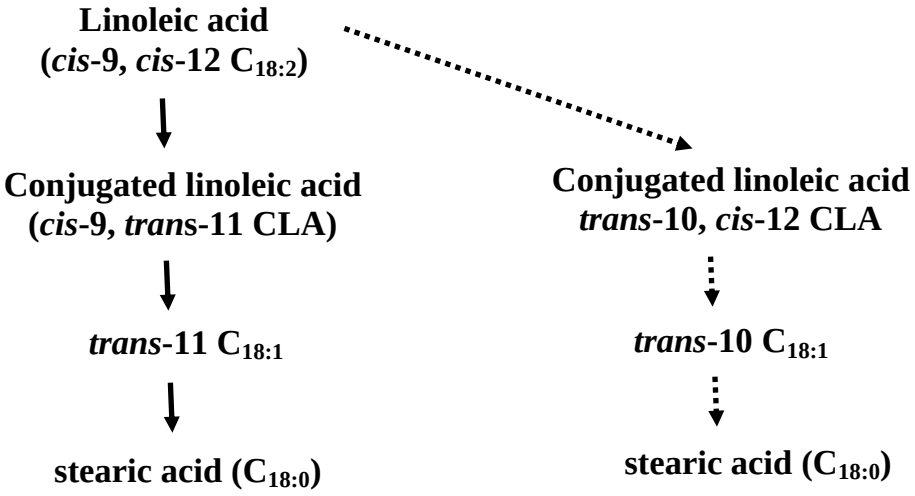


Figure 2.6 Pathways of ruminal biohydrogenation of linoleic acid
Source: Bauman and Griinari (2001)

2.2.2.3 **Effect of condensed tannin on milk composition**

Effect of condensed tannin supplementation on protein content of bovine milk

Supplemented-condensed tannins may increase milk protein synthesis by improving supply of amino acids required for milk protein synthesis. The ability of condensed tannins to form complexes with protein forages may protect these proteins from degradation in the rumen. This then may lead to increase in the flow of amino acids for milk protein synthesis to the duodenum, thus increasing yield of

milk proteins (Waghorn, 1990; Barry and McNabb, 1999; Woodward et al., 1999; Min et al., 2003). Furthermore, besides reducing degradation of protein forages, dietary condensed tannins may also alter population of rumen microbial, especially those that are involved in proteolysis (Molan et al., 2001). Increase in milk protein content was observed when cows were fed a birdsfoot-supplemented diet (Woodward et al., 1999). Effect of condensed tannins in increasing amino acid absorption in the small intestine is influenced by concentration and molecular weight of condensed tannins in diet and proanthocyanidin composition (McSweeny et al., 2001). It has been reported that condensed tannins in a moderate level i.e. 5.5% of dry matter (DM) in diets could improve nitrogen retention in rumen, decrease protein metabolism, and increase plant amino acid absorption in the small intestine (Waghorn, 1990; Barry and McNabb, 1999; Woodward et al., 1999; Min et al., 2003).

It was also been postulated that dietary condensed tannins may depress milk protein synthesis through their astringency as well as their bacteriostatic and bactericidal effects (Waghorn et al., 1990; Min et al., 2003). Due to their astringency effect, condensed tannins may reduce voluntary feed intake (VFI) of animal, and could lead to reduction in milk protein synthesis (Waghorn et al., 1990). In addition, by exerting their bacteriostatic and bactericidal effect, condensed tannins could inhibit microbial protein synthesis in rumen and thus, reduce production of amino acids required for milk protein synthesis (Min et al., 2003).

Effect of condensed tannin supplementation on fat content and fatty acid composition

Animal diet may affect milk fatty acid composition by influencing production of precursors for synthesis of milk fatty acids in the rumen and those available in the blood (Vasta et al., 2008). It was proposed that the ability of dietary condensed tannins to form complexes with fibre and cellulose may influence production and proportion of volatile fatty acids (VFA), which act as precursors for lipid synthesis in mammary glands (Dahlberg et al., 1988; Khiaosa-Ard et al., 2009). Diet containing birdsfoot-condensed tannins has been associated with an increase in VFA concentration and a shift in VFA proportion towards a decrease in the acetate concentration and an increase in the propionate concentration. These changes are proposed to be related with the reducing effect of condensed tannins in fibre degradation or suppression of cellulose digestion (Dahlberg et al., 1988). Similarly, an increase in a propionate proportion has also been reported by Khiaosa-Ard et al (2009) when cows were fed with sainfoin. Reduction in non-dietary fibre (NDF) degradation associated with sainfoin-condensed tannins might be responsible for this increase. However, in the latter study, concentration of VFA was unaltered. In contrast, a lack of effects of dietary condensed tannins on VFA concentration and proportion has been documented (de Oliveira et al., 2007). An increase in propionate proportion, but not accompanied with any changes in VFA concentration, has also been reported (Carulla et al., 2005; Bhatta et al., 2009). This change may be due to reduction in digestion or degradability of fibre by condensed tannins (Waghorn et al., 1994; Carulla et al., 2005).

Effects of dietary tannins on milk composition in ruminants have been reported previously (Piredda et al., 2002; Cabbidu et al., 2005). Dietary tannins increased concentrations of linoleic and linolenic acids but reduced CLA and trans-11 C18:1 in milk of sheep fed with a reproductive stage-sulla, despite the low concentration of PUFA in the sulla (Piredda et al., 2002; Cabbidu et al., 2005). According to the authors, the increased linoleic and linolenic concentration was likely due to the presence of tannins, which may have altered the activity of rumen microflora, and in turn reduced biohydrogenation of PUFA in the rumen. Thus, increasing flow rate of PUFA abomasal and intestinal absorption. Reduction in CLA may be attributed to the lower production of trans-11 C18:1. In addition, the presence of high PUFA may inhibit Δ^9-desaturase activity responsible for transforming cis9 C18:1/C18:0 (Piredda et al., 2002; Cabbidu et al., 2005).

2.3 EFFECT OF FAT AND TANNIN SUPPLEMENTS ON MILK COAGULATION PROPERTIES

2.3.1 Casein structure

Approximately 95% of caseins exist as a granular structure called casein micelles in milk (Fox and McSweeney, 1998). Various models of casein micelle have been proposed; however, the exact structure of casein micelle is still under debate (Phadungath, 2005). Basically, these proposed models can be categorised into three namely: coat-core models, sub-micelle (subunit) models and internal structure models (Phadungath, 2005). According to Walstra (1999), sub-micelle model can be

used to explain formation, structure and properties of the casein micelle, especially its reactions to changes in conditions, during milk processing.

Based on sub-micelle models, casein micelle consists of small uniform units, called sub-micelles, formed by αs-, β-, κ-caseins and small amount of inorganic matter. There are two types of sub-micelles: the first type of sub-micelle consists of αs- and β-caseins. These sub-micelles are more hydrophobic and buried in the centre of sub-micelle. The second type of sub-micelle primarily consists of αs- and κ-caseins. These sub-micelles are held together by hydrophobic interaction between proteins and calcium phosphate linkages. The κ-casein has two distinct regions: hydrophobic para-κ-casein (residue 1-105) and the hydrophilic macro peptide or glycomacropeptide (GMP or CMP, residue 106-169). The hydrophilic macro peptide protruding from the micellar surface interacts with a solvent to sterically stabilize the micelles (de Kruif and Zhulina, 1996). The stability of casein micelles also depends on the interactions between the micelles, electrostatic, hydrophobic, hydrogen bonding, and Ca-induced interactions (Fox and McSweeney, 1998). The casein micelle has a porous structure which may affect susceptibility to hydrolysis by pepsin. This structure allows water to enter the micelle (Jasiska and Jaworska, 1991).

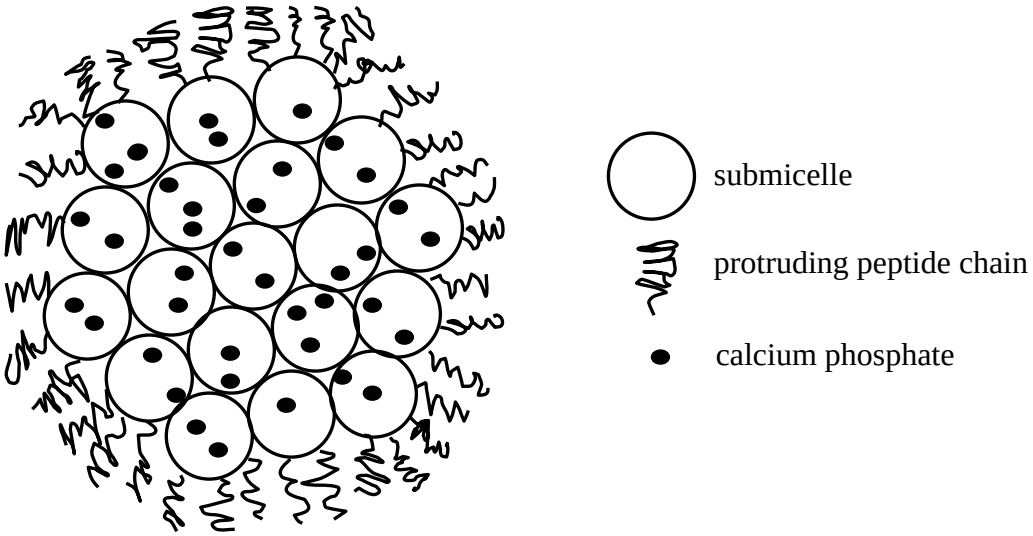


Figure 2.7 The structure of casein micelles based on sub-micelles model
Adapted from: Walstra (1999)

2.3.2 Milk coagulation process

Coagulation is one of the important milk physical properties since it is a key process in the transformation of milk to a range of solid or semi-solid dairy products (IDF, 2007). Milk coagulation occurs after the brush, which stabilizes casein micelles collapses, during acidification of milk or rennet-induced coagulation of milk (Lucey and Fox, 1993; Dejmek and Walstra, 2004). This process can be induced by acids, enzymes, or a combination of them (Horne and Banks, 2004). This review would focus on acid- and rennet-induced coagulation due to their importance during manufacturing dairy products such as yoghurt and cheeses.

2.3.2.1 **Acid-induced coagulation**

Acid-induced coagulation is the basic process during manufacture of yoghurt or fresh-acid coagulated cheeses such as cream cheese, tvorog, frais, cottage cheese, and quarg (Phadungath, 2005). In this type of coagulation, reduction of milk pH is

induced by production of lactic acid by lactic acid bacteria or by addition of hydrochloric acid (HCl) or glucono-δ-lactone (GDL) (Lucey et al., 1997), which is rapidly hydrolysed to gluconic acid (Lucey and Singh, 2003). Acid-induced coagulation of bovine casein micelles is a result of reduction in solvency of the κ-casein brush on the casein surface due to protonation of the negatively charged carboxylic acid groups of Glu and Asp (Lucey and Singh, 2003). Basically, there are three pH regions during milk acidification from pH 6.7 to 4.6:

1. pH 6.7 to ~6.0

Reduction in milk pH lowers the net negative charge of casein micelles, which consequently reduces the electrostatic repulsion. The micelle structure is unaltered since only a small amount of colloidal calcium phosphate (CCP) is dissolve above pH 6 (Lucey, 2004).

2. pH ~6.0 to ~5.0

As milk pH decreases, the net negative charge of casein micelles continues to get lower and the charged κ-casein brush may shrink. As a result, electrostatic repulsion and steric stabilisation of casein micelle decrease. Ionisation of acidic function of caseins (aspartic-glutamic-phosphoserine residues) is also lowered as pH decreases; and as a consequence, a surface potential is decreased. The molecular interactions between αs and β-casein is lessened, thus the solubility of the phosphocalcic salt in water increase. This causes transfer of calcium and ionorganic phosphate (CCP) from the micelle to the aqueous phase. When pH ~5 is reached, the CCP within the casein micelle is completely dissolved (Lucey and Fox, 1993), which

increases electrostatic repulsion between newly exposed phosphoserine groups (Brule et al., 2000; Lucey, 2004).

3. pH < 5

Reduction of pH from 5 to ~4.6 induces the disassociation of the calcium complexes by the phosphoserines. This process results in a disorganisation of the micelles and a reorganisation of the micellar sub units. When the isoelectric point (IP) is approached, the negative charge on the casein micelles declines, leading to a reduction in the amphiphilic characters of β- and κ-caseins. Calcium phosphate solubilisation strengthens electrostatic interaction, while the electrostatic repulsion is weakened, which allows the depolymerisation of the α-caseins. Thus, the hydrophobic interaction is increased. This causes aggregation and formation of chains and clusters linked together as a three dimensional network (Brule et al., 2000; Lucey and Singh, 2003; Lucey, 2004).

2.3.2.2 **Rennet-induced coagulation**

Coagulating properties of milk is important for cheese-manufacturing, cheese yield, and cheese quality (Lee and Lucey, 2004). Before rennet addition, casein micelles in milk have no tendency to aggregate. The stability of casein micelles arises from the negative charges on their surface and the macropeptide part of κ-casein, which covers the surface of casein micelles (Walstra, 1990). In addition, the steric stabilization prevents aggregation of casein micelles since the ‘hairy’ outer layers of the micelle cannot interpenetrate (Dalgleish and Holt, 1988). Cheese manufacturing mainly use natural enzyme chymosin to hydrolyse κ-casein and

induce destabilization of casein micelles to form gel (Herbert et al., 1999). Rennet acts on the caseins of milk in three phases. The first is primary (enzymatic) stage, in which the proteolytic enzyme in rennet hydrolyses the κ-casein amino acid chain at a bond between the 105th acid, phenylalanine, and the 106th acid, methionine. This process splits the macropeptides from para-κ-casein, thus removing the 'hairy' part of casein micelles. As a result, steric and electrostatic repulsion are greatly reduced. The second phase is coagulation or flocculation where the casein micelles approach one another closely and combine to form a coagulum (Dejmek and Walstra, 2004). The process of rennet coagulation is depicted in Figure 2.8.

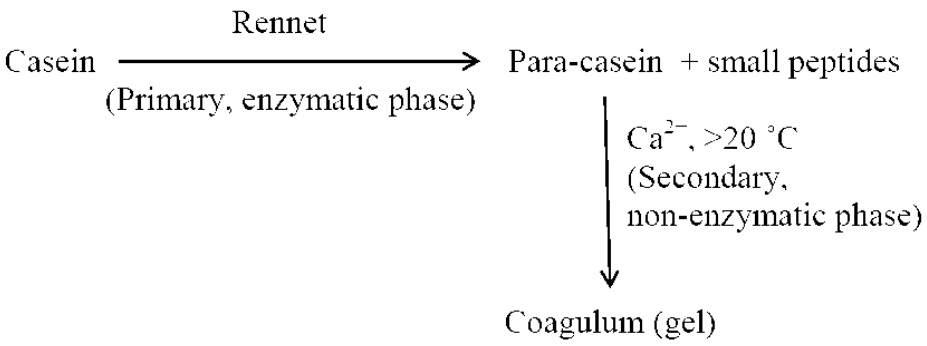


Figure 2.8 Steps involved in the enzymatic coagulation of milk
Source: Fox et al (2000)

2.3.3 Physical properties of milk gels

2.3.3.1 **Rheological properties**

Rheological and textural properties of acid and rennet gels can be studied using several methods such as small amplitude oscillatory rheology (SAOR), large amplitude oscillatory shear, penetration, and texture profile analysis (Lucey, 2004).

Gelation is the first step during manufacturing of acid- or rennet-coagulated cheeses; therefore an ideal technique to study these gels should be able to monitor development of the gels without destruction of gel structure (Lucey, 2004). The SAOR technique is a dynamic, non-destructive approach that has been used to study the development of milk gels (Van Marle and Zoon, 1995; Lucey and Singh, 1998). This technique applies an oscillating strain or stress within the linier viscoelastic region of the gel. Some parameters can be obtained using this technique include: elastic or storage modulus (G′), a measure of the energy stored per oscillation cycle; viscous or loss modulus (G″), a measure of energy dissipated as heat per oscillation cycle; and the loss tangent (tan δ), a ratio of the loss modulus to the storage modulus (Lopes da Silva and Rao, 1999). Loss tangent is associated with a degree of viscoelasticity of a sample. Change in tan δ is a more direct indication of changes in type and/or strength of bonds in a gel network. Another important parameter of milk coagulation is gelation time (GT), which is defined as the time when (1) a sharp increase in G′ is observed; (2) G′ increase is greater than 1 Pa; or (3) G′ and G″ cross over; or (4) the appearance of max tan δ before sudden decline; or (5) the time when G′ is equal to 1 Pa (Lucey et al., 1997; Mishra et al., 2005).

A typical curve of acid gel formation from unheated milk sample as a function of time monitored using SAOR technique is shown in Figure 2.9. After acid addition, pH of milk suspension decreases gradually. At the point of gelation, pH ~4.9-5.0, storage modulus (G′) started to increase, tan δ decreased rapidly to < 0.4 and flattened to about 0.2-0.3 during aging of the gel. After the gelation point, G′ increases rapidly and only starts to plateau during aging of the gel which is around

pH 4.6. Loss modulus (G″) also increases after gelation, however it increases at a lower magnitude compared to G′ (Lucey, 2004).

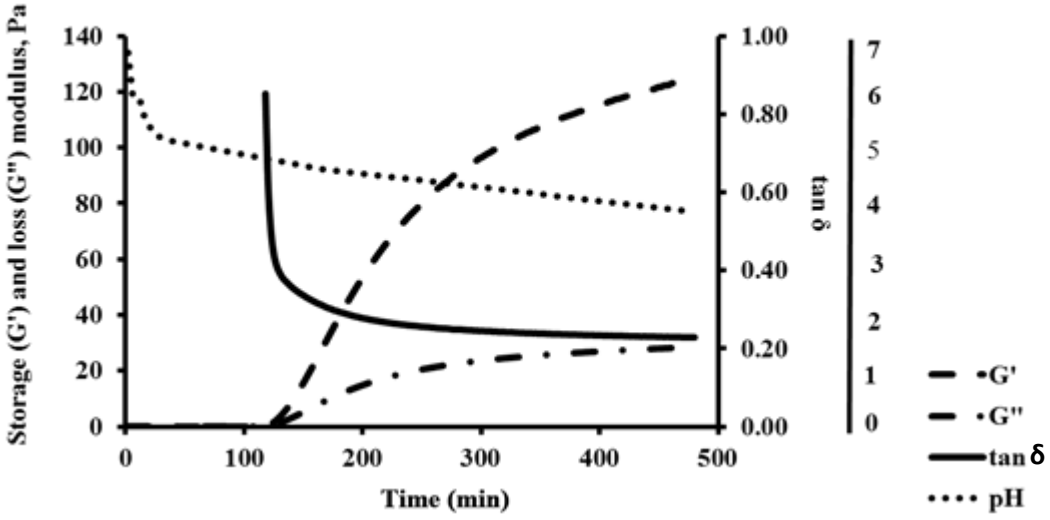


Figure 2.9 Development of acid induced gels as a function of time
Adapted from Lucey (2004)

Development of rennet gel as a function of time is illustrated in Figure 2.10. After rennet addition, storage modulus (G′) increases linearly with the time as more casein micelles get incorporated in the gel network (Zoon et al., 1988). Loss tangent (tan δ) decreases with aging and then is stabilized to a value of around 0.325 (Horne and Banks, 2004). Rheological characteristics of acid and rennet gels are influenced by milk pH, milk composition, storage condition and processing conditions such as incubation temperature, milk heat treatment, type and quantity of acid or rennet used to induce coagulation (Table 2.3).

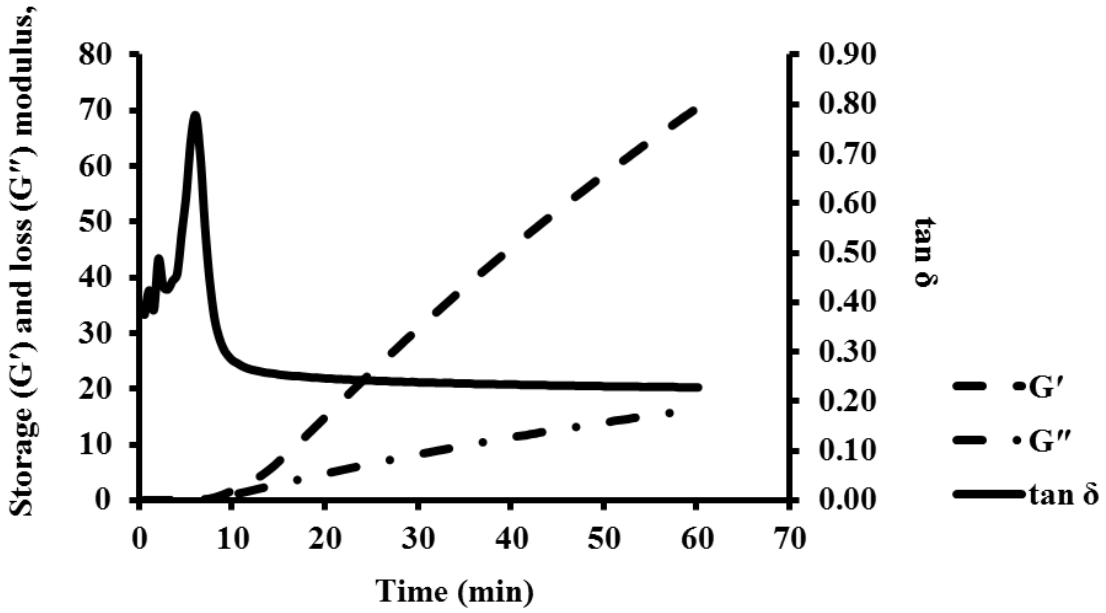


Figure 2.10 Development of rennet-induced milk gel as a function of time.
Adapted from Horne and Banks (2004)

Table 2.4 Summary of effects of milk composition and processing conditions on rheological properties of milk gels
(Adapted from: Phadungath, 2005)

Factors	Effects on rheological properties of milk gel	References
Milk pH	Milk pH may affect gel strength and renneting time of milk gel. Milk with a low acidity value i.e. < 4.2 may cause a weak gel.	Lucey (2004)
Milk composition		
Calcium ion	Due to interaction between calcium ion and negatively charged protein molecules (electrostatic interaction), high (10 mM) concentration of calcium ion may generate stronger interaction, thus result in a firmer gel. However, higher concentration of calcium ion (15-20 mM) may also cause interactions between proteins by bridging negatively charged carboxyl groups which increase the tendency toward aggregation rather than coagulation. This strong protein aggregation may cause a weaker gel considering the fact that large pores are the weakest part of a gel network.	Mulvihil and Kinsella (1988) Zirbel and Kinsella (1988)
Total solid	Increasing the concentration of total solid in milk sample, i.e. from 40 to	Gastaldi et al (1997) Lucey (2004)

	60%, may increase gel firmness and viscosity. In contrast, a low total solid concentration may cause a weak gel and increase whey separation.	
Fat content	During formation of a gel network, milk fat globules (MFG) may interact with casein matrix and act as a structure promoter in the gel network. In contrast, when MFG are not interacting with casein particles, they act as inert filler (structure breaker).	Houze et al. (2005) Guinee et al. (1997)
	Increasing fat content from 0.1 to 10% while maintaining protein content at 3.3% has been reported could reduce gelling time, increase gel firming rate, and increase curd firmness.	Guinee et al. (1997)
Protein content	Protein content may affect the level of gel-forming protein per se, which augments the rate of casein aggregation/fusion and contributes to the formation of coarser gel network. Therefore, increase in the content of milk protein, including casein fraction and casein number may reduce coagulation time and form a firmer curd.	Lucey et al. (1998) Van Hooydonk et al. (1987)
	Protein content ranging from 2.5-3.3% is required for gel formation during cheese making process. No gel	Guinee et al. (1997)

	formation occurs when protein content is lower than 2%.	
Casein content	Gel firmness is directly proportional to the number, strength, or both of bonds between casein particles. Thus the increase of casein content in milk may increase firmness of the gel. In addition, the increase of casein content may facilitate rapid formation of inter particle bond and preventing further reorganisation of the gel network. Approximately 2.75 g $100g^{-1}$ of casein is required for optimum milk coagulation.	Lucey (2004) Karlsson et al. (2007) Dalgleish (1992)
Casein types α_{s1}-CN, α_{s2}-CN β-CN, κ-CN	Firmer curd obtained if the proportion of α_{s2}-CN & β-CN are smaller or those of κ-CN is larger. The increase of β-CN in total casein leads to the formation of larger micelles & deterioration of rennet coagulation. The increase of αs_1-CN, αs_2-CN, β-CN, κ-CN in milk up to ~2; 2.5; 1.2; and 1 g L^{-1}, respectively, could increase the firmness of milk gel.	Joudu et al (2008)
Processing condition		
Heat treatment	Excessive heat treatment i.e. >78 ˚C for 15 min, may cause whey denaturation which subsequently may	Lucey (2004)

	reduce gelation time and increase gelation pH. Whey protein has higher isoelectric pH (~5.2) than casein (~pH 4.6) which initiates aggregation at higher pH than for casein. Whey denaturation may also increase gel firmness (G′ value) by increasing the disulphide cross-linking between casein strands. In addition, it may also increase the loss tangent value since solubilisation of CCP occurs in casein particles that are already participating in gel matrix, which trigger greater rearrangement. Insufficient heat treatment of the milk may cause a weak gel.	
Incubation temperature	A high incubation temperature may cause faster acid production, which result in reduces gelation times. At temperature higher than 30 ˚C, casein rearrangement increase and leading to formation of a weak acid gel. However, at very low incubation temperature, for example 4 ˚C, no casein coagulation will occur.	Lucey (2004)

2.3.3.2 **Permeability**

Permeability (*B*) provides information about the coarseness (inhomogeneity) of a gel network (Lucey, 2004). Permeability of milk gel is determined by measuring the flow rate of whey through the gel (Lee and Lucey, 2004). This technique is based on the fact that protein gel is a network of aggregates, and solvent can flow through the pores of this network if driving pressure exists (Lee and Lucey, 2004). Permeability value of a gel correlates to the square of the pore radius within the gel, which in turn correlates to the square of the aggregate radius (Bremmer et al., 1998). Therefore, gel with high permeability indicates that the gel has large pore size, more susceptible to syneresis (whey separation), has low water holding capacity (WHC) and less stable (Lucey et al., 1997). Acid- and rennet-induced gels have similar permeability values (~1.5 x 10^{-13} m^2 and ~2.5 x 10^{-13} m^2, respectively) (Roefs et al., 1990; Van Marle and Zoon, 1995; Lucey et al., 1998). However, due to microsyneresis resulting from larger pores in the network, permeability of acid gel does not change over time, while permeability of rennet gel increases with time (Walstra, 1993). Gel permeability may be affected by factors such as milk pH, protein content, salt concentration in milk, high incubation temperature, use of rennet and rapid acidification (GDL-induced gel) (Lucey, 2004).

2.3.3.3 **Water Holding Capacity**

Water holding capacity (WHC) is an estimation of water retention capacity of a gel and indicates the gel strength (Serra et al., 2007). This parameter is assessed based on percentage of whey expulsion after centrifugation (Remeuf et al., 2003). There are some factors that may affect WHC of a gel; these include milk

composition (calcium and fat contents in milk) and milk treatments (homogenization). Calcium acts as a cross-link between protein molecules. Therefore high calcium content may increase the strength of milk gel, thus increasing the ability of the network to retain water (Lucey, 2004). Fat content may exert its effects on WHC of a gel through its interaction with proteins. Protein-fat interactions are responsible for the increase of gel firmness and enhanced binding of water within the network (Serra et al., 2007). A high pressure homogenization may increase interactions between protein-protein or protein-fat, which lead to the formation of a stable network with high capacity to retain water (Lucey, 2004).

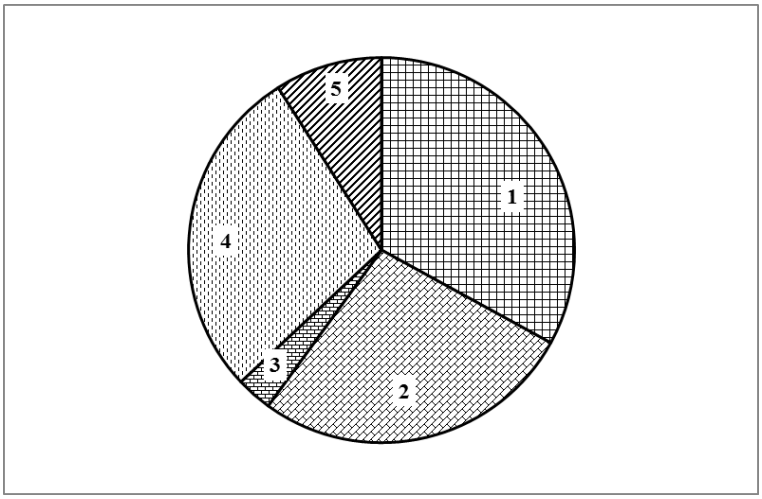
2.3.4 Effect of animal diet on milk coagulation

Animal diets not only affect milk composition but also its technological characteristics and quality of derived dairy products (Jaramillo et al., 2009). Milk coagulation is mainly affected by physicochemical properties of the milk, which include pH and chemical composition of milk such as proteins, casein, fat, and minerals as listed in Table 2.3. Improvement in dietary energy of dairy cows by feeding better forage and concentrates increased coagulation properties of cow milk as indicated by reduction in coagulation time and increase in curd firmness (Kreuzer et al., 1996). In contrast, inclusion of lupin seed (*Lupinus albus L*.) as an alternative protein source in ewes' diet had no marked effect on milk clotting properties (Masucci et al., 2006). According to the authors, a lack of an effect of this modified diet on milk coagulation properties might be partly attributed to unaltered content of milk proteins. Similar result has been reported by de Renobales et al. (2012), who found that different feeding regimes fed to sheep did not alter percentages of proteins

and fat in the milk as well as milk renneting time and curd firmness. However, rennet-induced gel made from milk with higher percentages of unsaturated fatty acids had lower resistance to compression (de Renobales et al., 2012). Inclusion of cow diet with fish meal rich in n-3 PUFA or n-3 PUFA precursors did not alter milk coagulation time or rate of acidification but reduce the rate of gel firming and curd firmness (Avramis et al., 2003). Based on discussion above, it appears that modification in animal diet may alter some properties of milk gels.

2.4 EFFECT OF FAT AND TANNIN SUPPLEMENTATIONS ON THE *CHEDDAR* CHEESE PROPERTIES

Cheese is a major product of Australia dairy industry (Figure 2.12) with total cheese production about 340,340 tonnes in 2011/2012 (Table 2.4). The *Cheddar* cheese is the most popular and most produced cheese in this country despite increasing trend in production of a non-*Cheddar* cheese type and decrease in the total cheese production volume in recent years due to reduction in milk availability. In 2011/2012 the *Cheddar* cheese production increased slightly from the previous year with little changes in non-Cheddar varieties (Dairy Australia, 2013).



1 Cheese (33%)

2 Drinking milk (27%)

3 Others (3%)

4 Skim milk powder (28%)

5 Whole milk powder (9%)

Figure 2.11 Utilisation of Australian milk in 2012/13
Source: Dairy Australia (2013)

Table 2.5 Production of cheese in Australia (tonnes) from 2007/2008 to 2012/2013 (Source: Dairy Australia, 2013)

Type of cheese	**Years of production**					
	2007/08	**2008/09**	**2009/10**	**2010/11**	**2011/12**	**2012/13**
Cheddar	71,260	178,360	164,220	154,720	160,683	157,996
Semi hard	73,854	61,754	82,504	68,176	67,023	57,190
Hard grating	16,908	17,924	12,238	13,591	13,871	14,681
Fresh	90,934	75,650	82,004	95,431	99,024	102,342
Mould	7,966	8,915	8,673	6,739	5,930	6,103
Total cheese	360,922	342,603	349,639	338,657	346,530	338,312

2.4.1 Manufacturing of the Cheddar cheese

The Cheddar cheese is a type of hard cheese, originally from England, made from standardized and pasteurized cow milk and coagulated using calf rennet or rennet substitute (McSweeney, 2004).

Cheese-milk treatment

Before being used for cheese manufacturing, cheese-milk is often standardized, homogenized, and heat treated. Milk standardization is aimed to achieve some consistent cheese parameters such as moisture, fat in dry matter (FDM), and moisture in non-fat substance (MNFS) which are regulated by law (Kosikowski, 1977). In addition, standardisation is required to compensate for a variation in milk composition due to seasonal variations. Variation in fat and protein content in milk may affect yield, composition, texture, flavour, and sensory attributes of the resulting cheese (Guinee et al., 2006).

After standardisation, usually cheese-milk for soft cheese or yoghurt manufacturing is homogenized to distribute the fat evenly in the milk. Homogenization is also aimed to increase interaction between fat globules and casein matrix which may improve coagulation. In addition, homogenization may increase cheese yield due to its effect in improving fat and protein recovery as well as reducing fat separation during storage (Fox et al., 2000). Moreover, when milk is cooled, indigenous immunoglobulin (cyoglobulins) precipitates onto the fat globules. These cyoglobulins induce a strong tendency of the fat globules to agglutinate, which then results in a rapid rate of creaming. In commercial dairy processing, to

prevent creaming of the fat globules, milk is usually homogenized at 10-20 MPa at 55-65 ˚C (Lee and Lucey, 2010). Homogenization can delay creaming rate of the fat globules by reducing the size of fat globules, increasing the fat surface area, replacing the natural milk fat globule membrane (MFGM) by caseins and denaturing cyoglobulins (Simpson, 2012). Microfluidization is a homogenization technique, in which the liquid is pumped at a constant high pressure into a chamber and split into two smaller streams, which are then projected against each other within an interaction chamber (Simpson, 2012). Similar to homogenization in reducing the size of milk fat globules, microfluidization can reduce the size of milk fat globules even further (Simpson, 2012).

Heat treatment of milk sample is aimed to eliminate bacterial pathogens, minimise damage to caseins due to proteolytic bacteria (Banks, 2000), and provide favourable environment for the starter culture (Lee and Lucey, 2010). Heat treatment at 65 ˚C for 30 min is usually chosen to avoid any side effects of severe pasteurisation such as denaturation of whey proteins, interaction between whey proteins and casein micelles, transfer of soluble calcium, magnesium, and phosphate to the insoluble colloidal state (Kelly et al., 2008), which subsequently may impair coagulation (Dalgleish, 1992) and syneresis of gel (Pearse et al., 1985) as well as flavour and firmness of cheese (Guinee et al., 1998).

Heat treatment of milk at high temperature (i.e. 80 ˚C for 30 min) may denature whey proteins, especially β-lactoglobulin. The denatured whey proteins are unfolded and thus expose the side chain groups, particularly the reactive thiol groups, which are originally buried in the native structure. Subsequently, some of the

unfolded whey proteins interact with the casein micelles through κ-casein via thiol-disulphide interchange (Mohammad and Fox, 1987). Interactions between denatured β-lactoglobulin and casein, then may affect rheological characteristics of acid and rennet gels differently. For acid gels, denatured whey proteins may aggregate as their isoelectric points (~5.3) are approached. Thus, isoelectric point would shift sooner from the isoelectric point of caseins (~4.6) to the one of whey protein (~5.3). Consequently, gelation would occur at a higher pH after a shorter time. In addition, complexion of β-lactoglobulin with casein micelle would result in an acid gel with a high G′ value (Lucey et al., 1999).

Association between β-lactoglobulin and κ-casein may prolong gelation time and reduce firmness of rennet gel (Walstra et al., 1985; Van Hooydonk et al., 1987; Guinee et al., 1998). Gelation time of rennet gel can be delayed due to presence of a β-lactoglobulin-κ-casein complex on the surface of casein micelles restricting accessibility of κ-casein to the chymosin (Van Hooydonk et al., 1987). In addition, this complex may reduce gel firmness by inhibiting reactivity of the casein micelle for aggregation and reducing the amount of micellar calcium (Van Hooydonk et al., 1987; Vasbinder et al., 2003). Increase in water holding capacity (WHC) and thus reduction in syneresis of rennet gel may also occur due to the reduction in the shrinkage of para casein-β-lactoglobulin complex, which may detrimentally affect properties of cheese (Walstra et al., 1985; Guinee et al., 1998).

Calcium chloride addition is a common approach during preparation of milk for cheese manufacturing to promote gel formation, curd firming and syneresis (Zoon et al., 1988). It was proposed that added calcium ions can reduce coagulation

time and increase firmness of rennet gel by binding to the caseins. These effects are partly due to the effect of calcium in lowering milk pH (Lucey, 2002). In addition, calcium can also bind to the casein micelles and reduce the net negative surface charge. This process then may enhance hydrophobicity of casein micelles and thus promote aggregation due to enzymatic coagulation. Ca^{2+} may also involve in specific bridging interactions that may induce reticulation of the gel (Zoon et al., 1988).

Basically, production of the Cheddar cheese is divided into two stages i.e. curd formation and curd ripening. Curd formation consists of several steps including addition of culture (starter), milk ripening, milk coagulation, cutting of cheese curd, cooking of cheese curd, draining off the whey, Cheddaring of cheese curd, milling, salting, and pressing of curd, packaging, and ripening as depicted in Figure 2.13 (Kosikowski, 1977; Bylund, 1995).

Addition of culture (starter)

The most commonly used starter bacteria during the Cheddar cheese manufacturing are mesophilic starter bacteria *Lacctococcus lactis* subsp. *lactis* and *Lacctococcus lactis* subsp. *cremoris* (Kosikowski, 1977). The addition of cultures of selected lactic acid bacteria to cheese milk is aimed to achieve uniform acidification from lactose during cheese manufacturing. Besides, inclusion of culture may contribute to biochemical changes of cheese during ripening since enzymes released from the culture are involved in proteolysis, lipolysis, and conversion of amino acids into flavours (Fox and McSweeney, 1998; Collins et al., 2004).

Milk ripening

During milk ripening, the starter cultures begin acid production by producing lactic acid to activate chymosin from the rennet, and aid the coagulation process. The rate of acidification may affect texture and flavour development of the cheese (Fox et al., 2000). A very rapid rate of acidification is not desirable since the high acid concentration may dissolve too much insoluble calcium phosphate as soluble calcium lactate into whey. A low pH would occur in cheese at pressing. As a result, cheese will develop an intense acid flavour, weak, and pasty texture (Fox et al., 2000). A slow acid formation is more preferable since it may preserve more calcium phosphate in its soluble form until it is required as a soluble buffering salt. This process would result in a good texture cheese.

Milk coagulation

Milk clotting enzyme, e.g. rennet, can be used to convert cheese-milk into cheese curd. In general, pH 6.3-6.6 and temperature at 30-32 °C are required for an optimum milk coagulation. In the presence of chymosin, the principal enzyme in calf rennet, milk clotting occurs in two phases. During the first phase, caseinomacropeptide (CMP) is gradually loss. This process is accompanied by a decrease in the micellar zeta potential and thus destabilisation of casein micelle. The secondary phase involves the aggregation of para-κ-casein and other caseins under the influence of Ca^{2+}, which leads to gel formation (Dejmek and Walstra, 2004).

Cutting of cheese curd

Proper cutting of coagulum is necessary since improper cutting and handling of curd may result in the loss of fines (small curd particles that are not recovered in the cheese). Generally, cheese curd are cut to ~0.8-1.0 cm in order to obtain the desired moisture content in the final Cheddar cheese (Kosikowski, 1977).

Cooking of the cheese curd

Cooking and stirring of cheese curd are required to induce contraction of the protein matrix. This process causes the curd to shrink and expel whey. As a result, the curd are firm up and ready for the next steps during Cheddar cheese manufacturing, i. e. texture formation, pressing, and salting. In general, cooking temperature for Cheddar cheese curd is 38 °C for ~30-60 min or until curd pH decrease to 6.1-6.2 (Kosikowski, 1977). Following this step, the curd and the whey are separated (Kosikowski, 1977).

Cheddaring the cheese curd

Cheddaring is a series of steps involving packing, turning, piling and re-pilling blocks of warm curd at ~38 °C, in the cheese vat. As the curd blocks are piled, their structure flattens and forms a rubbery sheet of curd. During this process, the amount of lactic acid in the curd increases. During the Cheddaring process, moisture control and the proper texture of the curd are also attained (Kosikowski, 1977).

Milling

Following the Cheddaring step, the Cheddared curd is mechanically cut into small pieces. The aim of this milling is to increase the surface area of the curd, which enables more uniform salt distribution into the curd and to increase whey separation (Lawrence et al., 2004).

Salting

After milling, certain amount of salt, ~2.3 to 3.5%, is added to the cheese curd. This step is aimed to promote further syneresis, slow down lactic acid fermentation, prevents the growth of spoilage microorganism, promote controlled ripening and flavour development of the cheese (Ong, 2007).

Pressing

Pressing of cheese curd is aimed to transform the loose curd particles into a compact curd as well as to expel any free whey (Lawrence et al., 2004). To prevent fat leaking into the whey, temperature of curd before pressing should be below temperature of the liquid fat i.e. ≤ 24 ˚C (Scott et al., 1998).

Packaging

Packaging of dairy products is aimed to protect them from spoilage caused by physical damage, chemical changes or microbial growth (Rajoria, 2013). Ease of opening for the consumer and preservation of freshness are the main goal of the cheese packaging industry (Rajoria, 2013).

Ripening

The ripening of Cheddar cheese usually performed at 4 to 16 °C for 2 to 48 months. Characteristics flavour and texture of individual cheese develop during the ripening period (; Kosikowski, 1977; Ong, 2007).

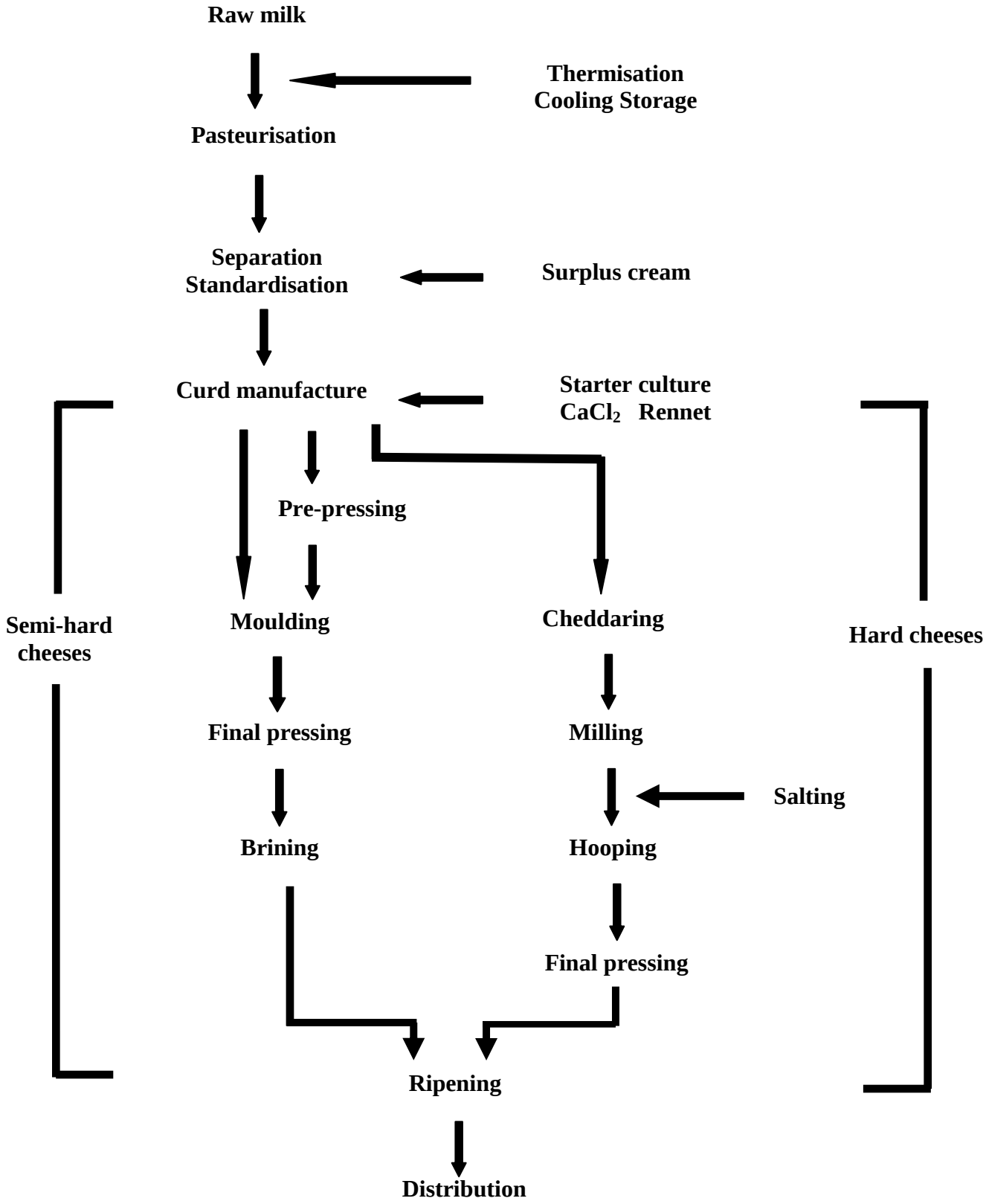


Figure 2.12 Process flow in production of hard and semi hard cheeses.
Source: Bylund (1995).

2.4.2 Yield and chemical composition of the Cheddar cheese

The quality of the Cheddar cheese is closely related to its pH and chemical composition especially moisture non-fat solid (MNFS), fat dry matter (FDM), ratio of salt to moisture (S/M), and cheese pH (Gilles and Lawrence, 1973). These parameters often used for cheese grading system are based on compositional analysis. For first grade the Cheddar cheese, suggested ranges of S/M, MNFS, FDM and pH are 4.7-5.7; 52-54; 52-56; and 5.1-5.3, respectively. While for second grade Cheddar cheese the ranges are 4.0-6.0; 50-56; 50-57; and 5.1-5.3 for S/M, MNFS, FDM and pH, respectively (Gilles and Lawrence, 1973). The first grade Cheddar cheese is then directed at the retail consumer market; while the second grade cheese can be used as a food ingredient or processed cheese (Bennett and Johnston, 2004).

2.4.3 Biochemical processes during the Cheddar cheese ripening

During the ripening period, a number of complexes microbiological, chemical and enzymatic reactions occur. Glycolysis, lipolysis, and proteolysis are the key processes involved in converting milk to the final value-added product: cheese. Ripening agents that are involved in these reactions can be derived from rennet, indigenous milk enzymes, starter bacteria and their enzymes, enzymes from secondary starters, and non-starter bacteria (Fox and McSweeney, 1998).

2.4.3.1 Proteolysis

Proteolysis is defined as a biochemical process during cheese ripening, which involves degradation of caseins into smaller peptides and free amino acids (McSweeney and Sousa, 2000). This process is catalysed by proteinases and

peptidases from the milk, coagulant, starter bacteria, non-starter bacteria, and secondary culture (McSweeney and Sousa, 2000). Generally, proteolysis is divided into 2 stages, primary and secondary proteolysis. During primary proteolysis, residual chymosin (enzyme from coagulant) and plasmin degrade caseins into large (water soluble) and intermediate-size (water insoluble) peptides (Shakeel-Ur-Rehman et al., 1998). Therefore, a degree of the primary proteolysis is influenced by activity of residual chymosin and plasmin. In addition, the amount of fat in cheese may positively affect degree of primary proteolysis, but not secondary proteolysis (Fenelon et al., 2000). During the secondary proteolysis, intermediate-size peptides are hydrolysed further into small peptides and amino acids by proteinase and peptidase from starter culture and NSLAB (Shakeel-Ur-Rehman et al., 1998). Degree of secondary proteolysis is quantified by measuring the levels of phosphtungstic acid-soluble nitrogen (PTA-SN) and free amino acids (FAA) (Jarrett et al., 1982).

Proteolysis may affect texture and sensory properties of cheese (Upadhyay et al., 2004). It contributes to the development of cheese texture by hydrolysing protein matrix of cheese, increasing pH through the production of ammonia (NH_3) from amino acids catabolism and by increasing the water-binding capacity of the curd through the formation of new α–carboxylic and α–amino groups produced during hydrolysis of peptide bonds (Fenelon et al., 2000). Proteolysis may contribute to cheese flavour through the liberation of short peptides and amino acids, some of which may have flavours and through the production of amino acids as precursors

for a range of catabolic reactions which produce many important volatile compounds (Fenelon et al., 2000; Upadhyay et al., 2004).

Basically, methods of assessment of proteolysis are categorised into (1) non-specific methods and (2) specific methods (McSweeney and Fox, 1993). The non-specific methods provide information about the extent of proteolysis and the activity of proteolytic agents; however, specific peptides produced or degraded during ripening period cannot be revealed. These methods include determination of soluble nitrogen using Kjeldhal method and measurement of reactive groups. Specific methods such as chromatography: capillary electrophoresis (CE) and Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) can be used to resolve and isolate peptides produced during ripening.

2.4.3.2 Lypolysis

Free fatty acids (FAA) produced from lipolysis during cheese ripening become precursors for many catabolic reactions resulting in flavour and aroma compounds. These FAA include methyl ketones, lactones, esters, alkanes, and secondary alcohols (Fox and McSweeney, 1998; McSweeney and Sousa, 2000). Lactones are cyclic esters formed by esterification of hydroxyl fatty acids. They confer a nutty, coconut, and buttery type character to cheese (Wallace and Fox, 1997). The presence of high concentration of lactones may cause rancid flavour in Cheddar cheese (Wong et al., 1975). Esters are highly flavoured and arise from reaction between short to medium fatty acids and alcohols derived from lactose fermentation. In Cheddar cheese, some LAB cultures hydrolyse milk fat and esterify certain short fatty acids with ethanol resulting in ester with fruity flavour notes such

as ethyl butanoate (Molimard and Spinnler, 1996). Secondary alcohols are product of enzymatic reduction of methyl ketones (Engels et al., 1977). In the Cheddar cheese, 2-propanol odours can be describes as fruity, green, fuel oil like, and earthy. However, their real contribution to the overall cheese flavour is limited since they have high odour thresholds.

2.4.3.3 Glycolysis

Lactic acid bacteria (LAB) are a diverse group of microorganism which produced lactic acid as the main metabolite of sugar metabolism (Upreti et al., 2006). This bacterial group include strains of *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Leuconostoc*, and *Bifidobacterium* genera (Vasiljevic and Shah, 2008). Based on mechanism of sugar utilisation, LAB are categorised into homofermentative and heterofermentative groups. Bacterial strains which are included in homofermentative group belong to *Lactococcus*, *Streptococcus*, and some *Lactobacilli*. This group of bacteria transform glucose to pyruvate, and eventually lactic acid, through the Embden-Meyerhof-Parnas (EMP) pathway (glycolysis). Meanwhile, heterofermentative bacteria such as *Leuconostoc* and remaining lactobacilli, use phosphoketolase pathway to transform glucose into lactate, carbon dioxide (CO_2), and either acetic acid or ethanol (Vasiljevic and Shah, 2008).

Due to their ability to transform ~95% of fermented sugar into L-lactate, *Lactococci (Lactococcus lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*) is the most common starter LAB used during manufacture of several cheeses (i.e. Cheddar, Colby, cottage, cream, and Blue cheeses) and fermented milk products such as

buttermilk and sour cream (Platt and Foster, 1958). Under normal growth conditions, at which cells are free of stress of pH, salt, or water activity, *Lactococci* are homofermentative bacteria and thus most of lactose is converted to lactate through EPS pathway. However, under certain conditions such as carbohydrate limitation, reduced rates of sugar metabolism, and aerobic condition, the bacteria use other pathways to metabolise lactose (mixed acid fermentation) (Cocaign-Bousquet et al., 1996). As a result, pyruvate is converted into formate, acetate, and ethanol through reactions which involves several enzymes such as pyruvate formate lyase or pyruvate dehydrogenase. In addition, some flavour compounds such as diacetyl and acetoin can be formed (Vasiljevic and Shah, 2008). Non-starter lactic acid bacteria (NSLAB) are milk microflora derived from adventitious microflora in milk and can survive pasteurisation (Crow et al., 2001). The main NSLAB in Cheddar cheese include *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Steptococcus thermophilus* (Swearingen et al., 2001).

Lactic acid is mainly produced from metabolism of residual lactose by starter LAB before salting and moulding process during cheese manufacturing (Upreti et al., 2006). However, nearly ~98% of lactose is then removed with the whey, and ~0.3-0.6% is retained in the cheese curd (Fox et al., 1990). This residual lactose is then metabolised quickly by starter and non-starter lactic acid bacteria (NSLAB). Then, non-starter lactic acid bacteria (NSLAB) isomerise the remaining lactose to DL-lactate and subsequently racemize it to L-lactate (Fox et al., 1990). This last step may affect texture and structure of cheese since racemisation support the development of calcium-D-lactate crystals. In addition, LAB and NSLAB could

metabolise the L-lactate to carbon dioxide (CO_2) and acetate. The high level of CO_2 and acetate may give off-flavour perception (Aston and Dulley, 1982). Lactate may also undergo anaerobic fermentation to butyrate, H_2, and CO_2 (Fox and McSweeney, 1998). Fermentation of lactose also reduces pH of cheese curd to a value that prevents the growth of pathogenic bacteria. During ripening, cheese pH increase due to the formation of alkaline nitrogenous compounds and/or catabolism of lactic acid. Another product of glycolysis, citrate, is also precursor of flavour compounds. Most of citrate is lost in whey during cheese making process, however ~0.2-0.5% of citrate is remain in cheese curd and can be metabolized to diacetyl, acetate, acetoin, 2,3 butandiol, and CO_2 by LAB and NSLSB (Fox et al., 1990).

A rate and extent of lactose metabolism by starter culture or NSLAB during ripening is dependent on the salt to moisture (S/M) ratio of the cheese (Thomas and Pearce, 1981). This ratio may affect water activity (a_w) and consequently bacterial activities in metabolising lactose (Grummer and Schoenfuss, 2011). At low S/M ratio (< 4.1%), residual lactose is metabolised within 8 days from the day of manufacturing. However, at high S/M ratio (> 6.3%), a relatively high amount of residual lactose was still present for several weeks after manufacturing (Cogan and Beresford, 2002; Beresford and Williams, 2004). Besides S/M ratio, other factors, such as the availability of residual lactose (Upreti et al., 2006), salt content in cheese, temperature of incubation, and pasteurisation of cheese-milk, would also influence the content of lactose during ripening period. Salt in cheese could affect lactose metabolism by controlling the growth and activity of microbial in cheese and

determine the S/M ratio, while pasteurisation may affect the growth and activity of starter bacteria (Upreti et al., 2006).

2.4.3 Physical properties of the Cheddar cheese

Textural properties of cheese are critical parameters and may determine cheese sensory attributes and functionality (Lucey and Singh, 2003). During ripening period, development of the Cheddar cheese texture can be divided into two phases: primary and secondary phase. The primary phase occurs during the first two or four weeks of ripening. Para-casein network of the curds are weakened, resulting in softening of cheese texture. The secondary phase occurs for the duration of ripening and involves more gradual changes in cheese texture due to continuing changes in pH and proteolysis (Lawrence et al., 1987).

Texture Profile Analysis (TPA) is a measurement of cheese texture involving primary characteristics of cheese such as hardness, cohesiveness, adhesiveness, elasticity (springiness), and brittleness (fracturability) (Szczesniak, 2002). According to Szczesniak (2002), hardness is defined as force (in Newtons) necessary to attain a given deformation. Hardness of cheese can be affected by the level of calcium (Ca) and phosphorus (P), residual lactose, and the salt to moisture (S/M) ratio. The high level of Ca, P or moisture content in cheese may lower degree of proteolysis which in turn may affect the cheese hardness (Upreti et al., 2006). Cohesiveness is a measure of the extent to which the cheese can be deformed before it ruptures (Upreti et al., 2006). According to Tunick (2000), cohesiveness in cheese is the measure of the strength of the internal bonds of protein mycelium. The cohesiveness of cheese is affected by the levels of Ca and P (Upreti et al., 2006). The cross-linkages of Ca and

P increase casein intermolecular associations in the cheese para-casein network (Upreti et al., 2006).

Springiness is defined as a measure of the recovery of the original undeformed condition after the first compression force removed during a TPA test (Szczesniak, 2002). The levels of Ca and P may positively correlate with cheese springiness. A low level of Ca and P may result in a soft and plastic Cheddar cheese (Upreti et al., 2006). Chewiness is energy required to disintegrate the cheese and to change it to a consistency suitable for swallowing (Szczesniak, 2002). Chewiness is influenced by others TPA parameters such as hardness, cohesiveness, and springiness (Szczesniak, 2002). It is negatively correlated with proteolysis, but positively correlated with the level of Ca and P, residual lactose, and S/M ratio (Upreti et al., 2006).

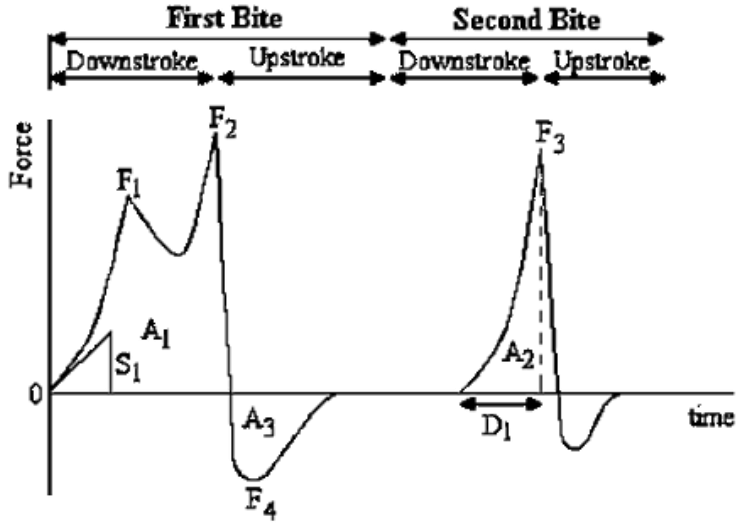


Figure 2.13 Typical TPA curve obtained from a double bite compression test.
A_1, Area 1; A_2, Area 2; A_3, Area3; D_1, the start of second bite; F_1, Force at the first peak, Fracturability; F_2, Force 2 Hardness; F_3, Force at the second bite.
Source: Bourne (1978).

2.4.4 EFFECT OF FAT AND CONDENSED TANNIN SUPPLEMENTATIONS ON THE CHEDDAR CHEESE PROPERTIES

2.4.4.1 **Effect of fat supplementation on the Cheddar cheese properties**

Depending on feeding level, types of fat, sources, and animal physiological stage, feeding fat to ruminants may affect content of fat, protein, lactose, and total solid in milk (Wu and Huber, 1994). Content of milk fat is the most vulnerable to any changes in diets (Bauman and Griinari, 2001). In contrast, protein content or lactose concentration in milk are more difficult to change (Wu and Huber, 1994). Being the main components in cheese, alteration in content of fat or proteins in milk used for cheese manufacture may impact yield, chemical composition, sensory, and textural properties of this dairy product (Guinee and Law, 2002).

Effects of supplemented fat on cheese yields reported in literature vary amongst studies (Avramis et al., 2003; Zhang et al., 2006). The Cheddar cheese manufactured from milk of ewes fed diet-supplemented flaxseed (*Linum usitatissimum*) had higher actual-, adjusted-, and theoretical yields compared to that of control which might be attributed to the higher total solid in the milk (Zhang et al., 2006). In contrast, no marked effect on yield efficiency of the Cheddar cheese manufactured from milk of cows fed fish meal-supplemented diet (Avramis et al., 2003).

Through its impact on altering rumen biohydrogenation, supplementation of free oil from plant or seed tends to reduce the content of milk fat and alter milk fatty

acid composition (Bauman and Griinari, 2001). Reduction in concentration of short- and medium-chain fatty acid and increase in concentration of long-chain fatty acids have been reported with addition of fats particularly those rich in PUFA (Bauman and Griinari, 2001). In general, processing of cow milk into cheeses does not alter fatty acid composition because the influence of the processing is negligible (Lucas et al., 2006). Similar results were observed in milk and cheese of sheep (Nudda et al., 2005) or ewes (Zhang et al., 2006). Baer et al. (1996) reported that cows fed flaxseed, rich in α-linolenic acid, produced milk with high concentration of CLA and linolenic acid. Processing the milk into cheese resulted in a cheese with high concentration of these fatty acids.

Together with cheese pH, fat in dry matter (FDM) content, mineralisation, and proteolysis, fatty acid profile may affect texture properties of cheeses (Lucey and Fox, 1993; Noel et al., 1998; Martin et al., 2005; Coppa et al., 2011). The influence of fatty acid composition on cheese texture relies mainly on C16:0 and cis-9-C18:1, which are the major milk saturated and unsaturated FA that have high and low melting points, respectively (Coppa et al., 2011). Milk with high concentration of PUFA used for cheese manufacturing may result in cheeses with creamy and less firm texture (Avramis et al., 2003; Coppa et al., 2011). Milk enriched with CLA can be used to produce the Edam cheese with a softer texture (Ryhanen et al., 2005).

However, Luna et al. (2005) reported that there was no significant difference in texture of cheese made from milk of sheep fed whole linseed and sunflower oil. Effects of PUFA in reducing firmness of cheese texture have also been reported for mozzarella cheese manufactured from milk fortified with either animal or vegetable

fat (Bermudez-Aguirre and Barbosa-Canovas, 2011). Cheese sensory properties are related with its fatty acid composition (Urbach, 1990). According to (Urbach, 1990), dietary linoleic acid may cause formation of sweet, raspberry flavour in cheese. Dietary fish meal caused the Cheddar cheese with stronger flavour (Avramis et al., 2003). However, other studies indicated no significant effect of supplementation of extruded soybean on flavour of the Cheddar cheese (Khanal et al., 2005) or butter (Ramaswamy et al., 2001).

2.4.4.2 **Effect of tannin supplementation on the Cheddar cheese properties**

Phenolic compounds in dairy products are mainly derived from animal feed and to a lesser extent form amino acid metabolism in these dairy products (Lopez and Lindsay, 1993). Phenolic compounds in dairy products derived from animal diet or amino acid metabolism are referred as indigenous phenolic compounds. Meanwhile, those added directly during processing are called exogenous phenolic compounds (O'Connell and Fox, 2001).

Even though there is evidence that the presence of condensed tannins in animal diets may affect milk composition, their significance on quality of dairy products has not been elucidated fully (O'Connell and Fox, 2001). Due to their astringency effect, phenolic compounds, including condensed tannins, have important role in sensory characteristic of dairy products (O'Connell and Fox, 2001). Astringency effect of these phenolic compounds is due to binding of these compounds with salivary glycoprotein and mucopolysaccharides on the tongue. This causes development of a feeling of constriction, roughness, and dryness on the palate (Haslam and Lilley, 1988).

The effect of indigenous phenolic compounds on sensory properties of dairy products is influenced by their concentration in these products. At a low concentration, these compounds may impart desirable sweet, smoky or caramel flavours to a range of dairy products. However, at a high concentration, they may cause undesirable sensory traits such as sharp, medicinal and sheepyard (Ramshaw, 1985). Indigenous phenolic compounds are also related to the off-flavour in the Cheddar cheese (Ramshaw et al., 1990) and milk (Lemieux and Simard, 1994). The impact of indigenous phenolic compounds on the sensory attributes of dairy products is, on most occasions, likely to be very subtle and when they are present at sub-threshold concentration they contribute to a delicate balance of flavour components (O'Connell and Fox, 2001).

From this review it appears that supplementation of cottonseed oil and condensed tannins in animal diet could impact yield and quality of cheese made of milk obtained from cow fed these modified diets. Diet exerts its effect on cheese properties mainly by altering composition of the resulting milk or directly transferring plant compounds into milk or dairy products (Walker et al., 2004). Effects of these supplementations are influenced by factors related with the supplement itself such as concentration, types, and source of supplements. In addition, animal factors, for example lactation period and breed may also influence the effect of these supplements on milk and cheese (Wu and Huber, 1994). Therefore, it is important to establish effect of addition of cottonseed oil, *A. mearnsii*-condensed tannins, or in combination on yield, chemical composition,

biochemical processes, texture, and sensory properties of the resulting Cheddar cheese.

3 Effects of dietary cottonseed oil and tannin supplements on protein and fatty acid composition of bovine milk

Chapter 3

3.1 INTRODUCTION

Supplementation of fat or plant secondary metabolites in ruminant diets is a promising strategy to reduce enteric methane (CH_4) emission. Despite an extensive body of published research into dietary manipulation to reduce enteric CH_4 emissions (e.g.: (Wu and Huber, 1994; Grainger et al., 2009; Grainger et al., 2010), little attention has been paid to the effects of modified diets on the resulting milk composition and its suitability for manufacturing purposes.

Feeding cottonseed oil to dairy cows could affect feed intake, milk fatty acid profile, and the content of fat and protein in milk (Mohamed et al., 1988; Zheng et al., 2005). High concentration of polyunsaturated fatty acid in ruminant diets could alter the pathways of rumen biohydrogenation. As a result, intermediates fatty acids, which could inhibit *de novo* synthesis of milk fat and alter milk fatty acid (FA) profile, might be produced (Palmquist et al., 2005). Effects of dietary vegetable oil on milk composition are variable and depend on the concentration of fat, forage type in diet, supplementation form, and stage of lactation (Grainger and Beauchemin, 2011).

Condensed tannins are plant secondary metabolites, which have antibacterial properties and capacity to bind protein, carbohydrate, and fibre (Waghorn, 1990). These compounds may affect dry matter intake (DMI), nitrogen digestibility, and microbial protein synthesis (Waghorn, 1990). *Acacia mearnsii* is a leguminous plant, which contains a substantial amount of condensed tannins (Khiaosa-Ard et al., 2009). An *in vitro* study showed that *A. mearnsii*-condensed tannin has a potency to

alter rumen biohydrogenation (Khiaosa-Ard et al., 2009). However *in vivo* studies showed inconsistent effects of added tannins on milk fat content and FA profile (Benchaar and Chouinard, 2009; Cabbidu et al., 2009). Differences in concentration, processing, and animal species might contribute to these inconsistent findings (Toral et al., 2011). Unsaturated fatty acid (Jenkins, 1993; Pantoja et al., 1994) and condensed tannins (Carulla et al., 2005) have a similar effect on inhibiting rumen microorganism and decreasing organic matter digestibility. However, information on effect of combination of fat and tannin supplement on milk composition is still limited.

In this study we hypothesized that supplementation of the diet of dairy cows with cottonseed oil, condensed tannin, or their combination would alter the composition of the resulting milk. Thus the objective of this experiment was to investigate the effects of supplementing a dairy cow diet with cottonseed oil, condensed tannin, or combination of cottonseed oil and condensed tannin on milk fat and protein composition.

3.2 MATERIALS AND METHODS

3.2.1 Cows and treatments

The experiment was carried out at the Department of Primary Industries (DPI) Ellinbank Research Centre, Victoria, Australia (38º14’ S, 145 º56’ E). The 13-week experiment was designed as a double 4x4 Latin square. Eight lactating, multiparous, rumen-fistulated (Rumen Cannula, www.rumencannula.com) Holstein-Friesian cows (32 ± 3.4 kg milk/days, 39 ± 13.0 days in milk, 4.1 ± 1.31 years of

age, 618 ± 55.9 kg body weight) were fed over 4 periods, such that a cow received each treatment once, and there were two of each treatment imposed in each period. Each period was of 21 days duration. Eight columns of the double Latin square were assigned to the eight cows with four rows assigned to the treatment diets, and these two sections were independently permuted at random. The treatment diets were: (1) a control treatment (CON), in which cows received a daily diet of approximately 6.0 kg dry matter (DM) of concentrates and ad libitum (~20 kg DM) alfalfa hay. The concentrate mix contained, on a dry matter basis, 68.3% crushed wheat, 25.0% cold-pressed canola meal, 2.0% mineral mix, 4.7% palabind molasses powder. The CON cows also received 800 ml/d of water, which was administered per fistula; (2) a treatment comprised CON diet, but instead of the water each cow was administered 800 g/d of cottonseed oil per fistula (CSO) (5 Ways Food Service, Dandenong, Victoria, 3175, Australia); (3) CON diet, but instead of the water each cow was administered 400 g/d of tannin from *A. mearnsii* per fistula (TAN) (Mimosa Central Cooperative Ltd, Pietermaritzburg, 3200, South Africa); (4) CON diet with water replaced by 800 g/d of cottonseed oil and 400 g/d of tannin from *A. mearnsii* (CPT). Treatment specific additives were administered per fistula, twice daily following the morning (~0700 h) and afternoon (~1500 h) milkings. The CON supplementation (water) was simply poured into the rumen. For the CSO, TAN and CPT supplements, ~5 kg of rumen material was removed and the supplement was thoroughly mixed with this material, after which the mixture was placed into the rumen. Alfalfa hay (~ 11 kg DM per feed) was offered at indoor feeding stalls (0800

– 1100 h and 1600 – 2000 h) after cows had been given their concentrate supplement.

Two additional, ruminally cannulated cows were fed the CON diet throughout the experiment so that they could act as donors of rumen fluid to enable refaunation of the experiment cows at each diet transition. During each treatment period, individual cows were gradually introduced to their dietary treatment so that on day 1 they were administered 50% of their putative dietary treatment. On day 2 and 3, 75% of their dietary treatment was administered followed by 100% of the full daily dose of the dietary treatment from day 4 to day 21. On day 2 of periods 2, 3 and 4, the rumens of all cows were inoculated with approximately 2 kg wet mass of rumen contents from the donor cows.

All quantities of feed offered and refused by individual cows were weighed and recorded. Feeds offered and refused were separately sampled and bulked for determination of DM concentration by drying in a forced draft oven at 105 °C for 24 h. Samples of the alfalfa hay, and the concentrates were bulked each day during the experiment and kept frozen at -18 °C. These samples were subsequently freeze-dried and ground to pass through a 0.5-mm screen and analysed for crude protein (CP), neutral detergent fibre (NDF), metabolizable energy (ME) and crude fat using near infra-red spectroscopy (#989.03; (AOAC, 2000)) at a commercial laboratory (Dairy-One Forage Laboratory, Ithaca, NY). Sub-samples of the freeze-dried ground feeds and supplements were analysed for concentration of total and individual fatty acids. Fatty acids were extracted from feeds using standard methods (IDF, 2001b, 2002b) and analysed for FA using a gas chromatographic procedure reported by

Ponnampalam et al. (2010). Calculations to estimate the total amounts of total FA and of individual FA that were eaten or administered, or estimated to have been absorbed from the intestines were made on the basis of the feed intake data, fatty acid composition of the dietary feed ingredients and supplements, and using the fat model in CPM-Dairy (Moate et al., 2004).

3.2.2 Milk sample analysis

Milk yield from each cow was recorded using a DeLaval ALPRO milk metering system (DeLaval International, Tumba, Sweden). The milk was collected into test buckets on days 20 and 21. Representative samples were chilled to 4 ºC prior to analysis for fat, lactose, total solids, fatty acid composition, protein composition and protein fractions within 48 hours. Milk samples for composition analysis were stored at -20 ºC. Prior to analysis, the frozen milk samples were thawed overnight at room temperature. Fat and lactose content in milk were measured using a near-infrared milk analyser (model 2000, Bentley Instruments, Chaska, MN, USA). Total solids concentration was determined according to method No. 925.23 (AOAC, 2006).

3.2.3 Protein composition

The thawed milk samples (50 mL) were centrifuged (1000 x *g*; 4 °C; 10 min) with a bench top centrifuge (Sorvall RT7, Newton, CT, USA) to remove fat. An aliquot of each skim milk sample was analysed on a Kjeltec System II (with Digestion System 2000 and Distillation Unit 1002; Tecator, Hoganas, Sweden) to determine the concentration of total nitrogen according to method No. 991.20

(AOAC, 2006). Proteins in milk samples were precipitated with 12% w/v trichloracetic acid, and following centrifugation (1000 x *g*; 4 °C; 10 min), filtrates were used to determine the concentration of non-protein nitrogen (NPN, #991.20; AOAC, 2006). Non-casein nitrogen (NCN, #998.05; AOAC. 2006) was determined using filtrates of skim milk samples after acidification with 10% v/v acetic acid and sodium acetate (1M) solutions at pH 4.6. Casein nitrogen (CN) was calculated as:

$$[CN] = [TN] - [NCN]$$

Nitrogen concentration was then converted to equivalent protein concentration using the coefficient of 6.38 (#998.07; AOAC. 2006). True protein (TP) concentration was calculated as:

$$[TP] = \{[TN] - [NPN]\} \times 6.38$$

Whey protein concentration was determined by NCN method (NCN, #998.05; (AOAC, 2006)).

Protein fractions

Content of individual protein fractions (αs_1-casein, αs_2-casein, β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin) was determined using a reversed phase-high performance liquid chromatography (RP-HPLC; Bonizzi et al. 2009). Skimmed milk samples (0.4 mL) were diluted with 1.6 mL of denaturing urea solution (8 M urea, 165 mM Tris, 44 mM sodium citrate and 0.3% v/v β-mercaptoethanol) and filtered through 0.45 μm pore cellulose membrane and directly analysed. Casein fractions were separated and quantified by a RP-HPLC with a Zorbax 300SB-C8

RP-HPLC column (silica-based packing, 3.5 micron, 300A, Agilent Technologies Inc., USA). Eluent A was HPLC-grade water containing 0.1% v/v trifluoroacetic acid (TFA) and eluent B was HPLC-grade acetonitrile containing 0.1% v/v TFA. The gradient elution programme was run at a constant flow rate of 0.8 mL/min and was set as follows: 0-40 min linear gradient from 30% B to 50% B; 40 to 42 min linear gradient from 50% B to 100% B; 42 to 43 min isocratic elution 100% B; 43-46 min linear gradient from 100% B to 30% B followed by a 5 min isocratic elution at the initial conditions.

Standard solutions were prepared according to (Bonizzi et al., 2009). Single-fraction mother solutions were prepared by adding respectively, 249.5 mg of purified αs-CN (purity 70%, catalogue number C6780, SIGMA, Castle Hill, NSW, Australia), 200.5 mg of purified β-CN (purity 98%, catalogue number C6905, SIGMA, Castle Hill, NSW, Australia), 51.2 mg of purified κ-CN (purity 70%, catalogue number C0406, SIGMA, Castle Hill, NSW, Australia), 201.2 mg of purified β-Lg B (purity 90%, catalogue number L8005, SIGMA, Castle Hill, NSW, Australia), and 201.6 mg purified β-Lg A (purity 90%, catalogue number L7880, SIGMA, Castle Hill, NSW, Australia) in 10 mL a denaturing solution. A mixed standard solution was prepared by mixing 1 mL of each single solution with 2 mL of the denaturing solution. Then, a set of four mixed concentration standards was obtained by adding 0.4 mL, 0.8 mL, 1.2 mL, and 1.6 mL of mixed mother solution to 1.6 mL, 1.2 mL, 0.8 mL, and 0.4 mL of urea solution, respectively. These standard solutions were used to construct αs1-, αs2-, β, and κ-CN as well as β-Lg, and α-Lac calibration curves. Since αs1- and αs2 are not available as single proteins, the

corresponding values were calculated from the αs by applying the 4:1 proportion known for cow milk (Fernandes et al., 2008).

3.2.4 Fatty acid composition

Milk fat was extracted and FA was methylated according to ISO14156-IDF172 and ISO15884-IDF182 (IDF, 2001b). Fatty acid methyl esters were quantified by a Varian CP-3800 gas chromatography (GC) (Varian Inc., Palo Alto, CA) equipped with Varian CP-8400 auto sampler and a 100-m (0.25mm ID×0.2µm DF) CP-Sil 88 column. The oven temperature was programmed following the method of Kramer et al. (2004): 45 °C (4 min), 13 °C/min to 175 °C (held for 27 min) and 4 °C/min to 225 °C (held for 10 min). The injector and flame ionization detector (FID) temperatures were 250 °C and 275 °C, respectively. Helium was used as the carrier gas at 29 psi. Fatty acids were identified using a standard mixture of 37 fatty acids C4-C24 (catalogue number CRM 47885, SUPELCO, Bellefonte, PA, USA). Rumenic acid (c9, t11 CLA) and vaccenic acid (t11 C18:1) were identified by linoleic acid, conjugated methyl ester (catalogue number 05632, SIGMA, Sigma-Aldrich, NSW, Australia) and trans-11-vaccenic acid (catalogue number 46905, SUPELCO, Bellefonte, PA, USA) respectively. Nonanoic acid methyl ester (catalogue number N5502, SIGMA, Castle Hill, NSW, Australia) was used as the internal standard. The Δ^9-desaturase index is ratio of Δ^9-desaturase products to Δ^9-desaturase substrates + products. This index was calculated as C14:1+C16:1+cis-9C18:1+cis-9,trans-11C18:2]/[C14:0+C14:1+C16:0+C16:1+C18:0+cis C18:1+trans-11 C18:1+cis-9, trans-11 C18:2] (Wales et al., 2009), while Δ^9-desaturase activities on selected milk FA were calculated using product-to-substrate ratios of

C14:1:C14:0, C16:1:C16:0; cis-9 C18:1:C18:0; and cis-9 trans-11 C18:2: trans-11 C18:1 (Lock and Garnsworthy, 2003). Fatty acids were extracted from feeds using standard methods (IDF, 2001a, 2002a) and analysed by a gas chromatographic procedure reported by (Ponnampalam et al., 2010).

3.2.5 Statistical analysis

Data were analysed using ReML in GenStat 13 to cope with imbalance caused by substitution of one cow after the first period. The statistical model was as follows:

$$y_{ij} = \mu + \alpha_f + \beta_t + \gamma_{ft} + C_i + P_j + \epsilon$$

where μ is the overall mean, αf is the main effect of fat treatment f, βt is the main effect of tannin treatment t, γft is the interaction effect of fat and tannin treatments f and t, Ci is an effect for cow i, Pj is an effect for period j and εij is a random error term. All terms were included as fixed effects except for the error term, ε.

Tannin and Fat main effects and interaction were tested by F-tests. Distributional assumptions of normality and constant variance were checked visually using graphs of residuals verses fitted values, histograms of residuals and normal quantile plots. Cell count data were log-transformed prior to analysis.

3.3 RESULTS AND DISCUSSION

3.3.1 Effect of CSO diet on milk yield and composition

In this experiment, inclusion of CSO in the diet did not affect ($P > 0.05$) total feed intake (Table 3.2). This result is consistent with the finding of Mohamed et al.

(1988) but in contrast to results of other studies which showed a decrease (Bremmer et al., 1998; Benson et al., 2001). This discrepancy could be related to the fact that the effects of fat supplementation on feed intake are influenced by degree of fatty acid saturation, feeding rate, palatability, fatty acid chain length, and form of fat (Drackley and Elliott, 1993; Bu et al., 2007).

Table 3.3 shows that CSO diet increased the yields of milk ($P < 0.05$). This increase in milk yield might be associated with the effect of dietary cottonseed oil in supplying more energy for the dairy cows to support milk production (Weiss et al., 2011). In addition, the increase in milk production might also be associated with the effect of the CSO diet in reducing *de novo* synthesis of FA (Table 3.4). Supplemented fat may increase energetic efficiency of milk production through its effect on decreasing *de novo* synthesis FA which requires acetate (DePeters and Cant, 1992).

The CSO diet also increased milk protein yields of cows fed this diet ($P < 0.05$). This result is in agreement with Wu and Huber (1994), who stated that supplementation of fat at concentration below 8% of DM may increase the yield of milk protein. The increase of available energy due to cottonseed oil supplementation might also lead to greater milk protein yield (Table 3.3) by improving the efficiency of microbial protein synthesis (Hoover and Stokes, 1991). Despite its positive effect on milk protein yield, CSO diet had no impact ($P > 0.05$) on the content of milk protein (Table 3.3). The lack of effect of the CSO diet on milk protein content observed in our experiment (Table 3.3) is consistent with the findings of Mohamed

et al. (1988) and Zheng et al. (2005), but contrasts with those of DePeters et al. (1985).

Table 3.1 Composition of main dietary constituents (g/kg DM unless otherwise stated).

Items[1]	Alfalfa	Wheat	Cold pressed canola	Cotton-seed oil	Tannin	Molasses
Crude protein	205	168	387	-	24	86
ADF[1]	327	72	182	-	2	81
NDF[2]	423	141	302	-	39	196
Lignin	74	10	81	-	2	16
NFC[3]	288	661	177	-	909	551
Starch	16	605	19	-	6	53
Ash	95	23	62	-	22.3	190
Total FAs[4]	12.0	26.8	104.9	992.0	0.23	-
Individual FA (% of total)						
C16:0	32.2	17.9	5.8	19.9	26.4	-
C18:0	5.5	1.5	1.9	2.4	0	-
C18:1 cis-9	3.5	20.8	56.6	17.9	13.2	-
C18:1 cis-11	1.2	0.9	6.6	0.9	0	-
C18:2 n6	17.8	54.7	19.2	58.4	60.5	-
C18:3 n3	33.3	3.4	8.1	0	0	-
C20:0	1.5	0	0.5	0	0	-
C20:1	0	0.7	0.9	0	0	-
C22:0	1.7	0	0	0	0	-
Other	3.3	0	0.5	0.5	0	-

[1]ADF, acid detergent fibre;
[2]NDF, neutral detergent fibre;
[2]NFC, non-fibrous carbohydrate;
[4]FA, fatty acid

Table 3.2 Effect of administration of tannin, cottonseed oil, and cottonseed oil plus tannin on the intake of concentrate mix and alfalfa hay, the calculated dietary intakes of crude protein, NDF, NFS, total and individual fatty acids.

Items	Dietary treatment[1]				
	CON	CSO	TAN	CPT	SED
Feed intake[2] (kg DM[3]/cow per d)					
Concentrate	5.8	5.9	5.9	5.9	0.11
Alfalfa hay	19.1	18.1	19.0	18.2	0.64
Total	24.8	24.0	24.9	24.1	0.66
Crude protein	5.15	4.82	5.10	4.77	0.137
NDF[4]	9.11	8.45	8.96	8.36	0.242
NFC[5]	8.51	8.04	8.79	8.29	0.226
ME[6] intake (MJ[7]/cow per d)	262.9	283.2	268.6	289.5	7.53
Fat	0.72	1.51	0.73	1.52	0.040
Fatty acids (g/cow per d)					
Total	487	1184	491	1185	32
C16:0	102	238	102	239	6.0
C18:0	17	33	17	33	0.9
C18:1trans	1	1	1	1	0.03
C18:1 cis	130	264	132	264	7
C18:2	128	539	129	539	14
C18:3	92	89	92	89	2.1

[a,b]Means in the same row within a category with different superscripts differ ($P < 0.05$)
[1]Dietary treatments: CON = control; CSO = cottonseed oil; TAN= tannin; CPT = cottonseed oil plus tannin
[2]Intakes are in kg/cow per day unless otherwise stated
[3]DM = dry matter
[4]NDF = neutral detergent fibre
[5]NFC = non-fibrous carbohydrate
[6]ME = metabolizable energy
[7]MJ = megajoules

Table 3.3 Yields of milk constituents and milk composition of cows fed diet supplemented with cottonseed oil, condensed tannins, and in combination

Items	Dietary treatment[1]				SED
	CON	CSO	TAN	CPT	
Milk yield (kg/cow per d)	32.3[a]	35.4[b]	31.1[a]	34.8[b]	1.18
Milk fat (g/cow per d)	1346[b]	1224[ab]	1249[ab]	1130[a]	67.5
Milk protein (g/cow per d)	1082[b]	1170[c]	1012[a]	1141[c]	28.5
Milk lactose (g/cow per d)	1668[ab]	1798[b]	1540[a]	1745[b]	62.1
Fat (%)	4.1[b]	3.4[a]	4.0[b]	3.2[a]	0.16
Total solid (%)	14.0	13.8	13.2	12.9	1.32
Lactose (%)	5.2[b]	5.1[ab]	5.1[ab]	5.1[a]	0.04
Total protein (%)	3.3	3.3	3.3	3.3	0.23
True protein (%)	3.4	3.3	3.3	3.3	0.11
Casein (%)	2.7	2.8	2.8	2.7	0.14
α_{s1}-CN (mg/mL)	12.7	13.1	12.9	12.9	1.45
α_{s2}-CN (mg/mL)	3.3	3.6	3.5	3.4	0.35
κ-CN (mg/mL)	2.7	2.7	2.6	2.6	0.26
β-CN (mg/mL)	9.7	9.6	9.9	9.8	1.07
α-lactoglobulin (mg/mL)	1.2	1.0	1.0	1.1	0.11
β-lactalbumin (mg/mL)	3.3	2.7	2.7	3.0	0.31
Casein: True protein (%)	79.4	84.8	84.8	81.8	0.04
NPN (% nitrogen)	0.3	0.4	0.3	0.3	0.03
Log SSC	1.7	1.8	1.8	1.9	0.11

[a,b]Means in the same row within a category with different superscripts differ ($P < 0.05$).

[1]Dietary treatments: CON = control; CSO = cottonseed oil; TAN= tannin; CPT = cottonseed oil plus tannin. Treatments of cottonseed oil and tannin were administered per fistula in two equal portions following each milking.

The CSO diet had little influence on the proportions of true protein, whey protein, casein, casein to protein ratio and individual protein fractions (Table 3.3). A slight effect of the CSO diet on proportions of whey protein, casein, casein to protein ratio and individual protein fractions (Table 3.3) was similar to those reported by other researchers who used different fat sources such as sunflower or safflower seeds (Stegeman et al., 1992; Bobe et al., 2008). In contrast, DePeters et al. (1985) reported that content of protein, casein, and whey protein were reduced when soybean oil was added to the diet of dairy cows.

The CSO diet had no significant effect on yield and content of lactose (Table 3.3). The absence of an effect of the CSO diet on milk lactose content is in agreement with the findings of Zheng et al. (2005), but contrasts with those of Uddin et al. (2013) who observed a decrease. According to Sutton (1985), the content of lactose in milk is remarkably constant due to its osmotically active properties and the fact that it cannot pass out the Golgi or secretory vesicles. The minimal effect of the CSO diet on lactose yield and content might also be related to the unaltered feed intake (Table 3.2).

Effect of this diet on fat yield was minimal. However milk fat content was reduced by 17.14% (Table 3.3). It might be argued that the lower fat content in the milk of cows fed the CSO diet compared to the CON diet (Table 3.3) was due to the alteration in rumen biohydrogenation of linoleic acid from the cottonseed oil leading to the production of t10, c12 CLA which is known as a potent inhibitor of milk fat synthesis (Bauman and Griinari, 2001, 2003). However, in the current study, t10, c12 CLA was only detected in the milk fat of cows fed the CSO diet, yet the CPT

diet also contained cottonseed oil and also reduced milk fat percentage (Table 3.4) suggesting that some agent or fatty acid other than t10, c12 CLA may have been responsible for the reduced milk fat content in cows fed the CSO and CPT diets. In support of this theory, in the milks from cows fed the CSO and CPT diets, there were enhanced concentration of three unknown fatty acids whose chromatogram peaks were in the region where the two trans isomers, elaidic acid and trans-vaccenic acid normally elute (Table 3.4). We assume that these unknown fatty acids are octadecenoic acid isomers.

The CSO diet decreased concentration of total saturated fatty acids (SFA) by 15.2% (Table 3.4). This diet also reduced the percentage of short- (C6:0 and C8:0) and medium- (C10:0-16:0) SFA ($P < 0.05$; Table 3.4) but it increased ($P < 0.05$) C18:0 concentration (Table 3.4). These results are in agreement with (Bauman and Griinari, 2001, 2003) who stated that fat supplementation lower the concentration of short- and medium-chain FA (*de novo* synthesised fatty acids) and greater concentration of long chain fatty acids (FA with more than 16 carbon atoms). The greater concentration of C18:0 in the milks of cows fed the CSO and CPT diets compared to the CON diet (Table 3.4) were probably related to an increased ruminal supply of linoleic acid from the cottonseed oil, which was transformed into stearic acid during ruminal biohydrogenation. The effect of the CSO diet on increasing long chain SFA (C18:0) and reducing short- and medium-chain SFA, especially C12:0; C14:0; and C16:0 FA in milk could improve the nutritional value of milk (Chilliard et al., 2000). In terms of milk processing properties, it would be useful to ascertain the effect of cottonseed oil supplementation on cheese fatty acid composition,

flavour and aroma since the milk concentration of short- and medium-chain FA, particularly C4:0-C12:0, have been reported to influence the flavour and aroma of cheese (Molimard and Spinnler, 1996).

In our study, dietary supplementation with cottonseed oil (in the CSO and CPT diets) resulted in reduced concentration of linear-odd chain FA such as C13:0; C15:0 and C17:0 ($P < 0.05$) in milk, a finding contrary to that of Loor et al (2004). It also appeared that the percentage of butyric acid remained unaltered due to the presence of cottonseed oil in the diet. This result is consistent with the findings of Chilliard et al. (2000) who reported that the concentration of butyric acid in milk was not changed by dietary supplementation with fat. Butyric does not require acetate for its synthesis since it is produced in the mammary gland directly from β-hydroxybutyrate, derived from the blood, which is in contrast to the de novo synthesised FA manufactured in the mammary gland by chain elongation using acetate units (Craninx et al., 2008).

The CSO diet also increased the concentration of total monounsaturated fatty acids (MUFA) by 42%, total polyunsaturated fatty acids (PUFA) by 67%, linoleic acid by 50%, rumenic acid (c9, t11 C18:2) by 350%, and vaccenic acid (t11 C18:1) by 164% ($P < 0.05$; Table 3.4). Cottonseed oil contains a high concentration of linoleic acid (Table 3.1). Therefore the increase of linoleic acid concentration in milk fat of cows fed the CSO and CPT diets may be related to a higher intake of this fatty acid. We speculate that this high ruminal supply of linoleic acid served as a substrate for the ruminal production by biohydrogenation, of rumenic acid and vaccenic acid (Moate et al., 2007). Rumenic acid can be absorbed from the intestines, transported

via the blood to the mammary gland and incorporated directly into milk fat (Moate et al., 2008). When vaccenic acid is absorbed from the intestines and transported to the mammary gland, it enhances the concentration of vaccenic acid in milk fat (Moate et al., 2008), and the enhanced supply of vaccenic acid to the mammary gland also serves as a precursor for the production and secretion of rumenic acid into milk fat (Mosley et al., 2006).

3.3.2 Effects of TAN diet on milk yield and composition

The TAN diet did not alter feed intake (Table 3.2) and milk yield (Table 3.3). According to Waghorn et al. (1990), the presence of tannins in ruminant diets may reduce feed intake, nitrogen digestibility and microbial protein synthesis. Generally, dietary condensed tannins reduce feed intake by decreasing palatability or affecting digestion due to their astringency (Landau et al., 2000). The effect of condensed tannins on feed intake is influenced by their concentration in the diet. A high concentration of condensed tannins (i.e. above 5–6% DM) has been associated with a reduction in feed intake (Barry and McNabb, 1999). However, other factors such as the source of condensed tannins, animal species, animal physiological state and diet composition may also influence the effect of CT (McSweeny et al., 2001). In the current experiment, the lack effect of the TAN diet on feed intake may be partly attributed to the low concentration of condensed tannins in the diet. The lack of effect of condensed tannins on feed intake was also observed when heifers (Baah et al., 2007) or cows (Benchaar et al., 2008) were fed quebracho condensed tannins.

The minimal effect of the TAN diet on feed intake is consistent with the unaltered milk yield of cows fed this diet (Table 3.3). The lack of effect of

condensed tannin on milk production has also been observed in early lactating ewes fed *Lotus corniculatu*s (Wang et al., 1996). However, effects of condensed tannins in both increasing (Woodward et al., 1999; Turner et al., 2005) and decreasing (Grainger et al., 2009) milk yield in dairy cows have been reported.

Table 3.4 Fatty acid composition (g/100 g of fatty acids) of milk fat from dairy cows fed diet containing cottonseed oil, condensed tannins, and their combination

Parameter[2]	Dietary treatment[1]				SED
	CON	CSO	TAN	CPT	
C4:0	4.4	3.9	4.3	4.1	0.26
C6:0	2.5[b]	2.0[a]	2.5[b]	2.0[a]	0.17
C8:0	1.4[b]	1.1[a]	1.4[b]	1.0[a]	0.11
C10:0	3.4[b]	2.3[a]	3.3[b]	2.3[a]	0.32
C12:0	3.9[b]	2.6[a]	3.8[b]	2.7[a]	0.36
C13:0	0.2[b]	0.1[a]	0.2[b]	0.1[a]	0.04
C14:0	12.8[b]	10.1[a]	12.5[b]	10.1[a]	0.79
C14:1	1.0[b]	0.7[a]	0.9[ab]	0.9[ab]	0.10
C15:0	1.4[b]	1.1[a]	1.4[b]	1.2[a]	0.10
C16:0	35.4[b]	28.9[a]	34.5[b]	28.5[a]	1.64
C16:1n7	1.8	1.6	1.6	1.5	0.17
C17:0	0.9[b]	0.7[a]	0.9[b]	0.8[a]	0.06
C18:0	8.6[a]	10.7[b]	8.9[a]	10.9[b]	0.57
C18:1unk	0.2[a]	0.6[b]	0.3[a]	0.6[b]	0.07
C18:1unk1	0.3[a]	2.2[b]	0.3[a]	1.2[b]	0.08
C18:1unk2	0.3[a]	0.8[b]	0.3[a]	0.8[b]	0.12
C18:1n9t	0.2[a]	0.4[b]	0.2[a]	0.4[b]	0.07
C18:1 11t (TVA)	1.7[a]	4.5[b]	1.8[a]	4.9[b]	1.08
C18:1n9c	17.5[a]	20.3[b]	16.9[a]	20.3[b]	0.08
CLA trans C18:2	0.5	0.4	0.5	0.4	0.03
C18:2n6c	1.8[a]	2.7[b]	2.0[a]	3.0[b]	0.26
C18:2 9c11t (CLA)	0.4[a]	1.8[b]	0.7[a]	2.0[b]	0.07
C18:3n3	1.069[ab]	0.919[a]	1.080[b]	0.995[ab]	0.070
C20:3n6	0.015	0	0	0	Insufficient data
C20:4n6 (AA)	0.036	0.077	0.023	0	Insufficient data
C22:5n3 (DPA)	0.108	0.053	0.155	0.028	Insufficient data
Total SFA	74.8[b]	63.4[a]	73.7[b]	63.6[a]	6.52
Total MUFA	21.9[a]	31.0[b]	22.3[a]	30.4[b]	0.15
Total PUFA	3.3[a]	5.5[b]	4.0[a]	6.0[b]	0.57
De novo synthesized FA	49.5[b]	39.1[a]	48.4[b]	39.3[a]	2.20
Total n-6 PUFA	1.8	2.7	2.1	2.9	
Total n-3 PUFA	1.2	1.0	1.2	1.0	
Ratio of n-6:n-3 PUFA	1.5	2.8	1.6	2.9	
Desaturation index	0.26[a]	0.31[b]	0.26[a]	0.31[b]	0.012
14:1/14:0	0.078	0.069	0.072	0.089	0.008
16:1/16:0	0.051	0.055	0.046	0.053	0.005
18:1/18:0	2.236[a]	2.681[b]	2.201[a]	2.552[b]	0.141

[a,b]Means in the same row within a category with different superscripts differ ($P < 0.05$).

[1]Dietary treatments: CON = control; CSO = cottonseed oil; TAN= tannin; CPT = cottonseed oil plus tannin.

[2]TVA = trans vaccenic acid; CLA = conjugated linoleic acid; AA = arachidonic acid; DPA = docosapentaenoic acid; SFA = Total saturated fatty acids; MUFA = Total monounsaturated fatty acids; PUFA = Total polyunsaturated fatty acids; *De novo* synthesized FA= the amount of C4, 6, 8, 10, 12, 13, 14, 15, and C16:1n7 fatty acids.

However, the TAN diet reduced the yields of protein and lactose (Table 3.3). Reports on the effect of dietary tannins on yield and content of milk protein are inconsistent (Toral et al., 2011). Even though the mechanism responsible for the reduction in milk protein yield associated with feeding the TAN diet is still unclear, we speculate that dietary tannins could reduce yield of milk protein by inhibiting microbial protein synthesis in the rumen and thus reducing amino acid production. According to Hoover and Stokes (1991), synthesis of microbial protein is influenced by the availability of energy derived from fermentation of carbohydrates. Condensed tannins are able to form stable complexes with proteins or carbohydrates (Barry and McNabb, 1999). Binding of condensed tannins to carbohydrates could reduce the extent of carbohydrate degradation and decrease the amount of energy available for microbial protein synthesis (Bach et al., 2005) and consequently cause reduction in the yield of milk proteins (Table 3.3).

Reductions in lactose yields in milk of cows fed the TAN diet might be due to the limited energy supply in the diet. In this study, the experimental cows were in early lactation and would have had a negative energy balance as they mobilised adipose tissue reserves to support milk production. Moreover, condensed tannin supplements might reduce starch digestion, which could then reduce the production of volatile fatty acids. In turn, a reduction in the amount of propionate would limit the synthesis of glucose, a precursor for lactose synthesis. In contrast, condensed tannins of *L. corniculatus* had no effect on lactose content in milk (Woodward et al., 1999).

Furthermore, the TAN diet had no effect ($P > 0.05$) on yield and content of milk fat. Similar result was also observed previously in studies that used condensed tannins from quebracho (Benchaar et al., 2008) and *L. corniculatus* (Woodward et al., 1999). In contrast, condensed tannins from *Hedysarum coronarium* have been reported to induce a reduction in fat content in sheep milk (Molle et al., 2009). The most abundant fatty acids in milk from cows fed the TAN diet were palmitic (C16:0, 34.5.8%), myristic (C14:0, 12.5%) and stearic acid (C18:0, 18.9%). The total concentration of SFA, MUFA, PUFA, rumenic, linoleic acid, vaccenic acid and oleic acid in milk fat were also unaltered ($P > 0.05$). The concentration of *de novo* synthesised FA and of SFA were also unaffected by the feeding the TAN diet, a finding similar to that of Benchaar and Chouinard (2009), who used quebracho-condensed tannins. Unlike short- and medium-chain saturated fatty acids, saturated fatty acids with more than 16 carbon atoms cannot be synthesised in the mammary gland. Thus the concentration of the latter in milk depends on a dietary source and subsequent synthesis in adipose tissue (Chilliard et al., 2000). However, Turner et al. (2005) found that when cows were fed birdsfoot trefoil (*L. corniculatus*), the condensed tannins in the lotus appeared to inhibit ruminal biohydrogenation of long chain fatty acids and the net effect was a reduction in the concentration of SFA in milk fat. In the current experiment, the concentration of all of the individual fatty acids in the milk fat of the cows fed the TAN diet were not different from the concentration of the corresponding fatty acids in the milk fat of the cows fed the CON diet. Thus, these findings suggest that the tannins from black wattle is

ineffective at influencing the bacteria that produce the enzymes which are responsible for biohydrogenation processes within the rumen.

The concentration of PUFA can be enhanced by feeding ruminants PUFA-rich feed or by decreasing biohydrogenation of PUFA in the rumen (Palmquist et al., 2005). However, the TAN diet had no effect on the concentration of total PUFA (Table 3.4), which could be attributed to the low level of PUFA in the TAN diet. Attempts to increase the concentration of CLA and its isomers in milk should be directed at increasing CLA synthesis during biohydrogenation in the rumen, or by increasing the activity of Δ^9-desaturase in desaturating vaccenic acid to CLA in the mammary gland (Chilliard et al., 2000). Therefore, the negligible effect of the TAN diet on CLA concentration could be attributed to the low amounts of CLA precursor (linoleic acid) in the diet and the lack of effect of this diet on the Δ^9-desaturase enzyme activity in converting stearic to vaccenic acid (Table 3.3). Our results are similar to the previous report of Dschaak et al. (2011) but contradict *in vitro* studies, which indicated that tannins had potential to increase CLA concentration in milk by inhibiting the growth of *Butyrivibrio fibrisolvens*, a rumen microbe which is responsible for transforming the major CLA precursor into rumenic acid (Jones et al., 1994) or by inhibiting the last step of rumen biohydrogenation (Khiaosa-Ard et al., 2009).

3.3.3 Effects of CPT diet on milk yield and composition

The CPT diet did not affect total feed intake (Table 3.2) ($P > 0.05$). The yields of milk and milk protein were increased ($P < 0.05$) while protein content was unaffected. This diet showed no effect on lactose yield, but the content of lactose in

milk was decreased (Table 3.3). In comparison to the CON diet, the CPT diet reduced yield and percentage of milk fat (Table 3.3). The CPT diet also induced changes in FA composition similar to those caused by CSO diet (Table 3.4). In milk samples of cows fed the CSO dietary treatment, t10, c12 CLA constituted 0.03% of the total fatty acids, but in all other milk samples for the other dietary treatments, the concentration of t10, c12 CLA, were below the level of detection. For all cows in all treatment groups, the concentration of C20:5 n3 (EPA) and C22:6n3 (DHA) in milk fat were below the level of detection.

The observed effects of the CPT diet in reducing *de novo* FA synthesis and altering milk FA composition were likely due to the presence of cottonseed oil in the CPT diet since the addition of tannins had no effect on milk fat content and FA profile. This result is similar to the findings of Toral et al. (2011), who reported that the inclusion of tannin to a diet containing sunflower oil did not increase the amount of vaccenic acid or rumenic acid above that achieved with a diet containing sunflower alone. Overall, results from our experiment showed that supplementation of cottonseed oil, condensed tannins or their mixture had a minimal impact on milk yield and composition. However, inclusion of cottonseed oil or in combination with condensed tannin (in the diet) reduced milk fat content but increased the proportion of CLA and its isomers in milk fat.

3.4 CONCLUSION

This study showed that inclusion of cottonseed oil or condensed tannin into cow diet could affect rumen metabolism and altered yield and composition of the milk. It is likely that supplementation of cottonseed oil increased available energy to dairy cows, thus increasing yields of milk and milk protein. The presence of cottonseed oil in the diets also altered rumen biohydrogenation, which caused a reduction in milk fat content and altered milk fatty acid profile. Dietary condensed tannins from *A. maernsii* appeared to affect nitrogen and carbohydrate metabolism, which led to reduced yields of protein and lactose in milk. However, condensed tannins appeared to have no impact on rumen biohydrogenation. Changes in fat content and fatty acid profile in milk of cows fed the CPT diet were most likely due to the cottonseed oil in the diet since the effect of condensed tannins were not significant. Octadecanoeic acid isomers which eluted between elaidic acid and trans-vaccenic acid might be responsible for milk fat depression in milk of cows fed diets supplemented with cottonseed oil alone or in combination with condensed tannin. Further research is required to identify these isomers due to their possible effect on milk fat depression, and to establish the effect of dietary cottonseed oil and condensed tannin on milk processing properties.

4 Coagulation properties of milk obtained from cows fed modified diets containing dietary fat and condensed tannins

Chapter 4

4.1 INTRODUCTION

Due to an increased awareness of consequences of methane (CH_4) emission, there has been an upsurge in research related to diet modifications for dairy cattle that could potentially reduce this negative impact (Grainger et al., 2009; 2010). Previous experiments have reported that the whole cottonseed (Grainger et al., 2010) or plant secondary metabolites, for example condensed tannins (Grainger et al., 2009) as dietary supplements, could reduce enteric CH_4 emissions from dairy cows. It is, however, evident that these strategies alter the composition of milk either by direct transfer of components from the diet or by affecting animal metabolism, which consequently may influence its technological properties (Chapter 3, Walker et al., 2004).

Diets enriched with whole cottonseed have been reported to alter milk fatty acids (FA) composition by reducing the proportions of short and medium chain while increasing the proportion of long chain FA (DePeters et al., 1985). Even though reports on the effect of added fat on milk protein content have been inconsistent, it has been postulated that dietary fat might affect milk protein levels by sparing amino acids used in protein synthesis (Wu and Huber, 1994). Effects of added condensed tannin on amino acid availability for protein synthesis in ruminants have also been documented (Waghorn, 2008) but again the direction of change was inconsistent.

As shown in Chapter 3, the addition of dietary cottonseed oil, *Acacia mearnsii*-extract tannins, or their combination had an impact on milk composition by increasing the yield of milk proteins but had no apparent effect on lactose yield.

These supplements also reduced yield and content of milk fat and substantially changed the fatty acid profile. On the other hand, inclusion of condensed tannins decreased the yield of protein and lactose in milk but had no effect on fat content or fatty acid profile.

Such changes in milk composition may affect its technological processability, for example coagulation properties or storage stability, which, in turn, can affect the quality of final products and process efficiency during manufacturing (Johnson et al., 2001). Coagulation of milk into various dairy products is based on the formation of an aggregated protein network, which mainly consists of the caseins. This process can be induced by acids, enzymes, or a combination of these two methods (Horne and Banks, 2004). Development of an acid gel by addition of acidifying agent such as glucono-δ-lactone (GDL) has been widely described (Lee and Lucey, 2004). This type of coagulation is mainly used for the production of fermented milk products (e.g. yoghurt, commercial buttermilk, and kefir) and some types of cheeses (e.g. cottage and quark cheese), while rennet coagulation plays an important part during cheese manufacturing (Lee and Lucey, 2004).

The current experiment focused on assessing the technological properties of milk obtained from cows fed a diet containing dietary fat and tannin supplements. Such milk was ascertained for its acid and rennet coagulation properties. While it is important to develop methane mitigation strategies, it is equally necessary to establish the effects of the proposed diets on the technological properties of milk. Based on the initial study, in which impact of diet modifications on the chemical composition of milk was established, in this chapter further testing of the hypothesis

was conducted, which in this particular case stated that supplementing the cow diet with fat, tannin, or a combination of these would not affect the coagulation properties of the milk.

4.2 MATERIALS AND METHODS

4.2.1 Cows and treatments

Milk samples used in this study were obtained from the same companion experiment conducted at Department of Primary Industries (DPI) (Ellinbank Research Centre, Victoria) as described in Chapter 3. The raw milk of cows fed treatment diets (Table 4.1) was collected into test buckets and representative samples kept at 4 °C overnight before used for any experiment.

Table 4.1 Composition of dietary treatments

Dietary treatments[1]			
CON	CSO	TAN	CPT
Six kg DM[2] of concentrates and ad libitum (~20 kg DM) lucerne hay.	CON diet plus 800 g/d of cottonseed oil.	CON diet plus 400 g/d of cottonseed tannin extracted from *A. mearnsii*.	CON diet plus 800 g/d of cottonseed oil and 400 g/d of *A. mearnsii*-condensed tannins.

[1]Dietary treatments: CON = Control; CSO = Cottonseed oil; TAN = Tannins; CPT = Cottonseed oil plus tannins
[2]DM = dry matter

4.2.2 Cheese-milk preparation and chemical analysis

The raw milk was separated at 40 ºC into cream (approximately 48% w/w fat) and skim milk using a centrifugal cream separator (Satellite, Donelly Machinery, Tullamarine, VIC, Australia). Milk was standardised by adding back previously collected cream to the skim milk to obtain a casein to fat ratio of 0.72. The standardised milk then was homogenised using a microfluidizer (Micofluidics^{TM}, Newton, Massachusetts, USA) at 20 MPa in a single cycle mode. Subsequently, the microfluidized milk was heat treated (65 ºC; 30 min) using a controllable water bath. The milk obtained by this process thus was termed the cheese-milk.

The cheese-milk samples were analysed for fat by Babcock method (No 933.05, AOAC, 2006), and protein content by Kjeldahl method (No 920.123, AOAC, 2006). Cheese-milk pH was measured using a calibrated pH meter (Hanna Instruments Pty. Ltd., Singapore), while total solid concentration was determined according to method No. 925.23 (AOAC, 2006).

Reversed phase-high performance liquid chromatography (RP-HPLC) was used to determine the concentration of individual protein fractions (αs_1-casein, αs_2-casein, β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin) in the cheese-milk samples according to method of Bonizzi et al. (2009) as described previously in section 3.2.3.

4.2.3 Gel preparation

Acid gels were prepared by adding GDL (2.2%, w/v), as an acidifier to the cheese-milk, followed by vigorous stirring for 2 min and incubation at 20 ºC until

pH 4.6 was reached. Rennet gels were prepared by adding calcium chloride ($CaCl_2$) (0.02%, w/w) to cheese-milk samples prior to the addition of single strength (140 IMCU/mL) rennet (Chr Hansen Pty. Ltd, Victoria) at the rate of 2 mL per 10 litre of milk followed by incubation at 30 ºC for 1 h. Prior to analysis, cheese-milk samples were incubated at the respective incubation temperatures for 15 min.

4.2.4 Rheological properties

The rheological properties of gels were studied using a controlled-stress rheometer (Physica MCR 301, Anton Paar GmbH, Ostfildern, Germany) equipped with a bob and cup geometry (CC27/T200/AL, Anton Paar). Data were analysed using Rheoplus/32 v2.81 software (Anton Paar). An aliquot (17 g) of acidified cheese-milk sample was transferred to the rheometer measuring cup and a few drops of low viscosity oil were added to prevent evaporation of the sample. The cup temperature was maintained at 20 ºC using a Peltier element (Anton Paar). The dynamic oscillatory measurements were carried out over a frequency of 0.1 to 10 Hz, to determine elastic properties (storage modulus, G′) of the samples. The strain was maintained at 0.5% and was inferred from the linear viscoelastic region determined by an amplitude sweep at a constant frequency (1 Hz). Gel formation was monitored for 8 h after GDL addition and pH of acid gel was monitored externally throughout the gelation process. To measure rheological properties of rennet gel, a 17 ml aliquot of renneted cheese-milk sample was transferred to the measuring cup. The same rheometer program was applied at a constant temperature of 30 ºC for 1 h. Gelation time was defined as the time when gel had G′ value $\geq$ 1 Pa (Lucey et al., 1997).

Elastic moduli (G′), loss moduli (G″), and loss tangent (tan δ) were monitored over time.

4.2.5 Permeability

Permeability of acid and rennet gels was determined by measuring a flow rate of whey through the gels (Dissanayake et al., 2010). Acidified cheese-milk or renneted cheese-milk mixture (10 mL) was placed in a Falcon tube (Falcon, Blue Max, Becton Dickinson and Company, Franklin Lakes, USA). A glass tube (inner diameter of 3.7 mm and length of 25 cm, open on both ends) supported with a rubber stopper was immersed in the Falcon tube that was filled with the milk mixture. The Falcon tube was then closed air tight by a fitting rubber stopper, and then incubated in water bath at 30 ºC for 1h for rennet gel or at 20 ºC until pH reached 4.6 for acid gel. After acidification, the glass tube containing now the gel inside was removed and the length of gel was measured. The glass tube then was immersed in another Falcon tube containing 35 mL whey. By osmotic pressure, the whey diffused up along the length of the gel and filled the surface of the gel. The height of whey on top of the gel was monitored at regular time intervals. Reference tubes were the same type of falcon tubes filled with the whey of the same volume (35 mL), in which a similar glass tube supported with rubber stopper was immersed. The height of whey was measured. Six tubes for each sample were assessed. Any imperfect gel, which was broken or detached from the glass wall, was excluded. Permeability coefficients of acid and rennet gels were calculated using the following equation:

$$B = -\ln\frac{(h\infty - h_{t2})}{(h\infty - h_{t1})}\cdot\frac{nH}{\rho g(t_1 - t_2)} \qquad (1)$$

Where B is the permeability coefficient, h_∞ is the height of whey in the reference tube, h_{t1} is the height of whey in tube at time t_1, h_{t2} is the height of whey in tube at time t_2, η is the viscosity of whey, h is the length of gel, p is the density of whey, and g is acceleration due to gravity.

4.2.6 Water holding capacity

Water holding capacity (WHC) of acid or rennet gels was measured according to a modified method of Remeuf et al. (2003). A 50 mL Falcon tube was filled with 35 mL of GDL acidified milk or renneted cheese-milk. For acid gel, these tubes were incubated at 20 ºC until pH 4.6 was reached, while for rennet gel the tubes were incubated at 30 ºC for 1 h. Subsequently, the weight of gel inside the tube was weighted and the tube was centrifuged (1,000 x g; 10 min; 20 ºC) with a bench top centrifuge (Sorvall RT7, Newton, CT, USA). After centrifugation, the expelled whey was removed and weighted (WW).

The WHC was defined as:

$$\text{WHC}\,(\%) = 100\times\left[\frac{(\text{GW} - \text{WW})}{\text{GW}}\right] \qquad (2)$$

GW is the weight of gel inside the tube before centrifugation, while WW is the weight of the expelled whey after centrifugation.

4.2.7 Gel hardness

Samples for texture analysis were prepared by placing a 30 mL aliquot of acidified cheese-milk sample or renneted cheese-milk sample into a plastic container, which then was incubated at the appropriate temperature until it reached pH 4.6 for acid gel or for 1 h for rennet gels. The strength of milk gels was determined by a texture analyser (TA-XT2 plus, Stable Micro Systems Ltd, Surrey, UK). The gels were penetrated with a 20 mm aluminium cylinder probe with a cross-head speed set at 1 mm/s and compression set at 50%. Gel hardness was expressed as the force (g) at the maximum peak of the force-time curve (Awad et al., 2005). Every determination was performed in triplicate.

4.2.8 Statistical analysis

A one-way analysis of variance (ANOVA) was used to study the difference between dietary treatments. Significant difference between mean of samples was determined using Tukey's HSD test. The level of significance was preset at $P < 0.05$. Simple linear correlation analysis was used to determined relationship within coagulation parameters or between mean scores of milk composition and coagulation parameters. Results obtained in this experiment were analysed using Minitab (version 16, Minitab Inc., State College, Pennsylvania).

4.3 RESULTS AND DISCUSSION

4.3.1 Composition of cheese-milk

Previous experiment (Chapter 3) showed that cows' diet containing cottonseed oil reduced content of fat and altered fatty acid composition in milk. Since these dietary diets did not alter milk casein concentration (Chapter 3), reduction in milk fat content due to CSO and CPT diets increased the ratio of casein to fat ratio in the raw milk samples (Table 3.3; Chapter 3). Therefore, in the present study, to reduce variations in the concentration of fat and casein, the cheese-milk samples were standardised to a casein to fat ratio of 0.72. This ratio was chosen to obtain an optimum Cheddar cheese production, i.e. optimal fat and protein recoveries (Guinee et al., 2000). After milk standardisation, the concentration of total solids, protein, individual casein fractions or their proportions in cheese-milk samples were relatively similar ($P > 0.05$).

Formation of cheese curd is a concentration process, in which protein and fat in milk are concentrated (Fox and McSweeney, 1998). Therefore, cheese-milk constituents, especially proteins and fat, play an important role during milk coagulation (Hallen, 2008). Optimal coagulation involves a short coagulation time and curd firming time and the formation of a firm curd (Lucey and Singh, 1998). Increase in the amounts of protein, especially some casein, such as α_{s1}-Cn and β-Lg in milk, is associated with shorter coagulation time and firmer curd (Joudu et al., 2008). A firmer curd is also associated with a higher proportion of κ-Cn and lower proportions of α_{s2}-Cn and β-Cn in the total casein (Joudu et al., 2008).

Table 4.2 Composition of cheese-milk samples of milk from cows fed diet enriched in cottonseed oil, condensed tannins, and their combination

Constituents[2]	Dietary treatment[1]			
	CON	CSO	TAN	CPT
Fat (% v/v)	3.7±0.5[a]	3.6±0.3[a]	3.9±0.2[a]	3.5±0.2[a]
Lactose (% w/w)	4.9±0.3[a]	5.0±0.3[a]	4.8±0.2[a]	4.9±0.3[a]
Total solid (% w/w)	12.8±1.3[a]	12.7±1.1[a]	13.0±1.4[a]	12.6±0.9[a]
Protein (% w/w)	3.5±0.3[a]	3.4±0.2[a]	3.6±0.2[a]	3.4±0.3[a]
Casein (% w/w)	2.8±0.2[a]	2.7±0.2[a]	3.0±0.2[a]	2.7±0.2[a]
Casein fractions				
α_{s1}-CN (%)	12.6±1.4[a]	12.9±1.0[a]	13.0±1.2[a]	13.1±1.1[a]
α_{s2}-CN (%)	3.4±0.4[a]	3.6±0.3[a]	3.5±0.2[a]	3.5±0.5[a]
β-CN (%)	9.9±1.2[a]	9.8±1.4[a]	10.3±1.1[a]	10.0±0.5[a]
κ-CN (%)	2.7±0.6[a]	2.9±0.5[a]	2.8±0.6[a]	2.6±0.3[a]
α-Lac (%)	1.9±0.4[a]	2.0±0.5[a]	1.8±0.4[a]	1.9±0.3[a]
β-Lg (%)	4.6±0.9[a]	4.3±0.9[a]	4.6±0.8[a]	4.3±1.3[a]
Casein proportion				
α_{s1}-CN:CN (%)	44.0±4.8[a]	44.3±3.0[a]	47.2±3.9[a]	44.8±2.4[a]
α_{s2}-CN:CN (%)	11.8±1.0[a]	12.5±3.0[a]	12.4±0.7[a]	12.0±2.0[a]
β-CN:CN (%)	34.7±4.2[a]	33.5±3.6[a]	30.6±2.3[a]	34.2±1.4[a]
κ-CN:CN (%)	9.5±1.7[a]	9.7±1.5[a]	9.8±1.7[a]	9.0±1.0[a]
Cheese-milk pH	6.5±0.1[a]	6.5±0.1[a]	6.5±0.1[a]	6.5±0.1[a]

[a,b]Means in the same row within a category with different superscripts differ ($P < 0.05$).
[1]Dietary treatments: CON = control; CSO = cottonseed oil; TAN= tannins; CPT = cottonseed oil plus tannins.
[2]Constituents: CN = casein; α-La = α-lactalbumin; β-Lg = β-lactoglobulin.

4.3.2 Effect of CSO, TAN, and CPT diets on acid gel properties

Destabilisation of casein micelles by lowering milk pH is the basic step during acid coagulation (Lucey and Singh, 1998). In the current study, milk acidification was induced by adding glucono-δ-lactone (GDL). Reduction in milk pH by GDL addition is due to the production of gluconic acid on hydrolysis of the internal ester bond (Lucey et al., 1998). Reduction in milk pH causes colloidal calcium phosphate (CCP) to dissolve from the casein micelles and reduction of the negative charge of the micelles. As the isoelectric point of the casein micelle (~4.6) is approached, aggregation takes place and a porous network is formed (Lucey and Singh, 1998). In the current study, formation of the acid gels as a function of time was observed using a dynamic oscillatory measurement. Results obtained from this measurement are summarised in Figure 4.1 and Table 4.3. In general, all acid gels had a similar pattern of acidification and acid gel formation (Figure 4.1). Initially, pH of milk gels reduced sharply from ~6.5 to ~5.0, followed by a gradual decrease from pH ~5.0 to ~4.6 (Figure 4.1). The sharp decrease in pH from ~6.5 to ~5.0 might be attributed to the fast hydrolysis of the GDL into gluconic acid (Lucey et al., 1998). Similar change in pH during milk acidification using GDL has been reported (Lucey and Singh, 2003).

According to Lucey (2004), reduction in gel pH is accompanied by physical and chemical changes of the casein micelles. Reduction in milk pH from ~6.5 to ~5.0 decreases the negative charge, and thus weakens the electrostatic forces holding the casein micelles together (Lucey, 2004). Reduction in milk pH also causes κ-casein “hairs” on the micelle surface to shrink, which results in a decrease in steric

stabilisation provided by the charged κ-casein "hairs". At pH ~5.0, the CCP in the casein micelles is completely dissolved. Loss of CCP increases electrostatic repulsion between the newly exposed phosphoserine groups. As pH is further reduced to the isoelectric point of the casein micelle (~ pH 4.6), the net electrostatic charge and steric interactions between the casein micelles would reduce, which subsequently allow aggregation of the micelles (Lucey, 2004).

Results show that for all acid gels, gelation occurred between 114 and 125 minutes after GDL addition, which coincided with pH of gels around ~4.7-4.8 (Figure 4.1). At the point of gelation, when storage modulus (G′) started to increase, tan δ decreased to ~0.29 and then flattened at ~ 0.23-0.24 during aging (Figure 4.1A, B). After gelation, G′ increased steadily, which may indicate reduced electrostatic repulsion and increased hydrophobic attractions as the isoelectric point of the casein (pH 4.6) was approached (Lee and Lucey, 2004). At the isoelectric point, G′ values ranged from 31.5 to 41 Pa, followed by an increase of 3-4 times during aging (after 8 h).

Rheological characteristics of these acid gels, particularly pH at gelation, the low G′ value at pH ~4.6, and absence of maximum tan δ during gel formation, resembled rheological characteristics of acid gels made from unheated milk (Lucey et al., 1997; Lucey, 2004). According to Lucey (2004), gelation of unheated milk usually starts to take place at pH ~4.9, near the isoelectric point of the casein (pH ~4.6). On the other hand, gelation of heated milk usually occurs at a higher pH, i.e. pH ~5.3, which is close to the isoelectric point of β-lactoglobulin. Heat treatment may denature β-lactoglobulin, which may results in formation of complexes between

β-lactoglobulin and caseins. This association may alter rheological properties of acid gel by reducing gelation time, increasing gelation pH and gel firmness (Lucey et al., 1999), as has been discusses previously. Therefore, results obtained in the current study indicated that the heat treatment (65 ˚C; 30 min) applied on cheese-milk had no observable effect on denaturation of whey proteins, especially β-lactoglobulin.

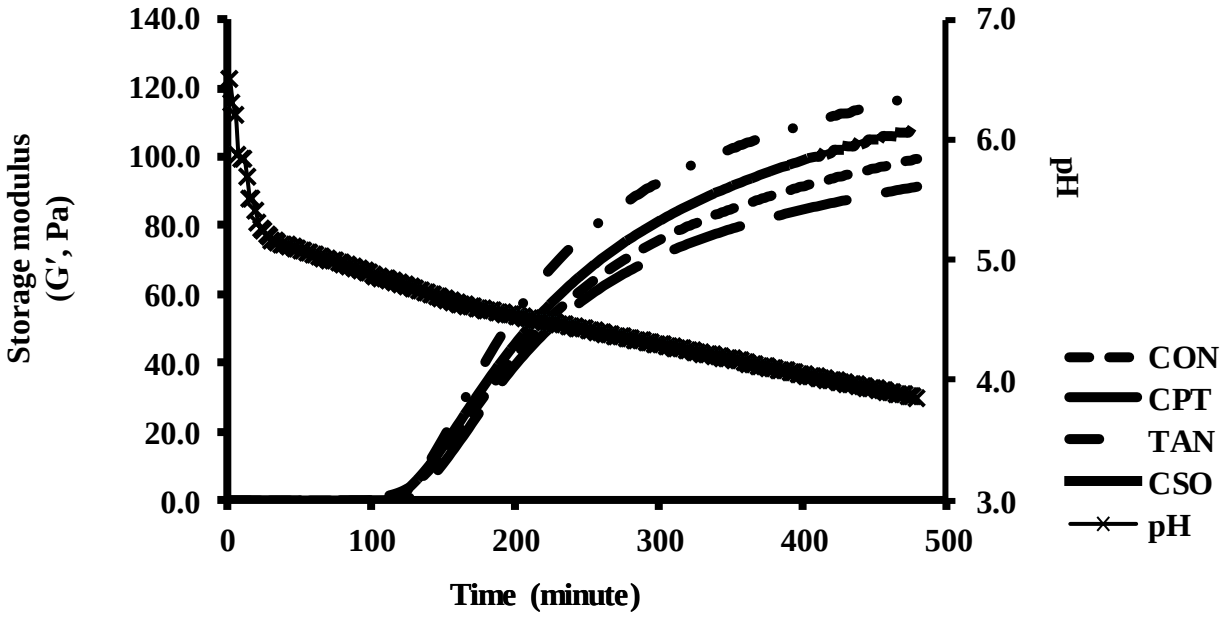
Rheology of acid gel is influenced by composition of the milk (Lucey et al., 1999). In the current experiment, G′ at pH 4.6 positively correlated with the concentration of caseins, total proteins, fat, and total solids (r = 0.53, 0.52 and 0.45, respectively, $P < 0.05$) in the cheese-milk samples. The positive correlation between G′ and the casein found in the current study can be linked to the role of the casein, which facilitates the formation of interparticle bonds and prevents further reorganization of the gel network (Karlsson et al., 2007). Protein content may increase G′ by increasing a level of gel-forming proteins per se (Guinee et al., 1997). A positive correlation between G′ of acid gel and concentration of total solids could be linked to the amount of proteins and fat, which comprise the bulk of the total solids. Increase in a total solid concentration may increase the casein concentration and interactions between casein particles; which consequently increase a number of bonds and rate of bonding (Gastaldi et al., 1997).

The positive effect of fat content on G′ value could be due to the incorporation of fat into protein network during gelation, which in the current study might be a result of microfluidization of the cheese-milk. According to Van Vliet and Dentener-Kikkert (1982), homogenization of milk sample may disrupt milk fat globules, which leads to partial replacement of native fat globules with other milk

proteins. This process allows for fat globules to be incorporated into a gel by cross linking them to the matrix, thus resulting in increased gel stiffness.

In contrast, a negative correlation was observed between gelation time of acid gel and content of the casein, total proteins, and fat in cheese-milk samples (r = -0.39, -0.39 and -0.45, respectively, $P < 0.05$). This negative correlation could be due to an increase of the casein or protein content, which may facilitate rapid formation of inter-particle bonds (Karlsson et al., 2007). This result was similar to another study, which showed that high protein content was related to a faster coagulation time (Van Hooydonk et al., 1987). Results from the current study also showed that within the coagulation parameters, gelation time (GT) was negatively correlated with G′ at pH 4.6 (r = -0.5, $P < 0.05$). This negative correlation may be due to a faster rate of gelation resulting in a longer stiffness time, which in turn led to higher G′ values (Mishra et al., 2005).

A



B

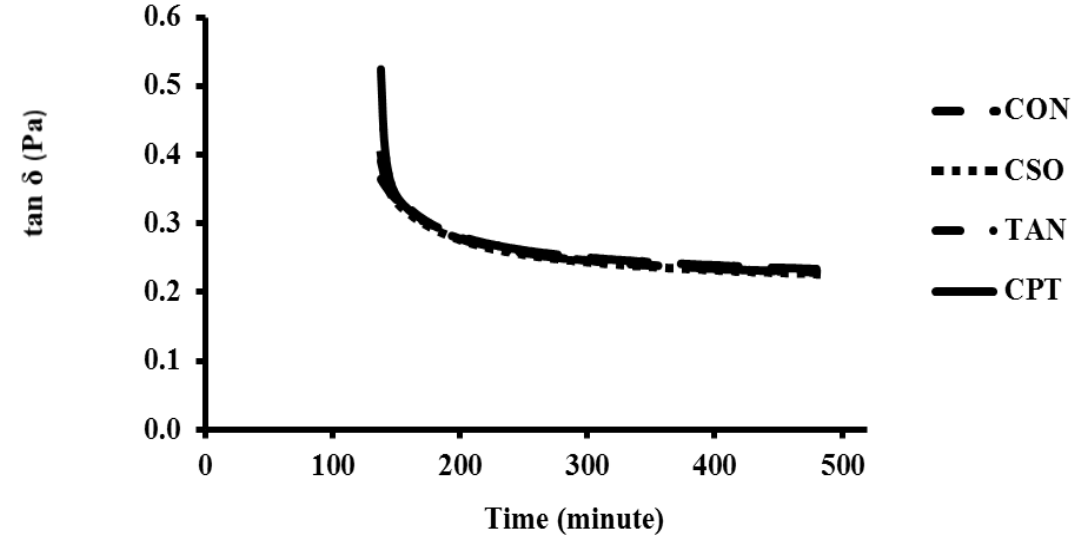


Figure 4.1 Development of storage modulus (G′) (A) and tan δ (B) of acid gels as function of time after GDL addition. Acid gel were made from milk of cows fed control diet (CON), control diet supplemented with cottonseed oil (CSO), condensed tannins (TAN), and in combination (CPT).

Results of the current experiment showed that CSO, TAN, and CPT diets did not alter ($P > 0.05$) G′, G″, and GT of acid gels (Table 4.3). The unaltered gelation time could likely be attributed to similar concentration of proteins, caseins, total solids and fat among the cheese-milk samples (Table 4.2). These findings were in agreement with a study of Masucci et al. (2006), who reported that a diet had no substantial impact on the acid coagulation mainly due to unchanged protein content. Major changes in the protein content appear crucial and may even modify milk clotting ability (Grandison et al., 1984). All acid gels had similar ($P > 0.05$) tan δ value at pH 4.6 or during aging. Tan δ value indicates degree of rearrangement of the gel network (Lucey et al., 2001). Thus, the similar value of tan δ of acid gels might indicate that the structure of a casein network and viscoelastic properties of these acid gels were similar. In addition, according to Lucey et al. (2001), tan δ is related to gel permeability, a parameter which indicates the size of pores in a gel system. Thus, it was anticipated that these acid gels would have a similar degree of permeability and water holding capacity, which are discussed later in this chapter.

Besides milk composition, rheological characteristics of acid gel can be affected by milk pH (Park et al., 2007) and incubation temperature (Mulvihill and Kinsella, 1988). Change in milk pH during acidification is accompanied by disassociation of salts, micellar calcium, inorganic phosphate, magnesium, and citrate as well as increase in volume of casein micelles (Van Hooydonk and Walstra, 1987). As pH is reduced to the isoelectric point of the casein micelle (~pH 4.6), the net electrostatic charge and steric hindrance between the micelles are reduced, which subsequently allow for aggregation of the casein micelles (Lucey and Singh, 1998).

Therefore, in regards to chemical and physical changes of the casein micelles resulting from the changes in milk pH, acidification process could affect acid gel rheological characteristics such as gel firmness, gelation pH, gelation time, and rearrangement between casein particles during gel development (Pyne and McGann, 1960; Park et al., 2007). Meanwhile, incubation temperature could affect rheology of acid gel by affecting acid production (Lucey, 2004). A high incubation temperature may cause faster acid production which subsequently may increase gelation time, gelation pH, gel firmness, and casein rearrangements (Lucey, 2004). In the current study, all cheese-milk samples had a similar pH (Table 4.1) and were incubated at the same temperature. Therefore, together with the comparable composition of cheese-milk samples, the similar milk pH and incubation temperature was expected to obtain similar rheological properties of these acid gels.

Others physical properties of acid milk gels such as permeability and water holding capacity (WHC) were also determined in this study. Permeability indicates the size of pores in a gel matrix, with a high permeability coefficient (*B*) indicating the presence of large pores in the gel matrix (Lee and Lucey, 2010). Thus, permeability may determine how water move to the gel surface (Serra et al., 2007). Table 4.3 shows that *B* of all acid gels was within the range (1-2 $x10^{-13}$ m^2) reported in the literature (Lucey et al., 1997). Compared with CON diet, all supplemented diets had no significant ($P > 0.05$) effect on acid gel permeability (Table 4.3). According to Lee and Lucey (2010), the pore size indicates the type of gel microstructure, which is formed during acidification process. Therefore, similar permeability coefficients among these acid gels may indicate that they had a similar

pore size and gel microstructure due to similar rearrangements that occurred during formation of these gels. The similar permeability among these acid gels was also reflected from their similar tan δ value (Table 4.2).

Permeability of acid gel is influenced by several factors such as milk composition, pH, and incubation temperature (Lakemond and Van Vliet, 2005). In the current experiment, permeability of acid gels was negatively correlated with the concentration of the casein, proteins, and fat in cheese-milk (r = -0.53, -0.52 and -0.45, respectively, $P < 0.05$). This result is in agreement with Lucey et al. (1998), who stated that milk with low protein content might result in a gel with larger pores and low interconnectivity between the protein clusters.

In addition to milk composition, permeability of acid gel is also influenced by pH and incubation temperature (Mellema et al., 2002; Lee and Lucey, 2004). A pH may affect rearrangement of the casein by influencing the bond energy of casein particles, which subsequently affect their mobility (Mellema et al., 2002). At lower pH, rearrangements of casein particles occur at a higher rate due to a higher mobility of casein particles influenced by a lower bond energy (Mellema et al., 2002). Since all cheese-milk had similar pH (Table 4.1), thus it was likely that these gels had a comparable rearrangement and a pore size, all of which could be reflected in similar ($P > 0.05$) permeability values (Table 4.3). A similar rearrangement of these acid gel networks was confirmed by their similar tan δ value (Table 4.3).

Water holding capacity is a measure of retained water within a protein matrix after milk centrifugation, and thus indicates the rigidity or pore size of a milk gel,

which allows water to be released when the gel is deformed (Huang and Kinsella, 1986). Weak gel with large pores usually has low ability to retain water, thus has low WHC (Mellema et al., 2002). Results showed that all acid gels had relatively high WHC values (Table 4.3) compared to WHC of GDL gel reported in the literature i.e. 82.12% (Gastaldi et al., 2003). High pressure homogenization has been reported to increase WHC of gel through its effect on denaturing whey proteins. In the current study, analysis of rheological characteristics of acid gel indicated that microfluidization at 20 MPa might have not caused the whey protein denaturation. However, due to its effect in reducing fat globule size, it is possible that this type of homogenization could increase interactions between fat and casein particles, which subsequently increased the gel rigidity and ability to retain water.

Table 4.1 also shows that all acid gels in the current study had a comparable ($P > 0.05$) WHC. Water holding capacity of a gel reflects gel rigidity or gel pore size, thus factors which affect rigidity and pore size of a gel such as milk composition, milk pre-treatments, incubation temperature, and pH, would also affect WHC. Therefore, the comparable milk composition especially the content of fat and protein may contribute to the similar WHC of acid gels. In addition, the identical condition of heat treatment and microfluidization applied on the cheese-milk might also contribute to the similar WHC.

Table 4.3 Physical properties of acid-induced gels achieved by addition of GDL to milk obtained from cows fed modified diets

Acid coagulation parameters[2]	Dietary treatments[1]			
	CON	CSO	TAN	CPT
G′ at pH 4.6 (Pa)	32.9±6.2[a]	34.4±6.6[a]	41.1±7.9[a]	31.5±4.8[a]
G″ at pH 4.6 (Pa)	9.6±3.7[b]	10.1±2.7[b]	12.5±2.8[b]	8.9±1.3[b]
GT (min)	124.0±9.1[a]	121.6±10.5[a]	114.8±9.3[a]	125.1±11.5[a]
Tan δ at pH 4.6	0.29±0.02[a]	0.29±0.02[a]	0.30±0.02[a]	0.28±0.03[a]
pH at gelation	4.7±0.1[a]	4.8±0.2[a]	4.8±0.1[a]	4.7±0.1[a]
Time to get pH 4.6 (min)	173.5±15.2[a]	170.0±15.3[a]	174.5±16.9[a]	169.8±16.8[a]
Cheese-milk pH	6.6±0.1[a]	6.6±0.1[a]	6.6±0.1[a]	6.6±0.1[a]
Gel hardness (*g*)	10.0±1.4[a]	10.7±1.9[a]	11.4±1.0[a]	9.9±1.5[a]
B (x 10^{-13} m^2)	1.1±0.3[a]	1.5±0.6[a]	1.3±0.3[a]	1.4±0.2[a]
WHC (%)	98.8±1.4[a]	98.9±0.7[a]	99.5±0.1[a]	99.3±0.3[a]

[a,b]Means in the same row within a category with different superscripts differ ($P < 0.05$).
[1]Dietary treatments: CON = control; CSO = cottonseed oil; TAN = tannin; CPT= cottonseed oil plus tannin.
[2]Acid coagulation parameters: G′ = storage modulus (Pa); G″ = loss modulus (Pa); GT= gelation time (min); tan δ= loss tangent; *B* = permeability (m^2); WHC = water holding capacity (%).

4.3.3 Effect of CSO, TAN, and CPT diets on coagulation properties of rennet-induced gels

Milk coagulation induced by rennet is an important step during cheese making. Enzymatic coagulation of milk involves destabilisation of casein micelles via hydrolysis of κ-casein by chymosin, the main protease in the rennet; followed by aggregation of the micelles induced by calcium (Fox and McSweeney, 1998). During coagulation, chymosin destabilises κ-CN by cleaving the peptide bond between Phe_{105}-Met_{106} residues. Basically, coagulation induced by chymosin consists of three phase: firstly, hydrolysis κ-CN by chymosin into para- κ-CN and

glycomicropeptide (GMP), which causes loss of negatively charged groups and decreases steric stabilisation. When most of κ-CN has been hydrolysed, colloidal stability of casein micelles reduces to a level which allows spontaneous aggregation to occur. Molecular chains connect through hydrophobic bonds to form a three-dimensional network. Calcium cross-linking further solidifies the network. In the last stage, whey is expelled from the network by syneresis (Fox and McSweeney, 1998).

In the present study, all rennet gels showed a typical viscoelastic development during renneting as described by Horne and Banks (2004). Initially, tan δ increased and reached a maximum, and then it decreased sharply and remained stable at ~0.23 Pa during the development of rennet gel (Figure 4.2; Table 4.4). The presence of maximum tan δ might be related to relaxation or loosening of intra-molecular forces within para-casein network formed initially due to solubilisation of colloidal calcium phosphate (CCP). Similar appearance of maximum tan δ has been reported during formation of rennet milk gel (Lee and Lucey, 2010). This phenomenon usually occurs in any milk system that form gels at pH value > 5.3 (Lee and Lucey, 2004). Decrease in tan δ after it reached maximum may indicate the transition from fluid milk into a viscoelastic rennet gel (Zoon et al., 1988). The sharp decrease of tan δ coincided with the visible gelation time (GT), which was ~9.8-10.3 min after addition of rennet. After the gelation point, G′ increased rapidly with time. According to Mellema et al. (2002), the increase of G′ during an early gel formation may be attributed to two types of structural rearrangements, which occur at this stage. The type A rearrangement is due to increase of contact area between casein micelles due to particle fusion which results in a greater number of bonds formed per

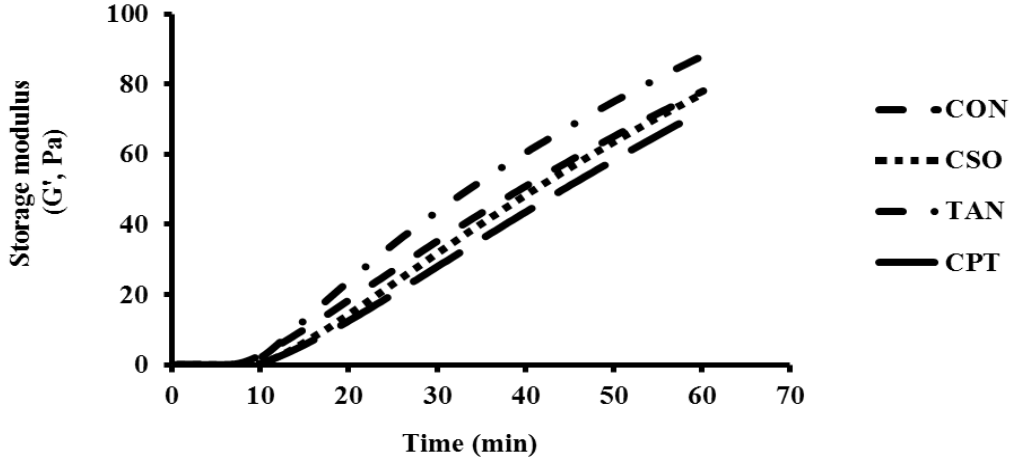
junction. The rearrangement type B is an interparticle rearrangement due to bond reversibility which results in more junctions (Mellema et al., 2002). For interparticle rearrangement to occurs there should be sufficient freedom for the micelle to move around, which may be in the case of the early stages of gel formation (Mellema et al., 2002). After a gel is formed, G′ continue to increase which could be attributed to further incorporation of the casein into the gel network, in addition to continuing rearrangement (rearrangement type B) (Mellema et al., 2002). The appearance of G′ was also accompanied with appearance of loss modulus (G″), but G″ increased at a lower magnitude compared to G′ (Figure 4.2 A, B). A higher magnitude of G′ than that of G″ indicated that predominantly elastic solid gel was formed (Mellema et al., 2002). Moreover, the relatively constant tan δ value during renneting process may indicate that the elastic and viscous components contribute in constant proportion to the G′ after the onset of gelation (Zoon et al., 1988).

Coagulation properties of a rennet gel are mainly affected by milk composition (Wedholm et al., 2006), even though sometimes a strong correlation cannot be drawn confidently (Auldist et al., 2002). In the current study, a negative correlation was observed between gelation time and casein or protein content ($r = -0.39$, -0.39, respectively; $P < 0.05$) in the cheese-milk. This result is in agreement with previous studies, which showed a decrease in gelation time as a casein (Sharma et al., 1994) or total protein content (Caron et al., 1997) increased. Delay in gelation time of rennet gel with a higher amount of casein or proteins in cheese-milk may be attributed to the increase of κ-casein needed to be hydrolysed by chymosin before gelation could take place (Sharma et al., 1994).

Furthermore, a positive correlation was observed between G′ of rennet gel with concentration of the casein, proteins, fat and the total solids in milk (r = 0.53, 0.40, 0.51, and 0.80, respectively; $P < 0.05$). Positive effect of protein content on G′ value is in agreement with results of Zoon et al. (1988), who showed that G′ and G″ values were proportional to a number of effective bonds in the network. According to Guinee et al. (1997), protein content might increase G′ by increasing the level of gel-forming proteins per se, which then enhances the rate of protein aggregation/fusion and thus attributes to the formation of coarser gel network.

Pre-treatments of cheese-milk may also affect rheological properties of rennet gel (Guinee et al., 1997). It has been reported that homogenization (Guinee et al., 1997) or microfluidization (Ciron et al., 2010) increased firmness of rennet gel by altering the role of fat globules from mainly filler particles to primarily active components during formation of gel network. Microfluidization reduced the size of milk fat globules, which thus resulted in numerous particles and increased the surface area. The small size fat globules were then incorporated within the protein network and acted as a reinforcing element in the new gel (Ciron et al., 2010). Thus, the positive correlation between G′ and fat content found in the current study may be linked to the effect of microfluidization on reducing particle size of milk fat globules, which allows for a better incorporation of fat into protein matrix during formation of the gel network (Guinee et al., 1997; Ciron et al., 2010).

A



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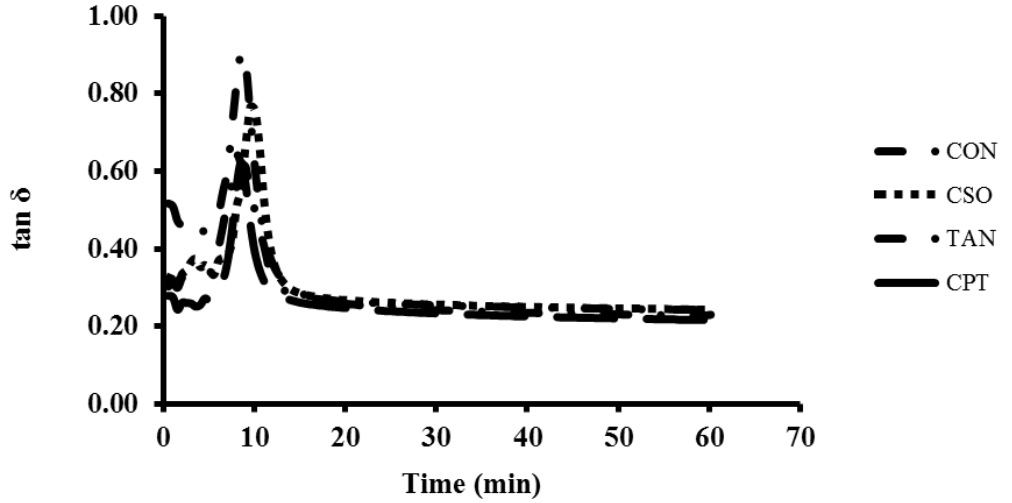


Figure 4.2 Development of storage modulus (G′) (A) and tan δ (B) of rennet gels made from milk of cows fed diet supplemented with cottonseed oil, condensed tannins, and their combination.
Dietary treatments: CON = control; CSO = cottonseed oil; TAN = tannin; CPT = cottonseed oil plus tannin.

Table 4.4 Coagulation properties of renneted-milk gel made from milk of cow fed diet containing cottonseed oil, condensed tannins, or their combination

Rennet coagulation parameters[2]	Dietary treatments[1]			
	CON	CSO	TAN	CPT
G′ after 1 h of rennet addition (Pa)	77.5±15.3[a]	76.9±8.6[a]	89.3±11.8[a]	74.3±14.4[a]
G″ after 1 h of rennet addition (Pa)	18.6±3.7[a]	18.1±2.1[a]	21.1±2.2[a]	18.0±4.2[a]
GT (min)	10.2±2.6[a]	9.9±1.9[a]	9.8±1.4[a]	10.3±1.3[a]
tan δ	0.24±0.01[a]	0.24±0.01[a]	0.24±0.01[a]	0.24±0.01[a]
Gel hardness	18.5±1.9[a]	18.4±0.9[a]	18.3±1.5[a]	17.9±1.4[a]
B (x10^{-13} m^2)	1.5±0.1[a]	1.6±0.1[a]	1.6±0.1[a]	1.5±0.1[a]
WHC (%)	99.0±0.7[a]	98.8±0.7[a]	98.5±1.5[a]	98.5±1.3[a]

[a,b]Means in the same row within a category with different superscripts differ ($P < 0.05$).
[1]Dietary treatments: CON = control; CSO = cottonseed oil; TAN = tannin; CPT =cottonseed oil plus tannin.
[2]Rennet coagulation parameters: G′ = storage modulus (Pa); G″ = loss modulus (Pa); GT = gelation time (min); tan δ = loss tangent; *B* = permeability (m^2); WHC = water holding capacity (%).

As indicated in Table 4.4, rheological properties (G′, G″, and GT) of rennet gels were unaffected ($P > 0.05$) by supplementation in the diets. This absence of the effects on rheological properties of rennet gels was likely associated with similar concentration of total proteins, the casein, fat, and total solids in the cheese-milk samples (Table 4.2). The importance of required protein content and its composition appears very relevant as it was noted by previous studies (Grandison et al., 1984; Verdier-Metz et al., 2001). In addition, pre-treatments of cheese-milk samples i.e. heat treatment and microfluidization, which were kept similar, may also contribute to the comparable properties of rennet gels in the current study.

Treatment diets did not change substantially ($P > 0.05$) the permeability and WHC of rennet gels (Table 4.3). Thus, it seemed that these rennet gels might have had similar gel pore size and microstructure. Permeability of rennet gels was ranging from 1.2 to 1.7 x 10^{-13} m^2. A similar permeability of these rennet gels might be due to the comparable milk composition and pre-treatment of cheese-milk since these two parameters influence microstructure and pore size of rennet gel as has been discussed earlier. Water holding capacity of rennet gel was positively correlated with cheese-milk composition, especially the casein, proteins, and fat (r = 0.44, 0.44, and 0.37, respectively; $P < 0.05$), and gel hardness (r = 0.44, $P < 0.05$). These results were in agreement with Serra et al. (2007), who proposed that interactions between fat and protein might enhance interactions between particles and formation of stable gel network with a high capacity to retain water. The positive effect of fat, protein and casein content on WHC might be related to the positive effect of these milk components on gel rigidity. The similar WHC of these rennet gels was confirmed by their comparable permeability and tan δ values. During cheese making process, the formed curd is normally cut after 30 min of rennet addition when the curd firmness is ~30 Pa (Horne and Banks, 2004). In the current study, all diets resulted in curd firmness ~30-40 Pa after 30 min of renneting, which indicates suitability of the resulting milk for cheese manufacturing.

4.4 CONCLUSION

Supplementation of cottonseed oil, condensed tannin, or their combination did not significantly alter gelation time, pH at gelation, time to reach pH 4.6, G′, G″, and tan δ of acid gels. Permeability, water holding capacity and hardness of acid gels were also unaltered. These supplemented diets also did not significantly alter gelation time, G′, G″, tan δ, permeability, and water holding capacity of rennet gels. Compared to that of control diet, minimal effect of cottonseed oil and condensed tannin-supplemented diets on coagulation properties of acid- and rennet-milk gels obtained in the current study can be linked to the minimal effect of these supplementation on content and composition of milk protein. In addition, the standardisation step during cheese-milk preparation, which eliminated difference in ratio of casein to fat of the cheese-milk, might have also contributed to the similar coagulation properties of these gels. Thus, it can be concluded that these diets could be used to mitigate methane emissions without any detrimental effects on milk coagulation properties.

5 Chemical composition, proteolysis, and textural properties of Cheddar cheese manufactured from the milk obtained from cows fed a modified diet supplemented with cottonseed oil and condensed tannins

Chapter 5

5.1 INTRODUCTION

The importance of reducing methane emission from dairy sector has been widely recognized. Novel feeding strategies by adding fat (Grainger et al., 2010) or condensed tannin (Grainger et al., 2009) into a cow diet could reduce enteric methane emission. Depending on a type of fat and its concentration, supplementation of fat to a ruminant diet could affect the content of protein and fat, as well as fatty acid composition in milk by altering rumen metabolism or providing precursor for synthesis of fatty acids (Palmquist et al., 2005; Chapter 3). Due to its ability to precipitate proteins and inhibit rumen microorganisms, tannins could furthermore affect the content of milk protein and alter rumen metabolism (Barry and McNabb, 1999). Changes in milk composition subsequently might alter technological and manufacturing properties of the milk and in turn compromise the quality of dairy products (Palmquist et al., 1993). In order to be accepted by farmers, methane mitigating strategy should have no detrimental effect on animal performance, productivity, and finally quality of dairy products (Beauchemin and McGinn, 2008). Therefore, while cottonseed oil and condensed tannin supplementation may be effective in reducing methane emission, the current study wanted also to establish if this dietary change would impact on the properties of an important dairy product, Cheddar cheese.

Cheese is likely a major product to the Australian dairy industry with the Cheddar occupying the largest proportion (Dairy Australia, 2013). Profitability of cheese manufacturing plants is determined by cheese yield and manufacturing efficiency i.e. protein and fat recovery in cheese (Guinee et al., 2006). Cheese yield

might be affected by milk characteristics and cheese processing conditions (Fenelon and Guinee, 1999), with milk fat and protein as two major milk components governing the yield. During processing, the casein micelles are destabilized by rennet creating a network (cheese matrix), which in turn entraps the fat (Lucey et al., 2003). Therefore, any change in fat or protein content in milk might affect in the first instance the yield of cheese and later other important sensory characteristics of cheese.

Proteolysis is one of important biochemical processes during ripening of renneted cheeses. This process may directly or indirectly affect cheese flavour via formation of peptides, free amino acid, or other compounds such as amines, acids, and thiols (Fenelon et al., 2000). In addition, proteolytic activity of released enzymes and surviving starter cultures affect the properties of the casein network and consequently change textural properties of cheese (Lawrence et al., 1999). This in turn may have implications on the sensory properties, functionality, and customer acceptances (Lucey et al., 2005). The rate of proteolysis, and thus the texture of Cheddar cheese, is influenced by the final pH, chemical composition of cheese i.e. fat, protein, salt, moisture (Lucey et al., 2005), and fatty acid (FA) profile (Baer et al., 1996).

Inclusion of fat into cow diet alters FA composition of milk (Jones et al., 2005; Chapter 3). When the milk is subsequently manufactured into cheese, these changes in FA profile may have no apparent effect (Luna et al., 2005) or alter cheese texture and flavour profile (Jones et al., 2005). In contrast to reported effects of fat supplementation on cheese quality, impact of phenolic compounds such as tannins

and lignin, on production and quality of dairy products has not been elucidated fully (O'Connell and Fox, 2001).

Analysis of the effects of feed supplementation by cottonseed oil, condensed tannins, and their combination on milk composition showed that these dietary supplements had no apparent effect on protein content and composition of individual proteins in milk (section 3.3.1 and 3.3.2; Table 3.3). However, milk fat content and the proportion of short- and medium-chain saturated FA were affected by inclusion of fat and tannins into the diet. Increase in the proportion of long-chain and polyunsaturated fatty acid in milk was also observed (section 3.3.1; Table 3.4). On the other hand, supplemented diets had no major impact on milk coagulation properties revealed by assessing milk renneting properties i.e. renneting time and gel hardness as has been discussed in section 4.3.3. Therefore, the objective of the current study was to establish the effects of feeding fat and tannin-supplemented cow's diet on yield, composition, proteolysis, and textural properties of the respective Cheddar cheese. Hypothesis tested in this study was that the supplementation of diets with cottonseed oil and condensed tannins would not substantially alter yield, composition, proteolysis, and textural properties of the Cheddar cheese.

5.2 MATERIALS AND METHODS

5.2.1 Cheese manufacture and sampling

Preparation of cheese-milk for Cheddar cheese manufacturing has been explained in section 3.2.1. The cheese-milk was held overnight at 4 ºC before being manufactured into a Cheddar cheese. On the following day, twenty litre of cheese-milk was transferred to a sanitized 20L rectangular stainless steel jacketed cheese vat (model IP67, Govan Weatherproof) and tempered to 31 ºC before inoculation with a freeze-dried Direct Vat Set (DVS) starter (R-704 Mesophilic Homofermentative culture, Chr Hansen Pty. Ltd, Bayswater, Victoria, Australia) at 0.5g/10L cheese-milk. Prior to inoculation of starter culture, calcium chloride ($CaCl_2$) at 0.02% was added into the cheese-milk. The milk was incubated for 45 min before the addition of a single strength (140 IMCU/mL) chymosin (Chr Hansen Pty. Ltd, Bayswater, Victoria, Australia) at the rate of 0.2 mL/L of cheese- milk. The cheese-milk was coagulated after about 45 min and the resulted curd was cut with a cheese wire knife to 8 mm cubes. The curd were cooked to 38 ºC by heating slowly at the rate of 1 ºC rise per 5 min with agitation until the pH declined to 6.1-6.2. The whey was drained and the curd was Cheddared at 38 ºC and turned every 15 min until the pH further declined to 5.4-5.5. The curd was milled and salted using NaCl (2%, w/w) before putting into a rectangular block lined with cheesecloth. The cheese was then pressed at 2.5 kg/100 cm^2 at room temperature overnight (Kosikowski, 1977). The fresh cheese was removed, packed in oxygen barrier Cryovac bags (Cryovac Pty. Ltd., Fawkner, Vic, Australia) and heat sealed with a Multivac vacuum packaging

equipment (Multivac Sepp Haggenmuller, Wolfertschwenden, Germany) and ripened at 4 ºC for 6 months.

5.2.2 Cheese chemical analysis

Moisture, fat, protein, total solid, and pH of cheese samples were analysed at day 1, and 6, 12, and 24 weeks after date of manufacturing. The cheese samples were shredded and analysed for moisture by oven drying method (No. 926.08, AOAC, 1990); fat by Babcock method (No 933.05, AOAC, 1990), ash by gravimetric method (AOAC, 2000), and protein by Kjeldahl method (No 920.123, AOAC, 1990) on a Kjeltec system II (with Digestion system 2000 and Distilling unit 1002, Tecator, Hoganas, Sweden).

Cheese pH was measured using a calibrated pH meter (Hanna Instruments Pty. Ltd. Singapore). Cheese was grated finely and blended with distilled water (20 g of cheese with 12 ml of distilled water) before pH measurement. Salt concentration in cheese was determined by the Volhard method (No 975.20 AOAC, 1990). Salt to moisture (S/M) ratio was calculated as % salt / % moisture. Fat in dry matter (FDM) was calculated as % fat / (100 - % moisture). Moisture in non-fat substance (MNFS) was calculated as % moisture / (100 - % fat) (Van Hekken et al., 2013). FDM, MNFS, and S/M value were determined on 1 day-old cheeses only. All analysis was done in duplicate.

5.2.3 Fat and protein recoveries and cheese yield

Protein and fat recovered in cheese (PR and FR, respectively) were calculated as weight of cheese multiplied by its percentage of fat or protein content

and then divided by the total weight of fat or protein present in the cheese-milk and multiplied by 100 (Lau et al., 1991).

$$\text{PR}\ (\%) = \frac{\text{cheese protein} \times \text{curd}}{\text{cheesemilk protein} \times \text{cheese milk}} \times 100 \qquad 1)$$

$$\text{FR}\ (\%) = \frac{\text{cheese fat} \times \text{curd}}{\text{cheesemilk fat} \times \text{cheese milk}} \times 100 \qquad 2)$$

The actual cheese yield (Ya) was calculated by dividing the weight of cheese after pressing by the total weight of cheese milk (Fenelon and Guinee, 1999). Predicted yield (Yp) was calculated according to the Van Slyke and Price (1936) formula:

$$\text{Yp}\ (\%) = \frac{[(0.93\text{fat} + (\text{casein} - 0.1)] \times 1.09}{100 - \%\ \text{moisture in cheese}} \qquad 3)$$

Moisture-adjusted yield (Yma) to 38.5% moisture was calculated as:

$$\text{Yma}\ (\%) = \text{Ya} \times \frac{100 - \text{Ma}}{100 - \text{Mr}} \qquad 4)$$

Ma and Mr correspond to actual and reference moisture content (38.5%), respectively.

5.2.4 Proteolysis assessment

In this study, protein breakdown of the Cheddar cheeses during ripening time was monitored by assessing proportion of nitrogen fractions and Reverse-Phase High Performance Liquid Chromatography (RP-HPLC). In brief, water soluble fraction

(WSF) was prepared by homogenizing a cheese sample with milli-Q water (ratio of cheese to water = 1:5) for 5 minutes at 10,000 rpm using a tissue-homogenizer (Polytron, Kinematica AG, Lucerne, Switzerland). The slurry was heat treated at 40 ºC for 1 h and then centrifuged at 3,000 x *g* for 10 min at 4 ºC (Sorvall RT7, Newton, CT, USA). The supernatant was filtered through Whatman no 42 filter paper. Twelve percent trichloroacetic acid (12% TCA) fraction was obtained by precipitating 5 mL of WSF with 5 mL of 24% (v/v) TCA (Sigma-Aldrich, St-Louis, Mo., USA). The mixture was held at room temperature for 1 h, and then filtered through Whatman No 42 filter paper (Kuchroo and Fox, 1982). Phosphotungstic acid fraction (5%) was prepared by precipitating 5 mL of filtered WSF with 5 mL of 10% (v/v) PTA (Sigma-Aldrich, St-Louis, Mo., USA). This mixture was then incubated at room temperature for 1h and filtered through filter paper (Whatman no 42) (Stadhouders, 1960). Nitrogen content in 9 mL of WSN, 12% TCA-SN, and 5% PTA-SN fraction was determined by Kjeldhal method (No 920.123, AOAC, 2000).

Peptide profile of all cheeses throughout ripening period was determined using RP-HPLC of WSN fraction (de Wit et al., 2005). An aliquot (25 mL) of WSN fraction of each cheese sample was freeze-dried and kept at -20 ºC until analysis. At the day of analysis, the freeze-dried sample was diluted with solvent A containing 0.1% (v/v) TFA (Sigma-Aldrich) in deionized water to a concentration of 70g/mL. The mixture was centrifuged (14,000 rpm, 30 min) using a bench top centrifuge (Sorvall RT7, Newton, CT, USA) and filtered through 0.45-µm filter (Millipore Corp., Belford, Mass., USA). The RP-HPLC consisted of a Varian 9012 solvent delivery system, a Varian 9100 auto-sampler, a Varian 9050 variable wavelength

ultraviolet-visible tunable absorbance detector, and a 730 data module. Wavelength to measure cheese peptides was 214 nm. A sample size of 30 µl was injected into the RP column (C18, 250 mm x 4.6 mm, 5 µm; Grace Vydac, Hesperia, California, USA). The effects of supplementation on peptide profile were assessed qualitatively by visually comparing the resolved chromatographs.

5.2.5 Cheese texture profile analysis

Texture Profile Analysis (TPA) of the cheese samples was conducted after 2, 4, 6, 12, and 24 weeks of storage using a texture analyser (TA-XT2 plus, Stable Micro Systems Ltd, Surrey, UK) with a load cell of 25 kg. Cheese samples (2.5 x 2.5 x 2.5 cm) were obtained from the middle of the cheese slice and prepared using a metal cutting device and stored at room temperature two hours before being tested (Halmos et al., 2002). A double-compression test was carried out using a stainless steel probe P/20, with a probe speed of 2.00 mm/s and a 25 kg load cell. Each sample was subjected to 30% compression. Force-time TPA curves generated by the instrument software were used to calculate texture parameters, which included hardness (maximum force in the first compression, in N), springiness (height of sample recovery after first compression and before the start of the second compression, in mm), cohesiveness (ratio of positive force area of 2^{nd} peak to 1^{st} peak), and chewiness (the product of hardness x springiness x cohesiveness) (Bourne, 1978).

5.2.6 Statistical analysis

A randomised, blocked, split plot in time design employing a corresponding general linear model (GLM) was used to study the difference between dietary treatments on yield, chemical characteristics, and texture properties of cheese samples at any ripening times. The diet supplementation was the main plot with the ripening time as the subplot. The repetitions served as blocks. Significant difference between mean of samples were determined using pdiff command, denoting a least significant difference (lsd test). The level of significance was preset at $P < 0.05$. Results obtained in this experiment were analysed using Minitab 16 (Minitab, 2010). Relationship between mean scores of composition of cheese-milk, which were obtained from an earlier experiment (section 4.2.2; Table 4.2), and Cheddar cheese was determined using simple linier correlation analysis. All results were expressed as mean ± SD.

5.3 RESULTS AND DISCUSSION

5.3.1 Effect of dietary treatments on composition and pH of Cheddar cheese

In Chapter 3 it was presented that the supplementation changed a profile of fatty acids in milk, while other characteristics remained fairly unaffected. Thus, the chemical composition of CON, CSO, TAN, and CPT cheeses during ripening period is summarized in Table 5.1. No marked difference ($P > 0.05$) in the cheese composition between control and CSO, TAN, and CPT cheeses was observed at any stages of ripening. For all cheeses, the content of fat and protein were relatively constant during ripening period. While concentration of cheese moisture decreased

and thus total solid increased during ripening. At the end of ripening period, content of fat, protein, moisture and total solids of cheeses were similar, which were in the range of 31.0-31.6%, 25.9-26.3%, 35.8-37.2%, and 62.8-64.2% respectively. These values were typical of a Cheddar cheese composition reported in literature (Fenelon and Guinee, 1999; Nelson and Barbano, 2005). Composition of Cheddar cheese obtained in the current study was also confirm with the composition of Cheddar cheese for local consumption or export according to Australian industry standards i.e. 36.5-37.0% moisture, 32-33% fat, 52.5-54% MFFS, 50.0-53.5% FDM, 1.5-2.0% salt, 4.5-5.0% salt in moisture and pH ~5.1-5.3 (Keating and Dennien, 1983).

Chemical composition of cheese is influenced by composition of milk, particularly protein to fat ratio, used to manufacture the cheese and cheese making practice (Guinee and McSweeney, 2006). Therefore, the comparable composition of CON, CSO, TAN, and CPT cheeses (Table 5.1) can be attributed to the similar composition of cheese-milk samples used to manufacture these cheeses (Chapter 4). Standardisation of fat to protein ratio (C/F= 0.72) during cheese-milk preparation has eliminated differences in the composition of the raw milk, which were introduced by the supplemented diets (Chapter 3). Earlier experiment showed that diet containing cottonseed oil i.e. CSO and CPT diets, reduced milk fat content and altered fatty acid composition but had no apparent effect on the protein content in milk. Meanwhile, condensed tannin supplementation had no impact on protein, milk fat contents and fatty acid composition (Chapter 3). The ratio of casein to fat of 0.72 was chosen because it is the most appropriate ratio for an optimum Cheddar cheese production i.e. optimal fat recovery and cheese yield (Fenelon and Guinee, 1999). Another

factor that can affect cheese composition is cheese making process (Guinee and McSweeney, 2006). Firmness of curd at cutting may affect the recovery of milk components in cheese and subsequently composition and yield of the cheese (Bynum and Olson, 1982).

Table 5.1 shows that the concentration of fat in cheeses was similar across dietary treatments. According to Guinee and McSweeney (2006), the level of fat in cheese is influenced by several factors including milk composition particularly protein to fat ratio and level of fat recovery in cheese (Guinee and McSweeney, 2006). Therefore, the comparable fat content among cheeses (Table 5.1) may be attributed to the similar fat to protein ratio in the milk (Chapter 4). The amount of fat in cheese subsequently may affect composition, yield, texture, biochemical process, proteolysis, and flavour of the cheese (Bryant et al., 1995). Effects of fat content on these aspects of cheese will be discussed further.

In the current study, moisture content of cheese was comparable to control at any ripening time (Table 5.1). Similar content of moisture might then cause similar pH value among the cheeses (Table 5.1). Cheese with high moisture content tends to have higher content of lactose but lower protein and phosphate content. This consequently might lower buffering capacity and thus lowered pH value (Perrie, 2012). Despite their effects in reducing fat content and altering fatty acid composition in the raw milk as has been discussed in section 3.3.1 of this thesis, overall, CSO and CPT diets had minimal impact on the cheese composition (Table 5.1). The minimal effect of these supplements on the cheese composition could be partly attributed to the standardization of casein to fat ratio at the early stage of

cheese-milk preparation. The standardisation process eliminated differences in casein to fat ratio of the cheese-milk (section 4.3.1), thus consequently eliminated variation in the cheese composition (Table 5.1). This lack of observable effect of fat supplementation on the cheese composition observed in our study was in agreement with results of previous studies when Cheddar cheese was made from milk of cows fed sunflower oil (Coakley et al., 2007) or extruded soybean (Baer et al., 1996). Supplementation of extruded cottonseed also showed no effect on the fat, protein, and moisture content of Mozzarella cheese (Dhiman et al., 1999).

The minimal effect of dietary condensed tannins on the cheese composition (Table 5.1) could be related to the absence of a substantial impact of this supplementation on the content of protein and fat in the milk (Table 3.3). Moreover, O'Connell and Fox (2001) stated that due to their simple structure, the presence of phenolic compounds in milk bear little significance on physicochemical properties of dairy products. In addition to initial composition of milk used for cheese manufacturing, the content of protein (Cooney et al., 2000) and fat (Mitchell et al., 1986) in cheese could also be influenced by level of somatic cell count (SSC) in the milk, however this obviously had no bearing in any regards due to stringent protocols during the dietary trial and milk sampling collection.

Cheese quality is examined through grading system based on compositional analysis (Lawrence et al., 2004). Due to their effects on biochemical processes, bacterial growth, enzyme activity, and acid production in cheese, the levels of salt-in-moisture (S/M), fat-in-dry matter (FDM), and moisture non-fat substance (MNFS) are more reliable indicators of cheese quality compared to the chemical composition

of cheese itself, such as salt, moisture, fat, or protein content (Lawrence and Gilles, 1980).

MNFS reflects the relative amounts of moisture and casein in the cheese (Lawrence et al., 2004). In the current study, MNFS levels of all cheeses were similar ($P < 0.05$), in the range of 56.7- 57.4% (Table 5.1). The similar MNFS values of these cheeses can be linked to the similar fat level in the cheese-milk samples (Chapter 4). According to Dejmek and Walstra (2004), the amount of fat in milk may determine degree of syneresis during manufacturing. A high milk fat content could impair syneresis, which is due to the effect of clogging of the fat globules in casein matrix, which impeded the outward flux of whey from the curd particles. Subsequently, reduction in syneresis may affect moisture content and MNFS level in cheese (Tunick et al., 1995)

Compared to the range of MNFS for first grade Cheddar cheese i.e. 52-54% (Lawrence et al., 2004), MNFS values of cheeses in the present study were slightly higher (Table 5.1). The higher MNFS value of Cheddar cheese in the current study compared to values reported by Lawrence et al (2004) could be due to the effect of microfluidization (20 MPa; single stage) on the cheese-milk used to make the Cheddar cheese. Microfluidization impairs syneresis of cheese curd, which consequently increases moisture and MNFS level of the cheese (Lemay et al., 1993). This impairment is achieved by a shearing effect that takes place during microfluidization resulting in smaller fat globules, which in turn increases interactions between casein and fat, which reduce the surface area of casein micelles for mutual interaction (Green et al., 1983).

Salt in moisture (S/M) is an important parameter for cheese quality due to its effect on controlling water activity (a_w), and thus activities of chymosin (Fox, 1987), plasmin (Richardson and Pearce, 1981), and starter proteinase (Martley and Lawrence, 1972). No significant ($P > 0.05$) difference was observed in S/M values of cheeses in the present study, the values ranged from 4.8-4.9% (Table 5.1). Based on cheese grading system proposed by Gilles and Lawrence (1973), S/M value of these cheeses may be classified as the first grade cheese. The comparable S/M value of cheeses in the current study can be linked to the similar fat and moisture content in the cheese-milk (Guinee and McSweeney, 2006).

In comparison to the effect of MNFS and S/M in determining cheese quality, the effect of FDM is less important. However, FDM value of cheese still needs to be considered since it may affect the cheese quality indirectly by affecting the level of MNFS (Whitehead, 1948). However, no significant ($P > 0.05$) difference in FDM value of cheese in the current study, the value ranging from 51.4-52.5% (Table 5.1). The similar FDM values among these cheeses could be linked to a similar ratio of casein to fat in the cheese-milk (O'Brien and O'Connor, 2004). The comparable level of MNFS, S/M, and FDM among cheeses (Table 5.1) was likely due to the similar fat, moisture and protein content of the cheeses which is in agreement with Guinee et al. (2006). Apparently the differences in milk fatty acid composition (Chapter 3) played no effect on these properties.

Another parameter of cheese ripening, which may determine the cheese quality, is cheese pH, which governs cheese stability and final properties (Pastorino et al., 2003). However, the analysis revealed that yet again there was no significant

($P > 0.05$) effect of dietary treatments on cheese pH at any points during the ripening time (Table 5.1). At day 1 after manufacturing, all cheeses had similar pH value, ~5.24-5.25 (Table 5.1). These results rather indicated that the cheeses had a similar activity of starter bacteria regarding acid production during cheese manufacturing process.

The trend of pH changes during ripening period is shown in Table 5.1. In general, at day 1 after manufacturing, pH of cheeses was ~5.24-5.25. During the first 6 weeks of ripening, cheese pH continued to decline ($P < 0.05$), while this trend was reversed towards the end of ripening period. The pH decline during the initial ripening stage (Table 5.1) was likely related to generation of lactic acid from remaining lactose by surviving cells of the starter culture (Fenelon and Guinee, 1999). The increase of cheese pH afterwards could be partly attributed to production of NH_3 as a product of proteolysis during ripening (Guinee and Law, 2002). These findings are in line with several previous reports (Fenelon and Guinee, 2000; Rynne et al., 2008), but contrasted findings of O'Mahony et al. (2005), which showed no pH changes during ripening.

Table 5.1. Composition of Cheddar cheese made from bovine milk of cow fed a diet supplemented with cottonseed oil, condensed tannin, and in combination

Items[2]	Dietary treatments[1]			
	CON	CSO	TAN	CPT
FDM (%)	51.9±2.7^{a}	51.9±1.7^{a}	51.4±1.3^{a}	52.5±1.2^{a}
SNFP (%)	60.4±1.0^{a}	60.3±1.4^{a}	60.4±0.6^{a}	60.0±1.0^{a}
MNFS (%)	56.7±1.2^{a}	56.8±1.6^{a}	56.4±1.1^{a}	57.4±1.2^{a}
S/M (%)	4.8±0.3^{a}	4.9±0.3^{a}	4.8±0.4^{a}	4.8±0.3^{a}
Fat (%)				
Day 1	31.9±1.9^{aA}	31.7±1.3^{aA}	31.6±0.8^{aA}	32.0±0.9^{aA}
6 wk	31.2±1.5^{aA}	31.4±1.0^{aA}	31.3±1.0^{aA}	31.4±1.0^{aA}
12 wk	31.4±1.1^{aA}	31.1±1.5^{aA}	31.7±1.1^{aA}	31.1±1.0^{aA}
24 wk	31.2±1.0^{aA}	31.6±0.9^{aA}	31.0±1.0^{aA}	31.3±1.0^{aA}
Protein (%)				
Day 1	25.8±1.7^{aA}	26.1±1.1^{aA}	26.8±0.9^{aA}	26.4±1.2^{aA}
6 wk	26.7±1.2^{aA}	26.9±1.7^{aA}	25.9±1.5^{aA}	26.2±1.0^{aA}
12 wk	25.9±1.6^{aA}	26.0±0.9^{aA}	25.8±0.9^{aA}	26.2±1.3^{aA}
24 wk	26.1±1.0^{aA}	25.9±1.4^{aA}	25.9±0.6^{aA}	26.3±0.8^{aA}
Total solid (%)				
Day 1	61.7±1.1^{aB}	61.9±1.0^{aA}	61.7±0.6^{aB}	61.0±1.0^{aB}
6 wk	62.0±0.7^{aAB}	61.7±1.8^{aA}	61.1±0.9^{aAB}	62.0±1.9^{aAB}
12 wk	62.5±1.2^{aAB}	62.5±1.5^{aA}	62.8±0.6^{aA}	62.3±1.0^{aAB}
24 wk	64.2±0.7^{aA}	62.8±1.6^{aA}	63.7±0.7^{aA}	63.3±0.9^{aA}
Moisture (%)				
Day 1	38.3±1.8^{aA}	38.1±1.2^{aA}	38.3±1.6^{aA}	39.0±1.4^{aA}
6 wk	38.0±1.8^{aAB}	38.3±1.7^{aAB}	38.9±1.0^{aA}	38.0±1.3^{aAB}
12 wk	37.5±2.1^{aAB}	37.5±1.0^{aB}	37.2±1.0^{aAB}	37.7±1.9^{aAB}
24 wk	35.8±1.4^{aB}	37.2±1.4^{aB}	36.3±1.1^{aB}	36.7±1.9^{aB}
pH				
Day 1	5.24±0.03^{aB}	5.24±0.04^{aB}	5.25±0.02^{aB}	5.25±0.02^{aB}
6 wk	5.06±0.03^{aC}	5.13±0.04^{aC}	5.17±0.03^{aC}	5.18±0.06^{aC}
12 wk	5.26±0.02^{aB}	5.27±0.04^{aB}	5.27±0.03^{aB}	5.23±0.03^{aBC}
24 wk	5.33±0.03^{aA}	5.36±0.04^{aA}	5.37±0.15^{aA}	5.34±0.04^{aA}

Means within a row with different lowercase superscript differ significantly ($P < 0.05$).
Means within a column with different capital superscript differ significantly ($P < 0.05$).
[1]Dietary treatments: CON = control; CSO = cottonseed oil; TAN = tannin; CPT = cottonseed oil plus tannin.
[2]Items: FDM = fat dry matter; SNFP = solid non-fat protein; MNFS = moisture non-fat solid; S/M = salt/moisture.

Cheese pH plays an important role on the yield and overall cheese quality through its effect on chemical composition, texture, flavour, and biochemical processes in cheese during ripening period (Fox and Cogan, 2004). The comparable moisture content in the cheeses can also be linked to similar pH in cheese (Table 5.1). Cheese pH may affect moisture content of cheese by its effect on increasing solubilisation of colloidal calcium phosphate (CCP) and thus decreasing attraction between proteins, which then increase hydration of protein matrix (Van Hooydonk et al., 1986). Effects on cheese pH on yield, texture, flavour of cheese will be discussed further in the next sections.

5.3.2 Effect of dietary treatments on recoveries and yield of Cheddar cheese

Fat and protein recovery of the Cheddar cheeses prepared from milk of cows fed CSO, TAN, and CPT diets were comparable ($P > 0.05$) to those of control (Table 5.2), and were in the range of 83.3 to 89.7% and 74.6 to 78.8%, respectively (Table 5.2). Fat recovery was positively correlated with the concentration of casein, total protein, and fat in the cheese-milk (r = 0.73, 0.72, and 0.43, respectively). According to Fenelon and Guinee (1999), increase in fat content in milk may reduce the amount of fat loss in the whey. Increase of fat content in milk may increase clumping of milk fat globules during cheese manufacture due to the shrinkage of casein matrix around the occluded fat globules. Thus, clumping of milk fat globules increases size of the milk fat globules, which then prevents the movement of these fat globules through para-casein matrix to the whey (Fenelon and Guinee, 1999). Protein recovery positively correlated with the casein and protein content (r = 0.36 and 0.37, respectively). Therefore, fat and protein recovery among the cheeses was similar

likely due to similar concentration of casein, protein, and fat in the cheese-milk as a result of standardization process (Table 4.2).

Many factors may affect cheese yield, these include milk composition, milk pre-treatment, curd firmness at cutting, vat design, and curd handling system (Fenelon and Guinee, 1999). The actual-, predicted-, and moisture adjusted-yield of the Cheddar cheeses are given in Table 5.2. Overall, actual yield (Ya) and moisture adjusted yield (Yma) for each cheese were similar; but slightly lower compared to predicted yield (Yp). This discrepancy could be related to a lower fat recovery from the samples prepared in the current study than the one that was used in Van Slyke formula to calculate Yp i.e. 93%. The Ya and Yma of TAN cheese were comparable ($P > 0.05$) to those of the CON cheese, but higher ($P < 0.05$) than those of CSO or CPT cheeses. The higher yield of TAN cheese in comparison to other cheeses can be linked to its slightly higher protein and fat recovery, even though the difference was not significant (Table 5.2). The higher fat and protein recovery thus increase the cheese yield through the increase of total solid amount recovered in cheese. In our study, cheese yield was positively correlated with the fat recovery (r = 0.78, 0.58, 0.79 for Ya, Yp, and Yma, respectively) and protein recovery (r = 0.71 for Ya).

According to Lucey and Kelly (1994), cheese yield may be affected by milk characteristics and cheese manufacturing conditions. Yields of all cheeses in our study were positively correlated with the concentration of casein (r = 0.81, 0.89, and 0.81 for Ya, Yp, and Yma, respectively), protein (r = 0.82, 0.89, and 0.82 for Ya, Yp, and Yma, respectively), and fat (r = 0.81, 0.89, and 0.82 for Ya, Yp, and Yma, respectively) in the cheese-milk. The positive effect of protein, casein, and fat

content in milk on cheese yields was rather expected since basically cheese is a product in which protein and fat of the milk are concentrated (Van Boekel, 1994). In addition, the positive correlation between casein content and cheese yield can be linked to the high proportion of caseins, which were entrapped in curd. The casein also forms structural network, which entraps fat and moisture (Van Boekel, 1994).

The positive correlation between cheese yield and fat content in milk can be attributed to the increase of cheese-recoverable solids, especially casein and fat (Fenelon and Guinee, 1999). Positive correlation between the actual cheese yields and the content of fat (Fenelon and Guinee, 1999) or protein in milk (Guinee et al., 2007) has been previously reported. In addition, milk fat content can indirectly affect cheese yield by increasing moisture content in the cheese curd. Milk fat globules, which are entrapped in the para-casein matrix, may restrict the contraction of para-casein network. This result in inhibition of syneresis and thus increase moisture content in the curd (Fenelon and Guinee, 1999). Moisture content may also increases cheese yield since it carries soluble components of whey such as whey proteins, caseinomacropeptide (CMP), lactate, and soluble milk salts (Guinee and McSweeney, 2006).

Another factor that may affect cheese yield is manufacturing process. This includes the appropriate cutting time to obtain curd firmness that would allow for maximum retention of proteins, fat and total solids as well as optimum moisture content (Bynum and Olson, 1982; Johnson et al., 2001). A curd that is too soft at cutting would reduce cheese yield since this soft gel may cause shattering of fragile network thus enhanced an amount of curd fines and fat losses (Lawrence, 1991). In

contrast, in a firm gel, the casein network is unable to rearrange, thus would increase a moisture content of curd (Castillo et al., 2006). Our study showed that firmness (storage modulus) of rennet-induced gel (Table 4.4) positively correlated with the cheese yield (r = 0.44, 0.50, and 0.42 for Ya, Yp and Yma, respectively). The current study also showed that gel firmness affected the yield of cheese by increasing protein recovery (r = 0.50). This result is in agreement with Bynum and Olson (1982), who found that increased in curd firmness lowered fat and protein losses thus increased the cheese yield. Increase in curd firmness may also enhance the ability of casein network to withstand breakage due to physical damages, thus reducing the amount of casein fines and increasing retention of nitrogenous compound in curd (Schultz-Collins and Senge, 2004).

A lack of an impact of fat supplementation on cheese yield found in the current study is similar with results of Sinclair et al. (2007), in which CLA supplementation did not affect cheese yield despite of its decreasing effect on milk fat content. According to the authors, improvement in casein to fat ratio was attributed to the lack of this effect. In contrast, Chen et al., (2009) reported that goats fed diet supplemented with conjugated linoleic acid produced milk with lower fat content; but with no effect on protein content or casein to fat ratio. Transformation of this milk into semi-hard cheese resulted in cheese with a lower yield. The discrepancy among studies on the effect of fat supplementation on cheese yield can be attributed to the fact that cheese yield was not only affected by milk composition but also by other factors such as milk pre-treatment, cheese making practice, and somatic cell count (SSC).

Table 5.2 Fat or protein recovery and yield of Cheddar cheese made of bovine milk obtained from cows fed a diet supplemented with cottonseed oil, condensed tannin, and in combination

Parameters (%)	Dietary treatments[1]			
	CON	CSO	TAN	CPT
Recovery in cheese				
Fat recovery	85.8±5.0^{a}	83.3±3.9^{a}	89.7±2.9^{a}	83.9±8.0^{a}
Protein recovery	75.9±7.8^{a}	74.6±3.4^{a}	78.8±3.5^{a}	74.7±4.8^{a}
Cheese yield[2]				
Actual yield (Ya)	10.5±1.4^{ab}	9.7±1.2^{b}	11.2±0.7^{a}	9.6±0.9^{b}
Predicted yield (Yp)	11.3±0.8^{a}	10.7±0.8^{a}	11.5±0.4^{a}	10.8±0.6^{a}
Moisture-adjusted yield (Yma)	10.5±1.4^{ab}	9.6±1.2^{b}	11.2±0.7^{a}	9.5±1.0^{b}

Means within a row with different lowercase superscript differ significantly ($P < 0.05$).
[1]Dietary treatments: CON = control; CSO = cottonseed oil; TAN = tannin; CPT = cottonseed oil plus tannin.
[2]Ya = actual yield; Yp = predicted yield; Yma =moisture-adjusted yield.

5.3.3 Proteolysis in cheese

There are two stages of proteolysis in cheese during ripening, primary and secondary proteolysis (Upadhyay et al., 2004). Primary proteolysis involves hydrolysis of the Phe_{23}-Phe_{24} bond of α_{s1}-casein by chymosin; and hydrolysis of β-casein by plasmin to a lesser extent. This process produces large (water soluble) and intermediate (water insoluble) size peptides (Shakeel-Ur-Rehman et al., 1998). During secondary proteolysis, intermediate-size peptides are hydrolysed further into small peptides and amino acids by proteinase and peptidase from starter culture and

NSLAB (Shakeel-Ur-Rehman et al., 1998). Degree of primary proteolysis can be monitored by measuring the concentration of soluble nitrogen in WSN fraction (Upadhyay et al., 2004). In the current study, no apparent ($P > 0.05$) variation was observed in the percentage of WSN/TN of CSO, TAN, and CPT cheeses compared to that of CON diet (Table 5.3) at any ripening point. This result indicated that, in comparison to control cheese, CSO, TAN, and CPT cheeses had a similar activity of chymosin and plasmin in hydrolysing casein.

Degree of proteolysis is influenced by several factors including pH, S/M, MNSF, and moisture content in cheese (Fox, 1987). The level of S/M, MNSF, and moisture may affect the rate of proteolysis in cheese through their effect in controlling water activity, which in turn may govern bacterial growth and enzyme activity (Fox, 1987). Meanwhile, cheese pH may influence proteolysis through its effect on calcium solubilisation, which in turn may determine relaxation of protein-protein interactions and thus enable proteolytic enzyme to better access sites for hydrolysis (Watkinson et al., 2001). Therefore, the comparable primary proteolysis in all cheeses obtained in the present study may be attributed to the similar level of pH, S/M, MNSF, and moisture content in these cheeses (Table 5.1) as well as the activity of endogenous bacterial proteases.

Table 5.3 The levels of soluble nitrogen (% total N) in water soluble, 12% trichloroacetic acid (TCA), and 10% phosphotungstic acid (PTA) fractions of Cheddar cheese made of bovine milk supplemented with cottonseed oil, condensed tannins, and in combination, during 24 weeks of ripening

Ripening time (week)	Dietary treatments[1]			
	CON	FAT	TAN	FPT
WSN/TN[2]				
1 week	4.02±0.30^{a,D}	4.05±0.29^{a,D}	4.29±0.16^{a,D}	3.99±0.16^{a,D}
6 week	8.42±0.62^{a,C}	8.77±0.68^{a,C}	8.89±0.40^{a,C}	8.34±0.05^{a,C}
12 week	12.60±0.87^{aB}	13.35±0.90^{aB}	13.68±0.63^{aB}	12.60±0.95^{aB}
24 week	16.42±1.20^{abA}	16.99±0.82^{aA}	17.79±0.95^{aA}	15.42±1.22^{bA}
TCA-SN/TN[3]				
1 week	1.08±0.13^{b,D}	1.20±0.11^{ab,D}	1.18±0.08^{ab,D}	1.30±0.22^{a,D}
6 week	2.35±0.18^{a,C}	2.46±0.17^{a,C}	2.50±0.11^{a,C}	2.34±0.11^{a,C}
12 week	3.51±0.24^{aB}	3.78±0.25^{aB}	3.81±0.17^{aB}	3.53±0.23^{aB}
24 week	4.56±0.35^{bcA}	4.76±0.22^{abA}	4.92±0.26^{aA}	4.37±0.19^{cA}
PTA-SN/TN[4]				
1 week	0.51±0.04^{a,D}	0.51±0.05^{a,D}	0.53±0.03^{a,D}	0.50±0.03^{a,D}
6 week	1.02±0.09^{a,C}	1.08±0.11^{a,C}	1.11±0.06^{a,C}	1.03±0.08^{a,C}
12 week	1.58±0.11^{bB}	1.69±0.12^{abB}	1.73±0.06^{aB}	1.58±0.08^{bB}
24 week	2.07±0.15^{abA}	2.15±0.15^{aA}	2.24±0.13^{aA}	1.94±0.18^{bA}

Means within a row with different lowercase superscript differ significantly ($P < 0.05$).
Means within a column with different uppercase superscript differ significantly ($P < 0.05$).
[1]Dietary treatments: CON, control; CSO, cottonseed oil; TAN, tannin; CPT, cottonseed oil plus tannin.
[2]WSN/TN = water soluble nitrogen/total nitrogen.
[3]TCA-SN/TN = trichloroacetic acid-soluble nitrogen/total nitrogen.
[4]PTA-SN/TN = phosphotungstic acid-soluble nitrogen/total nitrogen.

In the current study, the percentage of WSN to total nitrogen (TN) in all cheeses increased ($P < 0.05$) during the ripening period (Table 5.3), which is in agreement with results from other studies (Fenelon and Guinee, 2000; de Wit et al., 2005). After 6 months of storage, the level of WSN was ~4 times higher than that at day 1. However, these WSN/TN values were lower than other study when cheese was ripened at temperature higher than 4 ºC (Ong et al., 2006). This discrepancy likely indicates the importance of ripening temperature on the activities of plasmin and chymosin, which are primarily responsible for the formation of WSN (Folkertsma et al., 1996).

The TCA fraction mainly contains medium and small peptides of low and medium hydrophobicity derived from the N-terminal half of β-casein and the N-terminal half of α_{s1}-casein (Singh et al., 1994) as a result of the starter and NSLAB enzyme activity (O'Keeffe et al., 1978). The comparable proportions of TCA-SN/TN of CSO, TAN, and CPT cheeses in comparison to that of the CON cheese might indicate that these supplemented diets did not affect the activity of starter enzymes.

Similar to WSN fractions, percentage of soluble nitrogen in TCA fraction to total nitrogen (TCA-SN/TN) increased throughout the ripening time (Table 5.3). In general, at the end of the ripening period, concentration of TCA-SN/TN was ~4 times higher than those at the beginning. The level of TCA-SN/TN was 4 times lower than WSN-SN/TN (Table 5.3). This result was rather expected since TCA-SN fraction consists of small peptides and free amino acids (FAA). In addition, with the presence of 12% TCA, most peptides in WSN fractions precipitated (Kuchroo and Fox, 1982).

PTA-SN level is an indicator of secondary proteolysis (Jarrett et al., 1982). This fraction consists of very small peptides, less than 15 kDa, and amino acids of ~600 Da (Aston and Dulley, 1982). In the current study, degree of secondary proteolysis did not vary between the dietary treatments as indicated by a similar ratio of PTA-SN/TN among dietary treatments throughout ripening time (Table 5.3). This result might indicate that the supplemented diets did not affect peptidase activity in the cheeses. A lack of an effect of cottonseed supplementation on soluble nitrogen, and thus the proteolytic activity of the Cheddar cheese obtained in the current experiment is similar to findings of Baer et al. (1996) when cows were fed extruded soybeans.

As for WSN/TN and TCA-SN/TN percentages, PTA-SN/TN percentage increased during ripening time but to a lesser extent (Table 5.3). The lower proportion of PTA-SN/TN in comparison to its TCA-SN/TN and WSN/TN counterparts could be attributed to compositional differences. Namely, PTA-SN fraction consists of only free amino acids, while TCA-SN fraction corresponds to small peptides and free amino acids. Furthermore, WSN fraction accounted for whey protein, high-, medium-, and low-molecular weight peptides, and free amino acids (Kuchroo and Fox, 1982).

5.3.4 Peptide profile determined by RP-HPLC

Profiling WSN extract of cheese using RP-HPLC can be used to characterize secondary proteolysis in cheese (Parente et al., 2012). RP-HPLC chromatograms of water soluble fractions of CON, CSO, TAN, and CPT cheeses aged for 1, 6, 12, and 24 weeks are given in Figure 5.1. No obvious differences were observed between peptide profiles and peak intensity of CON cheese compared to those of CSO, TAN, and CPT cheeses. This result indicated that supplemented diets did not affect peptidolysis in the cheese substantially.

In general, all chromatograms changed and become more complex as the cheese matured. The peaks area increased during ripening time (Figure 5.1). These changes indicated a great extent of proteolysis taking place during ripening. Profile of peptide development during cheese ripening obtained in this study was comparable to results of other studies (Lau et al., 1991; de Wit et al., 2005). In our study, for all cheeses, the most remarkable changes in peak intensity were observed for peaks eluted at 9.45; 13.78; 18.10; 23.85; and 31 minutes. These peaks likely corresponded to peaks eluting at 14, 15, 18, 22, and 29 minutes found in previous studies (Lau et al., 1991; de Wit et al., 2005). For all cheeses, peptides that elute at 9.45 min were present at high amount throughout ripening time. This concentration was stabilized by week 12, and decreased slightly afterwards. For all cheeses, the intensity of peaks eluted at 13.78, 18.10 and 23.85 minutes increased from week 1 until week 12; afterwards, the intensity decreased or stabilized (Figure 5.1). Similar trends in development of these peaks during cheese ripening have been reported previously (de Wit et al., 2005).

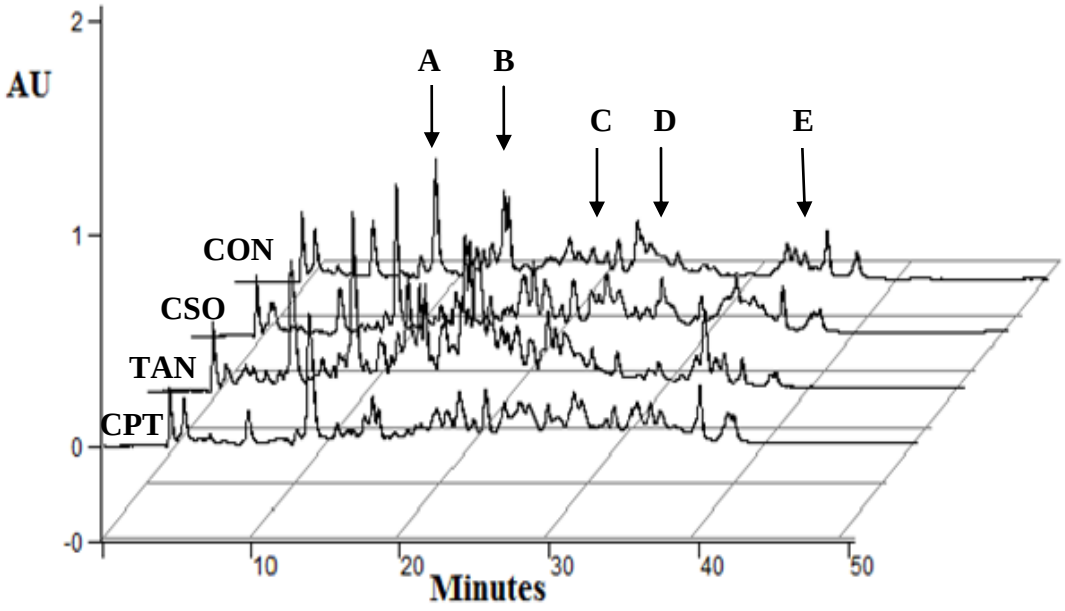
Peaks eluting at retention times < 35 minutes correspond to hydrophilic peptides, while those eluting above 35 minutes are attributed to hydrophobic peptides (Shakeel-Ur-Rehman et al., 2000). Thus, the increased intensity of hydrophilic peptides was rather expected as protein break down during proteolysis resulted in a release of amino acids and hydrophilic products.

Intensity of hydrophobic peptides increased throughout ripening time (Figure 5.1). A similar trend in development of hydrophobic peptides during ripening of ovine milk cheese made with calf rennet has been reported (Agboola et al., 2004). From the chromatograms (Figure 5.1), it also appeared that at any stages of ripening period, the amount of hydrophilic peptides was higher than that of hydrophobic peptides. The increase of hydrophilic peptides and decrease in hydrophobic peptides could be apparently attributed to degradation of hydrophobic peptides (Lau et al., 1991; Picon et al., 1994).

The WSN fraction contributes to the flavour of cheese (Barcenas et al., 1999). Separation of hydrophilic and hydrophobic peptides on water soluble fraction of cheese by RP-HPLC has been used to predict bitterness (Gaya et al., 1990; Lau et al., 1991). When present at a higher amount, hydrophobic peptides may cause a substantial bitterness in cheese (Cliffe et al., 1993). These bitter peptides are products of α_{s1}- and β-casein degradation mainly by non-starter bacteria and to a lesser extent by other proteases such as rennet and coagulant. On the other hand, hydrophilic peptides and amino acids are known as savoury peptides (Cliffe et al., 1993). Therefore, result from the current study which shows the increasing amount of hydrophilic peptides over hydrophobic peptides throughout ripening time was

important for development of acceptable cheese taste. Certainly these results need to be verified in the future by conducting a sensory analysis.

A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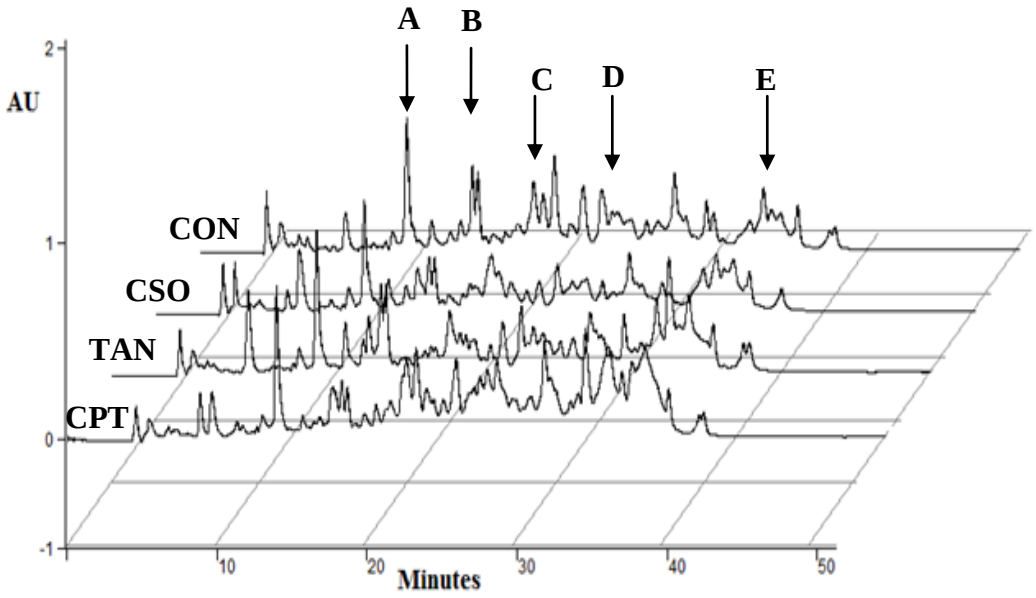
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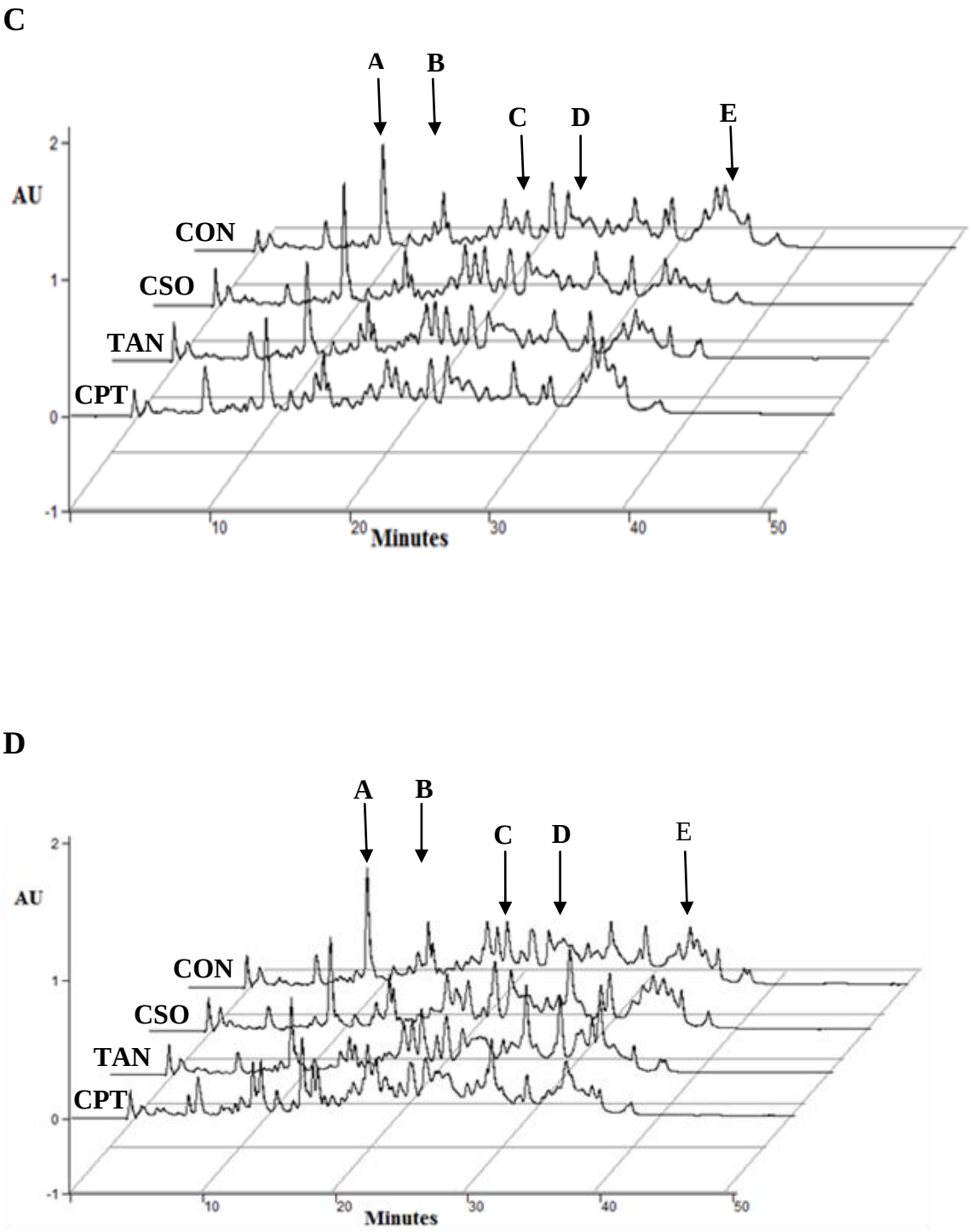

Figure 5.1 Peptide profiles obtained by RP-HPLC of Cheddar cheeses made of milk obtained from cows fed a diet supplemented with cottonseed oil and condensed tannins at week 1 (A), week 6 (B), week 12 (C) and week 24 (D) of ripening period.
CON = control; CSO = cottonseed oil; TAN = tannin; CPT = cottonseed oil plus tannin.
A, B, C, D, and E = peptides that elutes at 9.45; 13.78; 18.10; 23.85; and 31 minutes, respectively.

5.3.5 Cheese texture

Texture and flavour are important cheese characteristics, which determine cheese quality and consumer acceptability towards this dairy product (McEwan et al., 1989). Microscopy studies showed that cheese microstructure consists of a loose and open para-casein matrix with fat and moisture entrapped between the protein strands (Hall and Creamer, 1972). Para-casein matrix constituted ~82% of the cheese matrix, while fat and moisture/serum occupied the remaining i.e. ~18% (Pastorino et al., 2003).

Characteristic of cheese texture is influenced largely by its pH, degree of proteolysis, and chemical composition, particularly protein, fat, and moisture (Fox, 1993). Fat content of cheese may affect cheese hardness by influencing the amount of milk fat globules dispersed in the protein matrixes or by increasing moisture content (Bryant et al., 1995). Cheese with low fat content tends to have less milk fat globules embedded in the protein networks. This causes casein network to become more compact and dense with fewer open spaces, which consequently increases hardness of the cheese. Meanwhile high content of fat in cheese is related to a cheese with a softer texture (Bryant et al., 1995). A high fat content may increase moisture, thus weaken the protein framework as a volume fraction of protein decreases (Bryant et al., 1995). Cheese pH may affect textural characteristics of cheese by affecting solubilisation of colloidal calcium phosphate from the casein and influencing interaction between proteins (Pastorino et al., 2003). Cheese firmness also can be affected by the ratio of fat to protein in the milk (Coulon, 2004). Milk with a high fat

to protein ratio would produce a cheese with a higher level of fat in dry matter (FDM), thus result in a less firm cheese (Coulon, 2004).

Results from the present study showed that Cheddar cheese made from milk of cows fed the CSO, TAN, and CPT diets had a comparable ($P > 0.05$) hardness to the control cheese after 24 week of ripening (Table 5.4). These results were rather expected because there were no apparent differences in pH, proteolysis, and fat content among these cheeses (Table 5.1). While textural properties of cheese could be affected by the amount of fat, another very important feature in this regards is the composition of their fatty acids (Wong et al., 1973). Fatty acid composition could influence texture of dairy products by determining the amount of solid- or liquid-fatty acids (Baer et al., 1996; MacGibbon and Taylor, 2006). Thus, degree of saturation of milk fatty acids plays an important role in governing this effect (MacGibbon and Taylor, 2006). In contrast to saturated fatty acids, which have a high melting point and are in solid state at room temperature, unsaturated fatty acids have low melting points and are in liquid state at room temperature (MacGibbon and Taylor, 2006). The presences of high amount of unsaturated fatty acids in dairy products is associated with a softer texture and higher spreadability of butter (Chen et al., 2004); softer, less gummy, and less chewy of the Provolone cheese (Chen et al., 2004), and reduced hardness in the Cheddar cheese (Baer et al., 1996).

In the current study, despite the higher amount of unsaturated fatty acids in CSO and CPT cheeses compared to those in CON and TAN cheeses (Chapter 6), no significant differences ($P > 0.05$) were observed in cheese hardness at the end of ripening time (Table 5.4). This result was in agreement with Chen et al. (2004) who

argued that cheese textural parameters such as hardness, adhesiveness, chewiness, cohesiveness, gumminess, and resilience of Cheddar cheese were unaffected by the amount of unsaturated fatty acid in milk. However, our results contradicted reports in literature, which showed a reduction in Cheddar cheese hardness (Baer et al., 1996) associated with an increase amount of unsaturated fatty acid in the respective cheese. This discrepancy might be attributed to the difference in levels of polyunsaturation in cheeses. Difference in level of polyunsaturation, which exerted different effect on the cheese texture, has been reported by Wong et al. (1973), in which cheese containing 12-17% of linoleic acid had minimal texture and body defects. On the other hand, the cheese with more than 30% linoleic acid was characterised with texture and body defects.

Springiness is the extent at which a deformed material return to its undeformed shape after the deforming force is removed (Szczesniak, 1963). Cheese springiness is determined by elasticity of protein network with less milk fat globules entrapped in the network giving a more elastic network (Bryant, 1995). As a result, springiness increases with the lower amount of fat in cheese (Emmons et al., 1980). Cohesiveness is the extent to which a product could be deformed before it ruptures (Szczesniak, 1963). According to Bryant et al. (1995), cheese with a low fat content tends to be more cohesive since it resists deformation and does not rupture easily. In the current study, all cheese had comparable ($P > 0.05$) springiness at the end of ripening time (Table 5.4). This result might be associated with the similar fat content among these cheeses, which might lead to a comparable elasticity of protein network and thus the springiness. It also appeared that, TAN and CPT cheeses had similar (*P*

> 0.05) cohesiveness and chewiness to those of CON cheese after 24 week of ripening In contrast, CSO cheese had lower ($P < 0.05$) cohesiveness and chewiness compared to CON cheese (Table 5.4).

In general, all cheeses showed similar pattern of hardness development during ripening (Table 5.4). During the first 4-6 weeks, cheese hardness decreased significantly ($P < 0.05$). However, this trend reversed later and after 24 weeks of ripening, the cheese hardness was significantly ($P < 0.05$) higher than that of fresh cheese. Similar pattern has been reported in literature (Awad, 2007). According to Lucey et al. (2003), decrease in cheese hardness at early ripening period is likely associated with proteolysis of the casein network, which disrupts protein matrix or solubilisation of colloidal calcium phosphate due to pH decline. Recovery of the cheese hardness in the later stages of ripening might be related to reduction in the amount of free water, which increases cheese resistance to deformation (McMahon et al., 1999). Less available water is due to the proteolysis since for each peptide bond cleaved; a molecule of water is incorporated to resulting polypeptides. In addition, two ionic groups are generated and each of which compete for the available water in the system. Thus, water previously available for solvation of protein chains become tied up by the ionic groups, making the cheese more firm and less easily deformed (Creamer and Olson, 1982). In the current study, springiness of all cheeses decreased ($P < 0.05$) during ripening. According to Kanawjia et al. (1995) hydrolysis of para-caseinate molecules may cause reduction in cheese springiness. Cohesiveness of all cheese decreased significantly during ripening (Table 5.4),

which might be due to proteolysis that disrupted the protein matrix (Irudayaraj et al., 1999).

Table 5.4 Texture profile of Cheddar cheese made from milk cows fed diet with added cottonseed oil and condensed tannins during 24 weeks of ripening period

Ripening time (week)	Dietary treatment[1]			
	CON	CSO	TAN	CPT
Hardness (N)				
1	118.68±6.38^{a,B}	110.20±5.76^{a,B}	119.55±6.19^{a,B}	114.47±8.76^{a,B}
4	92.31±7.38^{ab,C}	88.97±6.12^{b,C}	99.11±5.08^{a,D}	90.60±5.47^{b,C}
6	100.37±9.50^{ab,C}	95.46±6.62^{b,C}	107.10±9.23^{a,CD}	93.08±5.63^{b,C}
12	118.86±9.29^{a,B}	106.54±3.33^{b,B}	112.96±6.57^{ab,BC}	109.23±5.40^{b,B}
24	132.16±5.39^{ab,A}	125.30±5.78^{b,A}	134.92±7.76^{a,A}	130.42±4.77^{ab,A}
Springiness (mm)				
1	0.76±0.08^{bc,A}	0.73±0.05^{c,A}	0.86±0.07^{a,A}	0.83±0.07^{ab,A}
4	0.64±0.06^{b,B}	0.61±0.04^{b,B}	0.74±0.05^{a,B}	0.66±0.03^{b,B}
6	0.53±0.05^{ab,C}	0.52±0.05^{b,C}	0.61±0.04^{a,C}	0.54±0.07^{ab,C}
12	0.49±0.03^{a,CD}	0.44±0.02^{b,D}	0.47±0.03^{ab,D}	0.45±0.02^{b,D}
24	0.42±0.04^{a,D}	0.39±0.04^{a,D}	0.43±0.02^{a,D}	0.41±0.03^{a,D}
Cohesiveness				
1	0.48±0.05^{a,A}	0.45±0.04^{a,A}	0.49±0.03^{a,A}	0.48±0.04^{a,A}
4	0.42±0.04^{a,B}	0.34±0.03^{b,B}	0.45±0.02^{a,B}	0.37±0.04^{b,B}
6	0.39±0.04^{ab,BC}	0.35±0.03^{b,B}	0.41±0.03^{a,B}	0.36±0.03^{b,B}
12	0.35±0.03^{a,C}	0.29±0.02^{b,C}	0.34±0.03^{a,C}	0.32±0.02^{ab,CD}
24	0.29±0.03^{a,D}	0.24±0.02^{b,D}	0.30±0.02^{a,D}	0.29±0.02^{a,C}
Chewiness (N)				
1	48.22±4.62^{a,A}	41.70±4.35^{b,A}	46.55±4.59^{ab,A}	45.26±5.02^{ab,A}
4	30.55±2.81^{bB}	25.53±1.08^{c,B}	36.58±2.88^{a,B}	26.74±2.89^{b,C}
6	31.65±5.06^{ab,B}	27.78±2.91^{b,B}	34.96±3.44^{a,BC}	27.45±2.91^{b,B}
12	33.24±3.34^{a,B}	25.14±1.70^{c,B}	31.04±4.08^{ab,BC}	29.03±2.66^{bc,B}
24	30.18±3.44^{ab,B}	24.48±1.25^{c,B}	32.18±3.29^{a,C}	27.85±2.95^{bc,B}

Means within a row with different lowercase superscript differ significantly ($P < 0.05$).
Means within a column with different uppercase superscript differ significantly ($P < 0.05$).
[1]Dietary treatment: CON = control; CSO = cottonseed oil; TAN = tannin; CPT = cottonseed oil plus tannin.

5.4 CONCLUSION

This study demonstrated that supplementation of cottonseed oil, *A. maernsii*-condensed tannin, or in combination to a normal bovine diet had a marginal effect on assessed properties of the cheese based on fat and protein recoveries, and cheese yield. These modified diets also had no effect on the concentration of protein, fat, moisture, and total solids of the cheeses. Except for decrease in moisture content, total solid increased whereas content of protein and fat remained unaltered during ripening. The ratio of salt-in-moisture (S/M), FDM, and MNFS values were also similar among the cheeses.

The comparable levels of WSN, TCA-SN/TN, and PTA-SN/TN among the cheeses indicated that these supplementations had no effects on primary and secondary proteolysis in cheeses during ripening. The levels of WSN, TCA-SN/TN, and PTA-SN/TN were kept increasing throughout ripening time. Overall, there was no significant effect of cottonseed oil and condensed tannin supplementations on hardness and springiness among the cheeses at the end of ripening period. However, CSO cheese had lower cohesiveness and chewiness compared to those of control cheese. Thus, this study showed that feeding dairy cows with diet supplemented with fat (cottonseed oil), tannin extracted from, or combination of fat and tannin can be used to produce Cheddar cheese without negative effects on its yield, composition, and proteolysis. In addition, supplementation of cottonseed oil can be used to produce Cheddar cheese with new textural characteristics.

6 Organic acid, fatty acids profile, and sensory characteristics of Cheddar cheese produced from milk obtained from cows fed diet supplemented with cottonseed oil and condensed tannins

Chapter 6

6.1 INTRODUCTION

Sensory characteristics of cheese play an important role in determining the overall cheese quality and customer acceptance. Any defect in sensory properties is thus objectionable to consumers (Soryal et al., 2005). Cheese sensory characteristics are affected by various factors related to chemical and microbial properties of cheese-milk and cheese-manufacturing technology (Coulon et al., 2004). In addition, biochemical events such as proteolysis, lipolysis and glycolysis, which occur in cheese matrix throughout manufacturing and ripening period, may also affect sensory characteristics of this dairy product (Fox, 1993).

Content of fat and protein in cheese-milk affect the content of fat and protein in the resulting cheese. Besides its function as the source of aromatic and sapid compounds, cheese fat may act as a solvent for products of cheese lipids and other sources (Fox, 1993). Metabolism of fat (lipolysis) and protein (proteolysis) may produce free fatty acids (FFAs) and free amino acids (FAAs), respectively, which could directly contribute to cheese flavours. Free fatty acids and free amino acids may also indirectly affect cheese flavour via production of substrates for synthesis of volatile compounds (Fox et al., 2000). In addition, organic acids, which are formed during hydrolytic lipolysis in cheese fat from normal bacteria growth or from the addition of acidulate during cheese ripening, are also another flavour compounds in aged-cheese (Akalin et al., 2002; Izco et al., 2002). In order to produce cheese with highly desirable texture, aroma and flavour, these enzymatic processes must occur in a coordinated way. However, when these processes are unsynchronized and

unbalanced, cheese with off-flavour characteristic and unwanted texture may be produced (Fox, 1993).

The properties of cheese-milk could be affected by upstream factors such as physiological stages, genetics, and dietary requirements of animals (Coulon et al., 2004). These factors may affect cheese sensory properties through several mechanisms including direct transfer of feed components, modification of fat and protein in milk, transfer of endogenous enzymes, which may affect proteolysis and lipolysis during cheese ripening, and modification of microbial ecosystem (Coulon et al., 2004). Recently, there has been an increase in consumers' concern on the impact of animal diet on cheese sensory properties (Coulon et al., 2004).

Cheese colour is another parameter that may influence consumer preference. According to Voigt et al. (2012), cheese colour depends on colour of fat and the presence of fat-soluble carotenes. Carotene, abundantly found in fresh forage, is a pigment, which contributes to the yellowing colour of dairy products such as cheese and butter (Fox et al., 2000). Thus, composition of animal diet may influence the colour of dairy products by affecting the amount of carotene in milk (Cornu et al., 2001; Houssin et al., 2002).

Effects of cottonseed oil and condensed tannins supplemented diets on milk composition have been discussed in Chapter 3 of this thesis. Diets containing cottonseed oil reduced yield and content of fat in raw milk without affecting protein yield and content. These diets also reduced content of saturated fatty acids (SFAs) and at the same time increased medium chain- and long chain-unsaturated fatty acids

(MUFAs and PUFAs, respectively). Furthermore diet containing condensed tannins had no apparent effect on milk fat content and fatty acid profile. Despite their effects on milk composition, these supplemented diets had minimal impact on coagulation properties of acid- and rennet-induced gels (Chapter 4). These diets also had minimal impact on cheese yield, chemical composition, proteolysis, and texture properties of the resulting Cheddar cheese (Chapter 5). In order to ensure that Cheddar cheese manufactured from milk of cows fed these supplemented diets can be accepted by consumers, the current study aimed to establish the effect of these diets on sensory properties of the cheeses. Effects of the diets on of fatty acid profile and organic acid concentration in cheese, which have been known to affect cheese sensory properties, were also studied. Moreover, effect of these diets on cheese colour was also determined. The hypothesis of this study was that cottonseed oil- and condensed tannin-addition to diets fed to dairy cows may affect colour, organic and fatty acids profile, as well as sensory properties of the Cheddar cheese. Whether these changes would be detrimental for consumers' acceptance was needed to be established.

6.2 MATERIALS AND METHODS

6.2.1 Cheddar cheese manufacture and sampling methods

Cheddar cheeses were manufactured according to the standard procedures of Kosikowski (1977) as described previously in section 5.2.1. All cheeses were packed in oxygen barrier Cryovac bags (Cryovac Pty. Ltd., Fawkner, Vic, Australia) and heat-sealed with a Multivac vacuum packaging equipment (Multivac Sepp Haggenmuller, Wolfertscgwenden, Germany) and ripened under controlled conditions at 4 °C for 24 weeks.

Cheese sampling was done at different stages during ripening period i.e. day 1, week 6, week 12, and week 24 for colour analysis and fatty acids profiling. The sensory analysis was performed after 24 weeks of ripening. On each sampling day, duplicate cheese samples (approximately 100 g of each) were cut out with a sterile knife, finely grated and frozen (-20 °C) prior to further analysis.

6.2.2 Analysis of cheese colour

A block of cheese samples (~300 g) was trimmed 1 cm on each side to eliminate any colour inconsistency due to packaging for colour analysis. The Hunter colour (*L*, *a*, and *b*) of Cheddar cheeses after ripening for 1day and 6, 12 and 24 weeks was measured in triplicate at random places on each cheese using a portable Hunter Minolta Colorimeter CR-300 (Minolta Camera Co. Ltd., Osaka, Japan). The *L* value corresponds to visual lightness; the value increase from 0 to 100. The *a* and *b* values correspond to redness to greenness, and yellowness to blueness, respectively, ranging from positive to negative values, respectively. The colorimeter was calibrated using a white tile as a standard before colour measurement (Drake et al., 2008; Rynne et al., 2008).

6.2.3 Organic acids analysis

Cheddar cheese samples were prepared for organic acid analysis using a modification of the method of Ong et al. (2006). The water soluble fraction was prepared by mixing 5 g of grated cheese samples with 25 mL of 0.009 N sulphuric acid (H_2SO_4) and 70µL of 15.5 N nitric acid. The mixture was homogenised with an Ultratorrax homogenizer (Jonke & Kunkel K.G., Staufen i. Breisgau, Germany) at

10,000 x *g*. Subsequently, the slurry was incubated for 1 h in 50 °C water bath and centrifuged at 4,000 x *g* for 20 minutes at 4 °C. Approximately 1.5 mL of the soluble fraction located between the upper layer (fat) and the precipitate casein was transferred to a tube. The soluble fraction was then further centrifuged at 14,000 x *g* for 10 minutes at room temperature with a bench top centrifuge (Sorvall RT7, Newtown, Conn., USA). Two millilitres of the supernatant was delivered to a 5 mL syringe and filtered through a 0.45 µm filters (Millex, Millipore, Bedford, MT, USA). Approximately 1 mL aliquot from each filtered sample was stored in HPLC vials at -20 °C until required for analysis.

The filtered samples were analysed for organic acid concentration using a high performance liquid chromatography (HPLC) system, which consisted of a Varian 9012 solvent delivery system, a Varian 9100 auto-sampler, a Varian 9050 variable wavelength UV/Vis tuneable absorbance detector and a 730 data module (Varian Inc., Agilent, Mulgrave, Australia). The column used was an Aminex HPX-87H column (300 mm x 7.8 mm, Bio-Rad Laboratories, Richmond, CA, USA) and a guard column with disposable cartridges H+ (Bio-Rad Laboratories) maintained at 65 °C. Solvent (mobile phase) was sulphuric acid (0.009 N), filtered through a 0.45 mm membrane filter (Millipore). The column flow rate was 0.6 mL/min^{-1}. The detection device was the ultraviolet-visible detector set at 220 nm with running time of 15 min (Ong et al., 2006).

Concentration of citric, acetic, and lactic acids in cheeses was determined using series of standard solutions. Lactic, citric, and acetic acids (all Sigma) were used to prapare stock solutions. Sulphuric acid (0.009 N; Sigma) was used to dilute

each stock solution to obtain a range of concentration for each standard. All standards were measured by HPLC under the same operating conditions. The standard curves were constructed by plotting the known concentration versus their peak heights. Concentration of organic acids was determined based on these standard curves.

6.2.4 Cheese fatty acid profile

Fatty acid analysis was performed on cheeses samples taken on day 1, week 6, week 12, and week 24 after manufacture. Fat was extracted from the Cheddar cheese by mixing 10 g of grated cheese with 20 ml milliQ water. The mixture was homogenised using an Ultratorrax homogenizer (Jonke & Kunkel K.G) at 10,000 x *g* for 30 minutes. Then, the mixture was incubated in a water bath at 40 °C for 1 h with occasional shaking. After removal from the water bath, the mixture was centrifuge at 4 °C at 4,000 x *g* for 30 min. The fat layer was removed and placed in a microcentrifuge tube, which then was centrifuge again at 14,000 x *g* for 30 min at room temperature. The fat was removed and stored at -20 °C prior to preparation of fatty acid methyl ester (FAME) for analysis with gas chromatograph (GC) (ISO 14156/IDF 176).

Fatty acid methyl esters of milk fat samples were prepared by weighting 100 mg of test sample in a screw cap test tube. Hexane (5 mL; Sigma-Aldrich Pty. Ltd, Castle Hill, NSW, Australia) was added into the tube to dissolve the milk fat by vigorous shaking. After the milk fat has been dissolved, 0.2 mL of trans esterification reagent (KOH, 2 mol/L) was added. The content of the tube was mixed vigorously by vortexing for 1 min. After an additional reaction time of 5 min, solid

sodium hydrogen sulphate (0.5 g) was added and content of the tube was mixed again. After that, the test tube containing the test portion was centrifuge at 350 x *g* for 3 min at room temperature. After centrifuging, aliquot of the clear supernatant was taken and filtered through a 0.45 µm filters (Millex, Millipore, Bedford, MT, USA) into a vial for the GC analysis. A 50 µL volume of 1000 ppm internal standard (C13:0) was also added to the GC vial.

The instrument used to analyse free fatty acid of cheese samples was a Varian 3440 gas chromatograph equipped with a flame-ionisation detector (Varian Inc., Agilent, Mulgrave, Australia). Fatty acid methyl esters from all samples were separated using a BPX70 column (120 m x 0.25 mm, SGE Analytical Science Pty Ltd, Ringwood, Victoria, Australia). The initial oven temperature was held at 70 °C for 1 min, then increased at a rate of 5 °C/min to 100 °C, held for 2 min, increased at 10 °C/min to 175 °C, held for 40 min, and then finally increased at 5 °C to a final temperature of 225 °C and held for 45 min. The injector and detector temperature were kept at 255 °C and 250 °C, respectively, and the injector part was equipped with a 1:30 split ratio. The flow rate of helium as carrier gas was adjusted to a head pressure of 158.6 kPa and a flow rate of 0.7 mL/min). Final fatty acid concentration was derived on fat basis of cow milk cheese (mg/g of fat).

6.2.5 Descriptive sensory analysis of Cheddar cheese

The 45 untrained panellists for sensory evaluation of Cheddar cheeses consisted of staff and students of the Victoria University aged between 18 and 60 years who have consumed cheese within the past two years. Prior to the sensory evaluation, all panellists signed a Victoria University human subject's consent form.

Definitions of sensory attributes i.e. appearance, aroma, flavour, firmness and overall acceptability were explained to the panellists before sensory evaluation.

In order to achieve optimum conditions for sensory evaluation and to obtain homogenous cuts, Cheddar cheese samples for sensory analysis were allowed to equilibrate at room temperature (22 ˚C) for 1 hour prior to the evaluation. Cheese samples were prepared by slicing into 2 x 2 x 2 cm cubes using a knife and placed into lidded 50 mL plastic containers at room temperature with a labelled 3-digit random number that was arranged in a different order for each participant. Each panellist tasted all four samples in the randomized order and evaluated it for appearance, aroma, flavour, firmness and overall acceptability on a 9-point hedonic scale that is anchored from the top with like extremely to the bottom with dislike extremely. Sensory evaluations were conducted in an enclosed room free from external aromas, noise and distraction. The responses from the sensory analysis were converted to numerical values between 1 and 9 for statistical analysis. Crackers were available to aid in the removal of any carryover taste between each sample. In addition, water was also provided for panellists to rinse their pallet between each sample (Donkor et al., 2007).

6.2.6 Statistical analysis

A randomised, blocked, split plot in time design employing a corresponding general linear model (GLM) was used to study the difference between dietary treatments on organic acids, fatty acid concentration, and consumer liking of cheese samples at any ripening times. The diet supplementation was the main plot with the ripening time as the subplot. The repetitions served as blocks. Significant difference

between mean of samples were determined using pdiff command, denoting a least significant difference (lsd test). The level of significance was preset at $P < 0.05$. Results obtained in this experiment were analysed using Minitab 16. All results were expressed as mean ± SD.

6.3 RESULTS AND DISCUSSION

6.3.1 Cheese colour

Colour is an important attribute of food quality since it is considered by consumers to be related to freshness, ripeness, desirability, and food safety of food products (Jelinski et al., 2007). Measurement of cheese colour can be achieved using colorimeter due to its higher sensitivity and reproducibility compared to human eye. In addition, results obtained from colorimeter measurement correlate well with human perception (Mahaut and Korolezuk, 2002).

In this study, in general, there was no significant ($P > 0.05$) difference in terms of Hunter *L*, *a*, and *b* values between control and CSO, TAN, and CPT cheeses at any ripening time (Table 6.1). The Hunter *L* value indicates the lightness (Mahaut and Korolezuk, 2002). The comparable Hunter *L* values of CSO, TAN and CPT cheeses to that of CON cheese indicated that supplemented cottonseed oil, condensed tannin or their combination did not affect whiteness or lightness of the cheeses. According to Medeiros et al. (2010), lightness is influenced by size of milk fat globules with smaller fat globule size causing a higher lightness. Due to its effect in reducing *de novo* fat synthesis, supplementation of unsaturated lipid in a cow diet produced milk with smaller milk fat globule size (Wiking et al., 2003), which thus

might increase the lightness. Increase of cheese lightness in butter with high polyunsaturated FA has been reported previously (Gonzales et al., 2003). However, results from the present study showed that CSO and CPT cheeses, which were made from milk of cows fed diet containing cottonseed oil, have comparable lightness with control and TAN cheeses (Table 6.1). The discrepancy between our results with report in the literature could be attributed to the effect of microfluidization applied on cheese milk as has been explained in Chapter 4. Microfluidization employs a dual effect termed collision impact shearing, which is very effective in reducing milk fat globule. Since this piece of equipment was used in preparation of the cheeses, it would be a fair assumption that the treatment has a similar effect, which was consequently reflected in the cheese lightness. In addition, the amount of fat in cheese also influences the lightness of cheese through the effect of fat in scattering light (Rudan et al., 1999). Cheese with a lower fat content has been reported to have a lower lightness (Kahyaoglu et al., 2005). Thus, the similar fat content among cheeses in the current study, might have contributed to comparable lightness of the cheeses.

Table 6.1 Colour attributes of Cheddar cheeses made from cow fed diet supplemented with cottonseed oil, condensed tannins and in combination

Colour attributes[2]	Dietary treatments[1]			
	CON	CSO	TAN	CPT
L				
Day 1	82.86 ± 1.30^{aA}	83.30 ± 1.12^{aA}	82.58 ± 3.47^{aA}	82.37 ± 2.84^{aA}
Week 6	82.15 ± 3.94^{aA}	82.90 ± 2.75^{aA}	81.63 ± 3.15^{aA}	82.06 ± 2.46^{aAB}
Week 12	80.05±3.99^{aAB}	80.45±2.54^{aAB}	79.98 ± 1.63^{aB}	80.70 ± 1.83^{aAB}
Week 24	79.15 ± 1.27^{aB}	79.91 ± 2.45^{aB}	79.56 ± 2.22^{aB}	79.20 ± 2.02^{aB}
a				
Day 1	-4.12 ± 0.32^{aB}	-4.26 ± 0.43^{aA}	-4.44 ± 0.51^{aB}	-3.92 ± 0.47^{aA}
Week 6	-3.97 ± 0.36^{aAB}	-4.08 ± 0.62^{aA}	-4.32 ± 0.46^{aB}	-4.49 ± 0.65^{aA}
Week 12	-4.46 ± 0.51^{bB}	-4.07 ± 0.48^{aA}	-3.59 ± 0.37^{abA}	-3.80 ± 0.50^{aA}
Week 24	-3.67 ± 0.51^{aA}	-3.91 ± 0.66^{aA}	-3.66 ± 0.36^{aA}	-3.94 ± 0.47^{aA}
b				
Day 1	16.45 ±0.53^{aBC}	16.36 ± 0.81^{aB}	16.60 ± 1.17^{aB}	16.25 ± 0.79^{aB}
Week 6	15.75 ± 0.80^{aC}	17.16 ±1.24^{aAB}	16.20 ± 0.54^{aB}	16.59 ± 1.28^{aB}
Week 12	17.07 ±1.26^{aAB}	17.10 ±0.99^{aAB}	17.24 ± 0.77^{aAB}	17.64 ± 1.11^{aAB}
Week 24	18.22 ± 1.11^{aA}	18.86 ± 0.96^{aA}	18.60 ± 1.11^{aA}	18.24 ± 1.01^{aA}

^{a,b}Means in the same row within a category with different superscripts differ significantly ($P < 0.05$).
^{A,B}Means in the same column within a category with different superscripts differ significantly ($P < 0.05$).
[1]Dietary treatments: CON = control; CSO = cottonseed oil; TAN = tannin; CPT = cottonseed oil plus tannin.
[2]Colour attributes: *L* = lightness, ranging from 0 to 100; *a* = redness to greenness, positive to negative values, respectively; *b* = yellowness to blueness, ranging from positive to negative values, respectively.

Cheese colour is also related to the initial milk composition and final composition of cheese. According to Carpino et al. (2004) compounds from pasture plants are frequently transferred to cheese and this may also include flavour and colour agents. Hunter *b* value, which indicates the yellowness of cheese, is affected

by the amount of carotene in animal diet (Fox et al., 2000). Cottonseed oil has been reported to contain a low amount of carotenoid (less than 100 ppm) (Jain et al., 1990), which apparently had no obvious effect ($P > 0.05$) on yellowness of the cheeses observed in the current study.

In all cheeses, the *L* and *a* values tended to decrease ($P < 0.05$), while *b* value increased throughout ripening time (Table 6.1). These results indicated that during ripening, the cheeses decreased in whiteness as indicated by decline in *L* value, but increased in greenness and yellowness as indicated by negative *a* value and positive *b* value, respectively. According to Sheehan et al. (2005), decrease in cheese lightness during ripening might be associated with the increase of the casein hydration, which reduces a number of free moisture droplets. As consequence, degree of light scattering decreases. Similar decrease in cheese lightness during ripening has been reported for Cheddar cheese (Voigt et al., 2012) and other cheese types such as Iranian white cheese (Khosrowshahi et al., 2006).

6.3.2 Profile of organic acids in Cheddar cheese

Organoleptic properties of cheeses, such as flavour and aroma, may determine acceptability of the cheeses by consumers. Cheese flavour is governed by a balance between volatile and non-volatile substances such as organic acids, sulphur compounds, free-amino acids, lactones, methyl ketones, alcohols, and phenolic substances (Urbach, 1993). Organic acids, such as lactic, citric, acetic, formic, pyruvic acids, are main products of carbohydrate metabolism by lactic acid bacteria (LAB) (Upreti et al., 2006). Determination of organic acids concentration in cheese

is important because besides their roles in governing cheese flavour (Califano and Bevilacqua, 2000), these compounds may reflect the activity of starter LAB, become the intermediates for many biochemical processes, and can be used to categorise different type of cheeses (Bevilacqua and Califano, 1992).

Results from the present study show that, overall, there was no observable ($P > 0.05$) difference in the lactic acid concentration among all cheeses at any ripening time point (Table 6.2). As shown in the previous chapter (section 5.3.1, Table 5.1), all cheeses had comparable S/M values, therefore likely affecting the activity of starter cultures in a similar fashion resulting in similar lactic acid concentration. Moreover, the similar pH of all cheeses at day 1 after ripening (Table 5.1) indicated the similar rate and extent of acidification, which subsequently might have led to a similar chymosin retention, and thus, contributed to a comparable lactic acid concentration.

In all cheeses, lactic acids were present at higher concentration in comparison to that of citric and acetic acids (Table 6.2). This result is rather expected since the main role of starter culture is to metabolise lactose to lactic acid at high rate in the early stages of ripening and thus decrease pH (Califano and Bevilacqua, 2000). The higher concentration of lactic acid compared to other organic acids in Cheddar (Buffa et al., 2004; Ong et al., 2006) and Gouda (Califano and Bevilacqua, 2000) cheeses have been reported and clearly related to the starter culture characteristics.

Production of lactic acid in cheeses throughout ripening time is depicted in Table 6.2. Overall, in all cheeses, lactic acid concentration increased rapidly during

first six weeks of ripening. Afterwards, the concentration tended to stable until the end of ripening period, when there was no significant ($P > 0.05$) difference in terms of lactic acid concentration among the cheeses. The increase of lactic acid concentration during the first six week of ripening was concomitant with reduced in cheese pH at the early ripening time (Chapter 5). It was likely that the activity of starter LAB and residual lactose were still sufficient enough to support high production of lactic acid during this early ripening time.

Table 6.2 Concentration of lactic, citric, and acetic acids in Cheddar cheese made from milk of cows fed diet supplemented with cottonseed oil, condensed tannin, and in combination during 6 month of ripening period at 4 ˚C

Organic acids (%)	Dietary treatments[1]			
	CON	CSO	TAN	CPT
Lactic acid				
Day 1	1.25±0.04^{aC}	1.28±0.03^{aC}	1.23±0.06^{aC}	1.27±0.09^{aB}
Week 6	1.42±0.06^{aB}	1.46±0.04^{aB}	1.31±0.05^{bB}	1.39±0.06^{aA}
Week 12	1.44±0.05^{aB}	1.45±0.03^{aB}	1.35±0.07^{aB}	1.44±0.05^{aA}
Week 24	1.50±0.03^{aA}	1.52±0.04^{aA}	1.40±0.04^{aA}	1.48±0.06^{aA}
Citric acid				
Day 1	0.20±0.01^{aB}	0.20±0.01^{aBC}	0.20±0.02^{AB}	0.19±0.01^{aB}
Week 6	0.22±0.03^{aA}	0.22±0.02^{aA}	0.20±0.02^{AB}	0.22±0.02^{aA}
Week 12	0.18±0.01^{aB}	0.17±0.22^{aC}	0.19±0.01^{aB}	0.18±0.01^{aB}
Week 24	0.23±0.01^{aA}	0.21±0.02^{aAB}	0.22±0.01^{aA}	0.22±0.02^{aA}
Acetic acid				
Day 1	0.03±0.004^{aC}	0.04±0.004^{aC}	0.03±0.004^{aD}	0.03±0.006^{aD}
Week 6	0.05±0.006^{aB}	0.05±0.004^{aC}	0.04±0.006^{aC}	0.04±0.005^{aC}
Week 12	0.05±0.006^{aB}	0.06±0.005^{aB}	0.05±0.006^{aB}	0.05±0.004^{aB}
Week 24	0.06±0.008^{aA}	0.07±0.007^{aA}	0.07±0.007^{aA}	0.07±0.006^{aA}

^{a,b}Means in the same row within a category with different superscripts differ ($P < 0.05$).
^{A,B}Means in the same column within a category with different superscripts differ ($P < 0.05$).
[1]Dietary treatments: CON = control; CSO = cottonseed oil; TAN = tannin; CPT = cottonseed oil plus tannin.

Cheese manufacturing involves acidification and concentration of milk (Kosikowski, 1977). These processes may not support the growth and activity of starter culture, especially *L. lactis* spp. *cremoris*, which is more salt tolerant than *L. lactis* spp. *lactis* (Turner and Thomas, 1980). As a result, after 6 weeks of ripening, it was likely that the activity of starter LAB in metabolising lactose decreased and

NSLAB replaced the role of LAB in metabolising lactose (Turner and Thomas, 1980). In addition, residual lactose in cheese might be depleted after 6 weeks. Thus, these might cause a stable concentration of lactic acid during the prolonged ripening (Table 6.2). Stabilisation of lactic acid concentration due to depleted lactose content during ripening has been reported in Serra cheese (Macedo and Malcata, 1997). Similar trend in lactic acid production during ripening has been reported in Cheddar cheese-liked products (St-Gelais et al., 1991). Production of lactic acid is important for cheese production because it may affect desirable quality of the final product due to its effect on flavour, proper ripening, and optimal manufacture of the cheese (McSweeney and Sousa, 2000).

Table 6.2 indicates no apparent ($P > 0.05$) differences in citric acid concentration among cheeses observed at each sampling time. During ripening period, the concentrations of citric acid were inconsistent in all cheeses. In general, concentration of citric acid increased ($P < 0.05$) from 1-day to 6-week of ripening, then decreased ($P < 0.05$) from 6-week to 12-week. After this point, concentration of citric acid increased slightly ($P < 0.05$) until the end of ripening time (24-weeks). The inconsistence concentration of citric acid might be related to its biochemical metabolism. Reduction in concentration of citric acid may be because it may serve as substrate in the Krebs or citric acid cycle for production of pyruvic acid, carbon dioxide, and acetic acid by starter bacteria (Akalin et al., 2002; Ong and Shah, 2008). In addition, plasmid localisation of genes that encodes citrate permease may contribute to the unstable ability of LAB in metabolising citrate (Hugenholtz, 1993).

The increase of citric acid concentration can be linked to the ability of starter LAB to fix carbon dioxide using pyruvate carboxylase, and thus convert pyruvate to oxaloacetate. Oxaloacetate then can be converted to citrate by citrate synthase or to isocitrate by aconitase (Upreti et al., 2006). The inconsistence changes of citric acid concentration in Cheddar cheese (Phadungath, 2011), Pickled White cheese (Akalin et al., 2002), plain soft and Monterey Jack goat cheeses (Park et al., 2005) have been reported. In the current study, citric acid was the second most abundant organic acid after lactic acid (Table 6.2). This result is in line with the result of previous study on Cheddar cheese (Izco et al., 2002).

Acetic acid is an important cheese flavour compound, even though its presence in high concentration may cause off-flavour in cheese (Aston and Dulley, 1982). Table 6.2 shows that concentration of acetic acid increased from 1-day to 24-week of ripening time. At the end of ripening, acetic acid concentration was higher than that of day 1. All cheese samples showed similar pattern. The gradual increase of acetic acid throughout ripening time might be because this organic acid can be produced either from citrate by heterofermentative LAB (Califano and Bevilacqua, 2000) or from amino acids by lactobacilli (Lues and Bekker, 2002; Buffa et al., 2004). In addition, when oxygen (O_2) is present at a high concentration, lactate can be oxidised by NSLAB, mainly pediococci, into acetate. Per mol lactate utilised, this bacterium uses 1 mol of O_2 producing 1 mol of acetate and 1 mol of CO_2 (Thomas et al., 1985). This reaction depends on lactate concentration, O_2 availability, and NSLAB population (McSweeney and Fox, 2004). During cheese ripening, unfavourable conditions for LAB to grow may shift utilisation of lactate from LAB

to NSLAB, which support oxidation of lactate by NSLAB to produce acetate (Califano and Bevilacqua, 2000). Due to the low level of O_2 in plastic wrapped-cheese, generally, lactate oxidation in this cheese is very limited (McSweeney and Fox, 2004).

Similar trend in increase of acetic acid concentration in Cheddar cheese during the first 6 month of ripening has been reported (Ong and Shah, 2008; Phadungath, 2011). Furthermore, Phadungath (2011) reported that the increase in acetic acid concentration is followed by a sharp decrease from 6 to 12 months of ripening which indicated the role of acetic acid as intermediate in biochemical pathway. No significant ($P > 0.05$) differences were observed between all cheeses in terms of their acetic acid concentration in each sampling time (Table 6.2). According to Phadungath (2011), the amount of acetic acid in Cheddar cheese was influenced by the amount of added salt during cheese manufacturing, which subsequently would affect the level of S/M in cheese and the activity of starter bacteria in producing acetic acid. Therefore, the similar acetic acid concentration among cheeses in the present study could be related to the similar amount of added NaCl, i.e. 2% of curd weight, during cheese manufacture (Chapter 5).

A lack of effect of cottonseed oil and condensed tannins supplementations on organic acid concentration in Cheddar cheese also indicated that these supplements did not affect the activity of LAB in the cheeses, which are responsible for metabolism of these organic acids. Results of the current study are in agreement with the negligible effect of dietary rapeseed on organic acid concentrations in hard cheese manufactured from bovine milk (Jaros et al., 2001). Production of organic

acids during cheese ripening is important, since lactic acid produced by starter culture reduced pH of cheese during manufacturing and preserves the cheese from bacteria and moulds. In addition, along with small peptides, free fatty acids, and amino acids, organic acids contribute to development of cheese flavours and aromas (Fox et al., 1990).

6.3.3 Cheese fatty acid composition

Overall, FA profile in the cheeses (Table 6.3 and Figure 6.1) reflected FA profile in the respective raw milk samples before standardisation, which indicated that manufacturing process did not alter FA composition of the cheeses (section 3.3; Table 3.4). This result confirmed those of other studies, which showed that cheese manufacturing process did not alter fatty acid profile (Jones et al., 2005; Mele et al., 2011; Caroprese et al., 2013).

Cheddar cheeses manufactured from milk of cows fed cottonseed oil supplemented diet or combination with condensed tannin, i.e. CSO and CPT cheeses, respectively, had an elevated ($P < 0.05$) concentration of monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), compared to that of control. Increase in concentration of these FAs was accompanied by reduction in concentration of short-chain saturated fatty acids (SCFA) such as C4:0, C6:0, C10:0, and C16:0 ($P < 0.05$; Table 6.3). Fatty acid composition of CSO and CPT cheeses can be linked to the effect of cottonseed oil supplementation on fatty acid profile of the raw milk (Chapter 3). Supplementation of cottonseed oil in cow diet reduced concentration of fat in raw milk and changed milk FA profile towards the increase in concentration of long chain- and unsaturated FAs but decrease in SFA concentration

(section 3.3.1; Table 3.4). These changes were proposed due to the increase supply of linoleic acid from diet and alteration in rumen biohydrogenation, which produced intermediate FAs that had the capacity to inhibit *de novo* synthesis FAs (Bauman and Griinari, 2001).

Increase in concentration of PUFAs and reduction in concentrations of SFAs, especially C14:0 and C16:0 FAs, in CSO and CPT cheeses would bring health benefits to humans by decreasing the risk of atherosclerosis and coronary thrombosis (Caroprese et al., 2013). It is known that PUFAs have positive effects on preventing atherosclerosis, cardiovascular-related diseases and cancer (Calvo et al., 2007). Meanwhile, C16:0 FA is related to an increase of low-density lipoprotein (LDL) cholesterol in plasma (Denke and Grundy, 1992).

Besides its nutritional effect, fatty acid profile in cheese may also influence other aspects of the cheeses such as flavour (Urbach, 1990), lipolysis (Wendoff, 2001), colour, and texture (Avramis et al., 2003; Coppa et al., 2011). Short chain (C4:0-C12:0) and medium chain (C14:0-C16:1) FAs, play important roles in cheese flavour since they have a low perception threshold. In contrast, long chain FAs (> C18:0) have a high perception threshold, thus have less important roles (Molimard and Spinnler, 1996). In addition, milk fats are subjected to lipolysis by lipoprotein lypase (LPL), enzyme that responsible for lipolysis in raw milk. Lipolysis of milk fatty acid by LPL enzyme produces free fatty acids (FFAs) during ripening, which then could be catabolised to produced flavour compounds (Wendoff, 2001).

Lipolysis depends on the sensitivity of milk fat globules (MFG) towards LPL enzyme (Wiking et al., 2003). The sensitivity of MFG towards LPL activity is influenced by several factors such as MFG size, fat content, the amount of fatty acid attached at position sn-1 and 3 of triglyceride, temperature, and unsaturated feed (Wiking et al., 2003). Changes in milk fat concentration may affect the size of MFG (Wiking et al., 2003). Increase of MFG diameter with higher concentration of fat in milk is attributed to the limited production of MFG membrane material when milk fat synthesis increases (Wiking et al., 2003). Compared to milk with a low concentration of fat, milk with a high fat concentration contains MFG with a large diameter size. Subsequently, the size of MFG may affect stability of MFG towards LPL enzyme, which responsible for lipolysis. The bigger MFG tend to be more susceptible to lipolysis, because they have a lower surface potential, and thus presumably increase the amount of LPL attacking the MFG (Wiking et al., 2003).

In the current study, degree of lipolysis, as indicated by the differences in concentration of milk FFAs among the cheeses, was not determined. However, based on fat concentration and the amount of fatty acids which are attached at position sn-1 and 3 of triglycerides, it was likely that CSO and CPT milk, which had lower fat content, might have smaller MFG size. As consequence, this milk might have higher stability towards LPL enzyme; and thus could have lower degree of lipolysis and FFAs compared to that of control. Condensed tannin supplementation, which unaltered milk fat content (Chapter 3) might cause comparable size of MFG, stability of MFG towards LPL enzyme, degree of lipolysis, and FFA content to those of the control milk. Degree of lipolysis can also be affected by the amount of fatty acids

which are attached at position sn-1 and 3 of triglycerides, such as C4:0, C6:0, and C18:1 as the LPL enzyme is very reactive towards FAs at this position (Deeth and Touch, 2000). Our results showed that CSO and CPT cheeses had lower concentration ($P < 0.05$) of C4:0 and C6:0 FAs but were slightly higher in C18:1 concentration (Table 6.4). Therefore, it was likely that, compared to CON cheese, CSO and CPT cheeses had lower extent of lipolysis.

Degree of lipolysis also can be affected by microfluidization technique. This technique may reduce the fat globule size and thus reduce the ability of the native milk fat globule membrane (MFGM) to protect fat globule from lipolysis (Fox, 2000). The cheese-milk sample used for Cheddar cheese manufacturing used in this study was homogenized through microfluidization (Chapter 4). Therefore, it was likely that all cheese-milk samples in the current study had a similar size of MFG, and thus caused similar degree of lipolysis in the cheeses. A further study is required establish the effect of the supplemented diets on degree of lipolysis in these cheeses. It is also interesting to examine the extent of lipolysis and fatty acid concentration in cheese made using cheese-milk that composes of native fat globule.

Fatty acid profile of milk and dairy products may also influence the risk of lipid oxidation. Milk contain high amount of polyunsaturated fatty acids (PUFAs) is prone to spontaneous oxidation during storage (Fox et al., 2000). Oxidation of pentadienyl groups in PUFAs led to the formation of primary product of oxidation i.e. hydroperoxides which are very unstable. Oxidation also produces secondary oxidation products such as saturated and unsaturated aldehydes, ketones, hydrocarbons, semi aldehydes and alcohol (Fox and McSweeney, 1998). The

presence of these compounds may cause oxidative rancidity and cause a flavour defect, which is described as metallic, cardboard, tallow, or fishy in dairy products (Timmons et al., 2001). Therefore, increase in concentration of PUFA in CSO and CPT cheeses and milk, could possibly increase the risk of lipid oxidation. In the current study, oxidation in the cheeses was not measured; however, the effect of CSO and CPT diets on cheese flavour will be discussed in more detail later in this chapter.

Table 6.3 shows that, there were no significant ($P > 0.05$) differences in the concentration of short-chain FAs, medium chain FAs, long chain FAs, and individual FAs between TAN cheese and that of control (CON) cheese. The comparable fatty acid composition between CON and TAN cheeses was most likely due to the absence of any effect of condensed tannin supplementation on the fatty acid composition of the milk used to manufacture the TAN cheese as has been reported in section 3.4.2. Inclusion of condensed tannin has no effect on milk fatty acid profile, thus increase in concentration of PUFA and reduction in SFA concentration in CPT cheese were more likely due to the increasing effect of dietary cottonseed oil on the amount of PUFA in milk fat.

Overall, throughout ripening time, the concentration of short-, medium-, and long-chains FAs tend to remain stable ($P > 0.05$) (Table 6.3). Dodecalactone can be produced from long chain unsaturated FA through the activity of *P. roquoforti*, and thus causes rancidity in cheese (El-Hofi et al., 2011). In the current study, concentration of long-chain unsaturated FAs remained stable, which indicate that the long chain unsaturated FAs did not serve as a precursor for dodecalactone formation.

Thus, this might cause the absence of rancid flavour in the cheeses. This study also showed that palmitic (C16:0) and oleic acid (C18:1) were the major components of FAs in all cheeses (Table 6.3, Figure 6.1). The higher concentration of palmitic and oleic acids compared to other fatty acids in all Cheddar cheeses in the current study was also found in other types of hard cheeses such as Greek hard cheese (Kondyli and Katsiari, 2001) and Kefalograviera cheese (Lalos et al., 2001).

Table 6.3 Fatty acid concentration in the control (CON) and cottonseed oil-supplemented (CSO), condensed tannin-supplemented (TAN) and cottonseed oil-condensed tannin supplemented (CPT) Cheddar cheeses at various time points during ripening time.

Time	Fatty acids										
	C4:0	**C6:0**	**C8:0**	**C10:0**	**C12:0**	**C14:0**	**C16:0**	**C17:0**	**C18:0**	**C18:1**	**C18:2**
CON											
Day 1	4.39±0.3^{aA}	2.51±0.2^{aA}	1.32±0.1^{aA}	3.02±0.3^{aA}	3.50±0.3^{aA}	12.06±0.9^{aA}	33.64±1.4^{aA}	0.94±0.1^{aA}	8.89±0.6^{bA}	18.32±1.0^{bA}	1.82±0.2^{bA}
6 week	4.21±0.4^{aA}	2.28±0.2^{aA}	1.39±0.1^{aA}	3.22±0.2^{aA}	3.43±0.3^{aA}	12.77±0.8^{aA}	34.54±2.3^{aA}	0.88±0.1^{aA}	8.1±0.6^{bA}	18.79±1.0^{bA}	1.96±0.2^{bA}
12 week	4.24±0.4^{aA}	2.29±0.2^{aA}	1.33±0.1^{aA}	3.22±0.3^{aA}	3.23±0.3^{aA}	12.96±0.9^{aA}	34.29±1.9^{aA}	0.93±0.1^{aA}	8.75±0.6^{bA}	17.24±1.1^{bA}	1.96±0.2^{bA}
24 week	4.48±0.4^{aA}	2.48±0.2^{aA}	1.41±0.1^{aA}	3.15±0.2^{aA}	3.65±0.3^{aA}	13.25±0.8^{aA}	34.72±2.4^{aA}	0.91±0.1^{aA}	9.42±0.9^{bA}	17.88±0.1^{bA}	1.91±0.2^{bA}
CSO	**C4:0**	**C6:0**	**C8:0**	**C10:0**	**C12:0**	**C14:0**	**C16:0**	**C17:0**	**C18:0**	**C18:1**	**C18:2**
Day 1	3.98±0.3^{aA}	2.05±0.2^{bA}	1.19±0.1^{bA}	2.08±0.2A	2.56±0.2^{bA}	10.05±0.9^{bA}	27.43±1.0^{bA}	0.69±0.1^{bA}	10.36±0.8^{aA}	20.55±0.1^{aA}	2.89±0.3^{aA}
6 week	3.69±0.3^{aA}	2.01±0.1^{bA}	1.16±0.1^{bA}	2.84±0.2A	2.75±0.3^{bA}	10.57±0.8^{bA}	29.96±1.8^{bA}	0.73±0.1^{bA}	10.14±0.7^{aA}	20.81±0.1^{aA}	2.83±0.2^{aA}
12 week	3.59±0.4^{aA}	1.94±0.2^{bA}	1.16±0.1^{bA}	2.81±0.2A	2.26±0.2^{bA}	10.76±0.9^{bA}	30.63±1.6^{bA}	0.70±0.1^{bA}	10.45±0.6^{aA}	20.37±1.5^{aA}	2.88±0.2^{aA}
24 week	4.06±0.4^{aA}	2.24±0.2^{bA}	1.25±0.1^{bA}	2.11±0.3A	2.57±0.2^{bA}	11.23±0.6^{bA}	29.63±2.6^{bA}	0.67±0.1^{bA}	10.51±1.1^{aA}	20.45±1.2^{aA}	2.92±0.2^{aA}
TAN	**C4:0**	**C6:0**	**C8:0**	**C10:0**	**C12:0**	**C14:0**	**C16:0**	**C17:0**	**C18:0**	**C18:1**	**C18:2**
Day 1	4.29±0.3^{aA}	2.55±0.2^{aA}	1.28±0.1^{aA}	3.10±0.2^{aA}	3.46±0.3^{aA}	11.76±0.8^{aA}	33.74±1.7^{aA}	0.92±0.1^{aA}	9.25±0.8^{bA}	17.72±0.1^{bA}	1.83±0.2^{bA}
6 week	4.11±0.4^{aA}	2.34±0.2^{aA}	1.32±0.1^{aA}	3.25±0.3^{aA}	3.46±0.3^{aA}	12.47±1.0^{aA}	35.64±1.4^{aA}	0.84±0.1^{aA}	8.49±0.9^{bA}	17.19±0.1^{bA}	1.87±0.2^{bA}
12 week	4.14±0.4^{aA}	2.31±0.2^{aA}	1.35±0.1^{aA}	3.14±0.3^{aA}	3.62±0.3^{aA}	12.66±1.2^{aA}	33.69±1.7^{aA}	0.93±0.1^{aA}	9.12±0.5^{bA}	17.61±0.9^{bA}	1.96±0.2^{bA}
24 week	4.34±0.4^{aA}	2.48±0.2^{aA}	1.38±0.1^{aA}	3.06±0.3^{aA}	3.55±0.3^{aA}	13.61±0.9^{aA}	33.82±2.8^{aA}	0.89±0.1^{aA}	9.72±0.7^{bA}	17.26±0.1^{bA}	1.83±0.1^{bA}
CPT	**C4:0**	**C6:0**	**C8:0**	**C10:0**	**C12:0**	**C14:0**	**C16:0**	**C17:0**	**C18:0**	**C18:1**	**C18:2**
Day 1	4.39±0.3^{aA}	2.11±0.2^{bA}	1.16±0.1^{bA}	2.10±0.2^{bA}	2.64±0.2^{bA}	10.20±0.7^{bA}	27.79±1.2^{bA}	0.70±0.1^{bA}	10.56±0.7^{aA}	20.78±1.0^{aA}	3.01±0.3^{aA}
6 week	3.65±0.3^{aA}	2.01±0.1^{bA}	1.10±0.1^{bA}	2.84±0.3^{bA}	2.82±0.2^{bA}	10.43±0.9^{bA}	30.32±2.0^{bA}	0.76±0.1^{bA}	10.34±0.1^{aA}	20.62±1.2^{aA}	2.94±0.2^{aA}
12 week	3.79±0.3^{aA}	1.91±0.2^{bA}	1.16±0.1^{bA}	2.92±0.3^{bA}	2.36±0.2^{bA}	11.02±0.9^{bA}	28.99±2.5^{bA}	0.75±0.1^{bA}	10.65±0.7^{aA}	20.46±1.7^{aA}	3.10±0.2^{aA}
24 week	4.32±0.4^{aA}	2.27±0.2^{bA}	1.28±0.1^{bA}	2.18±0.3^{bA}	2.63±0.2^{bA}	11.05±0.8^{bA}	29.05±2.7^{bA}	0.72±0.1^{bA}	10.86±1.1^{aA}	20.37±1.0^{aA}	3.12±0.2^{aA}

^{a,b}Means in the same row within a category with different superscripts differ ($P < 0.05$).
^{A,B}Means in the same column within a category with different superscripts differ ($P < 0.05$).
[1]Dietary treatments: CON = control; CSO = cottonseed oil; TAN = tannin; CPT = cottonseed oil plus tannin. SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

6.3.4 Sensory properties of Cheddar cheese

The aim of any food processor is to produce a product with a high quality with acceptable and preferable sensory attributes such as aroma, colour, flavour, texture, and taste. The new product undergoes several sensory evaluations to ensure it can be accepted by the consumers (Meilgaard et al., 2007). Table 6.4 shows consumer panel scores of Cheddar cheese made from milk of cows fed a diet supplemented with cottonseed oil (CSO), condensed tannin (TAN), or in combination (CPT). Overall, appearance of all cheeses was rated as like moderately, and no difference among the cheeses in terms of their appearance (Table 6.4). Appearance of cheese might be influenced by fat contents in the cheese (Sameen et al., 2008). Cheese with a higher fat content tends to have a better appearance compared to cheese with a low fat content. The appearances of a low fat cheese usually poor, rough and lacking lust (Sameen et al., 2008). Therefore, in the current study, the comparable scores of appearance amongst the cheeses might be partly due their similar content of fat in the cheeses as has been reported in section 5.3.1.

Fatty acid composition of dairy products may influence texture of the products by affecting the amount of solid- or liquid fatty acids (Baer et al., 1996; MacGibbon and Taylor, 2006). Saturated FAs have high melting point and are in solid state at room temperature; in contrast, unsaturated FAs, which have low melting point, are related with the liquid FAs (MacGibbon and Taylor, 2006). Basically, structure of dairy products is determined by lipid–lipid and protein–lipid interactions. Therefore, when the amount of solid FAs in the products is insufficient due to the high amount of unsaturated FAs, these products can lose their structure at

high temperature (MacGibbon and Taylor, 2006). Furthermore, FA composition may affect texture of dairy products by influencing the size of milk fat globule. Unsaturated FAs are related with the smaller size of fat globule, while saturated FA related with the larger fat globule size (Wiking et al., 2004). Therefore, the higher amount of unsaturated FA may cause the larger fat globule size, which has attenuated interactions with each other and with proteins (MacGibbon and Taylor, 2006).

Table 6.4 Mean consumer liking of Cheddar cheese made from milk of cow fed diet containing cottonseed oil (CSO), condensed tannin (TAN), or in combination (CPT) after 6 month incubation at 4 ˚C

Sensory Characteristics[2]	**Dietary treatments**[1]			
	CON	**CSO**	**TAN**	**CPT**
Aroma	6.9 ± 0.7[a]	7.1 ± 0.5[a]	6.8 ± 0.5[a]	6.8 ± 0.5[a]
Appearance	7.4 ± 0.6[a]	7.4 ± 0.3[a]	7.7 ± 0.5[a]	7.6 ± 0.6[a]
Firmness	6.9 ± 0.8[a]	7.1 ± 0.7[a]	7.1 ± 0.7[a]	6.9 ± 0.4[a]
Flavor/Taste	6.7 ± 0.7[a]	6.5 ± 0.6[a]	6.3 ± 0.4[a]	6.6 ± 0.4[a]
Overall acceptance	6.8 ± 0.8[a]	7.2 ± 0.5[a]	6.7 ± 0.6[a]	6.7 ± 0.7[a]

[a,b]Means in the same row within a category with different superscripts differ significantly ($P < 0.05$).
[1]Dietary treatments: CON = control; CSO = cottonseed oil; TAN = tannin; CPT = cottonseed oil plus tannin.
[2]Nine-point hedonic scale, where 9 = like extremely, 8 = like very much, 7 = like moderately, 6 = like slightly, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike moderately, 2 = dislike very much, and 1 = dislike extremely.

In the current study, sensory analysis on Cheddar cheese conducted after six months of ripening showed that firmness of all cheeses was rated as firm moderately by the panellist and no significant ($P > 0.05$) difference was observed among the

cheeses (Table 6.4). This result confirmed data obtained from analysis of hardness of the 24 week-cheese using Texture analyser, which showed that hardness of all cheeses were comparable after ripening for 24 weeks. During the first 12 weeks of ripening, hardness of CSO and CPT cheeses tended to be lower ($P < 0.05$) than that of control cheese (section 5.3.5). Similar result has been reported by Luna et al. (2005), in which cheese made of milk high in CLA content, had a lower score for texture based on sensory analysis during the first two months of ripening. Afterwards, cheese texture improved and was comparable to that of control cheese.

Flavour is another important sensory characteristic of cheese, which is governed by balance of a range of components (Fox and McSweeney, 1998). Formation of cheese flavour during ripening is influenced by three catabolic pathways i.e. in proteolysis, lipolysis, glycolysis in the cheese (Fox and McSweeney, 1998; Yvon and Rijnen, 2001). Proteolysis produces free amino acids which subsequently can be catabolised into aroma compounds which important for cheese flavour (McSweeney and Sousa, 2000). For examples, metabolism of tyrosine, tryptophan, and phenylalanine could result in fruity or putrid flavour (Yvone et al., 2000); while metabolism of arginine may related with the putrefactive flavour in cheese (Crow and Thomas, 1982). Metabolism of sulphur amino acids, such as cysteine and methionine, produces volatile sulphur compounds (VSCs), especially, methanethiol which is a principle component responsible for Cheddar cheese aroma (Weimer et al., 1999).

In the present study, CON, CSO, TAN and CPT cheeses had comparable ($P > 0.05$) flavour/taste (Table 6.4). This result may indicate that diet treatments partly

did not affect catabolism of amino acids into flavour compounds in cheese during ripening. PTA-SN and TCA-SN fractions contain peptides and free amino acids, which subsequently can be catabolised into flavour compounds (McSweeney and Sousa, 2000). Thus, similar degree of proteolysis as indicated by the comparable concentration of PTA-SN and TCA-SN fractions (section 5.3.3) among these cheeses may produce similar amount of amino acids, and thus may contribute to the comparable flavour among the cheeses.

Metabolism of lactate and citrate during glycolysis may result in flavour compounds such as acetate, butyrate, acetoin, diacetyl, 2, 3 butandiol, and $H_{2,}$ and CO_2 (Fox et al., 1990). Therefore, the comparable cheese flavour in the current study might also associated with a lack of an effect of cottonseed oil and condensed tannin supplementation on the concentration of organic acids i.e. lactic, acetic, and citric acids (Table 6.2). Citrate metabolism in milk by lactic acid bacteria produces flavour components such as acetate, acetaldehyde and diacetyl (Buffa et al., 2004). Acetic acid plays important roles for flavour development of cheese (Buffa et al., 2004), while lactic acid is important for assuring the good quality and proper cheese ripening process (Califano and Bevilacqua, 2000).

Moreover, lipid fraction could directly and indirectly affect flavour development in cheese. The direct effect of lipid fraction in cheese flavour is through its effect in providing short-chain FAs, which have strong and characteristic flavours. Cheese flavour is related with fatty acid composition in the cheese (Urbach, 1990). When present in excessive amounts, short chain fatty acids such as C4:0, C6:0 and C8:0 may impart a rancid, bitter and unpleasant taste (Soryal et al., 2005).

Our study showed that, CSO and CPT cheeses, which had lower concentration of short chain FAs than CON and TAN cheeses (Table 6.3) had a comparable flavour scores to that of CON and TAN cheeses (Table 6.4). Thus, it is likely that the difference in concentration of short chain FAs among the cheese could not exert significant changes in cheese flavour.

Meanwhile, lipid may indirectly affect development of cheese flavour by acting as solvent for sapid and aromatic compounds and producing free FAs during lipolysis (Collins et al., 2004). Free fatty acids produced during lipolysis then may undergo subsequent catabolism to produce volatile compounds such as ketones, lactones, alkanes, and ester volatile FA (Akin et al., 2003). Degree of lipolysis in the milk is influenced by several factors such as the stability of milk fat globules (MFG) toward LPL enzyme and the amount of FA attached at sn-1 and 3 position of triglycerides such as C4:0, C6:0, and C18:1 (Deeth and Touch, 2000). As it has been discussed earlier, CSO and CPT milk, which had higher concentration of PUFAs including C18:1, but lower amounts of C4:0, C6:0 fatty acids, were predicted to have lower degree of lipolysis, and thus had different flavour compared to those of the control cheese. However, the comparable flavour score among these cheeses might indicate that differences in FA composition in these cheeses could not cause a shift in FAA catabolism that would produce flavour compounds exceeding the threshold perception of the consumers. In addition, no difference in flavour score among these cheeses was detected by the panellists during sensory evaluation (Table 6.4), which might have been expected due to their inexperience.

The comparable flavour of CSO and CPT cheeses compared to the control cheeses in the present study (Table 6.3) may also indicate that CSO and CPT milk did not undergo lipid oxidation, despite higher levels of PUFAs in CSO and CPT milk. Thus, no oxidised flavours in milk were transferred to the cheeses. According to Timmons et al. (2001), lipid oxidation depends on the concentration oxidisable substrates, prooxidants, and antioxidants. In the current study, the concentration of Cu in milk was not measured; however, it was likely that the concentration of this mineral in milk was not high enough to induce oxidation. In addition, it was possible that proper packaging and exclusion of oxygen prevented PUFAs in CSO and CPT cheeses to undergo lipid oxidations. Moreover, Fox (1993) stated that lipid oxidation seems to be very limited in cheese due to its low redox potential. A lack of effect of lipid supplementation on development of oxidised flavour in the current study is contradicted with increasing effect of dietary whole roasted soybean in oxidised flavour formation in milk of dairy herds (Timmons et al., 2001). It was predicted that in addition to the possibility of lower Cu concentration, this discrepancy might also because the concentration of linoleic acid in the current study is lower than result obtained by Timmons et al. (2001).

In addition to proteolysis, glycolysis, lipolysis, and lipid oxidation during ripening, cheese flavour can be affected by milk pre-treatment such as pasteurisation (Fox and McSweeney, 1998) and homogenization (Geurts et al., 2003). Therefore, the comparable cheese flavour found in the current study might be related to heat treatment of raw milk before manufacturing into Cheddar cheese. Heat treatment such as pasteurisation or heat shock may change microflora and inactivate

endogenous enzymes in milk, and thus eliminate differences in cheese flavour caused by microbial ecosystem or result in cheese with weaker flavour and slower ripening (Fox and McSweeney, 1998; Verdier-Metz et al., 2002; Collins et al., 2004). Heat treatment used in the current study (65 ˚C for 30 min) might have played a similar role in all the cheeses and thus ensuring proper conditions for the starter cultures to grow and proper biochemical processes to take place during ripening. According to Geurts et al. (2003), homogenization of cheese milk may alter the flavour balance of the cheese since it could increase sensitivity of fat for lipolysis. However, in the current study, it appears that homogenisation did not affect flavour balance of the resulting cheese as all cheese were rated as like moderately by the consumers.

Overall acceptance of all cheeses was rated as liked moderately (Table 6.4). No difference ($P > 0.05$) was observed in overall acceptance of all cheese (Table 6.4). This result is in agreement with other studies, in which Cheddar cheese was manufactured from milk of cows fed dietary fat such as extruded soybean (Khanal et al., 2005) and sunflower oil (Coakley et al., 2007). These studies showed no apparent effect of fat supplementation on cheese sensory attributes i.e. flavour, body, and consumer acceptance. Luna et al. (2005) also did not observe differences in appearance, aroma, taste, texture, and acceptability in sheep cheese made from milk of animals fed whole linseed and sunflower oil in comparison to the control cheese. In contrast, another study reported that dietary linoleic acid may cause formation of sweet and raspberry flavour in cheese (Urbach, 1990).

Lack of any effect of dietary condensed tannins on cheese flavour found in the current study could be attributed to absence of the concentration of short chain fatty acids especially C4:0, C6:0 and C8:0 in cheese (Table 6.3). In addition, according to Ramshaw (1985), effect of plant secondary metabolites on sensory properties of dairy products depends on their concentration in these products. These plant compounds may cause desirable flavours when present at low concentration. In contrast, they may cause undesirable sensory traits when present in high concentration. O'Connell and Fox (2001) stated that when plant compounds were present at a sub-threshold concentration, they contributed to a delicate balance of flavour components. Thus, it is likely that the minimal impact of dietary condensed tannin on cheese flavour found in the current study might be partly due to concentration of condensed tannins in the cheese below a threshold to cause a significant effect on cheese flavour.

6.4 CONCLUSION

This study demonstrated that inclusion of cottonseed oil and condensed tannins into cow diet had minimal impact on organic acid production of the resulting Cheddar cheeses. Cheeses manufactured from milk of cows fed a diet containing cottonseed oil had higher concentration of medium- and long-chain FAs but lower in short-chain FAs. In contrast, supplementation of condensed tannin in cow diet resulted in cheese with comparable FA composition to that of control. In general, composition of FA in these cheeses reflected FA composition of the raw milk before standardisation. This indicated that transformation of milk into Cheddar cheese did not alter the composition of FAs. Throughout ripening time, concentration of short-,

medium- and long-chain FA remained stable. These supplemented diets also had no effect on cheese lightness and yellowness. These modified diets did not affect consumer preferences of for appearance, firmness, flavour, aroma, and overall acceptance. Thus, this study demonstrated that dietary cottonseed oil and condensed tannin can be used to produce Cheddar cheese with a new property without compromising acceptability of the cheeses.

7 Overall Conclusions and Future Research Direction

Chapter 7

7.1 OVERALL CONCLUSION

This study demonstrated that supplementation of cottonseed oil, condensed tannins, or in combination into a cow diet, which was aimed at reducing methane emission from dairy cows, affected yield and composition of the milk in a different way. Milk production increased with the presence of cottonseed oil alone or in combination with condensed tannin in the diet. Since inclusion of cottonseed oil did not affect animal's feed intake, increase in milk yield was more likely associated with an effect of dietary cottonseed oil in increasing supply and energy efficiency, which was required to support milk production. Dietary cottonseed oil also increased protein yield, which might have been due to an increase in available energy required for milk protein synthesis. However, dietary cottonseed oil did not significantly impact on content and proportion of milk proteins, casein, and whey protein. This diet also had minimal effect on yield and content of lactose in milk. Decrease in content of fat in milk was also observed with the presence of cottonseed oil in animal diet. It appeared that added cottonseed oil altered biohydrogenation in rumen and produced some fatty acids, which were likely to be octadecenoic acid isomers, which then induced milk fat depression (MFD). In addition to reduced milk fat content, added cottonseed oil also altered fatty acid composition in milk. Reduction in a concentration of short- and medium chain saturated fatty acids (SFAs), but an increase in long-chain SFA was observed.

Supplementation of *Acacia mearnsii*-condensed tannins had minimal impact on milk yield, which might have been due to a lack effect of this supplement on feed intake. The low concentration of condensed tannins in diet might be responsible to

absence of an effect of added condensed tannin to the diet. This supplement also reduced yield and content of milk proteins. These effects might have been associated with ability of condensed tannins to form complexes with carbohydrates in forages, thus preventing carbohydrate degradation and subsequently reducing energy available for milk protein synthesis. Reduction in lactose yield with condensed tannins supplementation might have been also associated with a limited supply of energy. This study also showed that dietary condensed tannins had no apparent effect on milk fat content and fatty acid composition. This minimal impact on milk fat might be due to the low concentration of condensed tannins in the diet and absence of an effect of condensed tannins on the activity of microorganism involved in the rumen biohydrogenation.

Analysis of the effect of cottonseed oil and condensed tannins supplements on milk coagulation properties indicated that these supplementations had a minimal impact on acid- and rennet-induced milk gels. In general, rheological properties (storage modulus, loss modulus, and gelation time), permeability, water holding capacity, and hardness of acid- and rennet-induced milk gel were unaltered due to these supplementations. A lack effect of an added cottonseed oil and condensed tannins on the properties of milk gel might be due to similar content and compositions of proteins in the cheese-milk samples. It also appeared that initial differences in fat concentration and fatty acid composition in the milk were eliminated due to standardisation thus fatty acid (FA) profile had a minimal impact on milk coagulation properties. Curd firmness and gelation time of rennet-induced

milk gels found in this study indicated that these renneted-induced gels were suitable for the cheese manufacturing.

Analysis on the Cheddar cheese made of the cheese-milk demonstrated that supplementation of cottonseed oil and condensed tannins did not significantly affect fat and protein recoveries in the samples cheese. Actual yield, moisture adjusted yield, and theoretical yield were also unaffected. This lack of an effect of these supplements on fat and protein recoveries and cheese yields was likely associated with a similar composition of the cheese-milk. Despite difference in casein to fat ratio in raw milk samples, standardisation eliminated this difference in the cheese-milk. Furthermore, no significant differences in fat, proteins, moisture, and the total solids were observed among the cheeses throughout ripening time. At the end of ripening period (week 24), concentration of fat, proteins, and the total solids remained unchanged, while moisture concentration decreased slightly. The Cheddar cheese manufactured in this study also had comparable ratio of salt-in-moisture (S/M), fat in dry matter (FDM), and moisture non-fat solid (MNFS) values. Analysis of fatty acid composition of the Cheddar cheeses indicated that it resembled composition of fatty acids in raw milk samples. Thus, the Cheddar cheeses made of milk of cows fed diet containing cottonseed oil had a lower concentration of short-chain SFA, but were higher in concentration of medium- and long-chain SFA. Supplementation of condensed tannins had no significant effect on fatty acid composition of the cheese. The concentration of fatty acids in all the cheeses remained stable throughout ripening time.

Supplementation of cottonseed oil and condensed tannins also resulted in the cheeses with a comparable degree of the primary proteolysis as indicated by similar concentration of soluble nitrogen in WSN fraction of the cheeses. Extent and rate of the secondary proteolysis were also unaffected as reflected from similar concentration of soluble nitrogen in phosphotungstic acid (PTA) fractions and similar peptide profile of water soluble nitrogen (WSN) fractions. Overall, observed differences in terms of cheese hardness and springiness appeared to be slight among the cheese at any ripening time point. Except for cottonseed oil (CSO) cheese, which had a lower cohesiveness and chewiness, all other cheeses had a comparable cohesiveness and chewiness. In general, all the cheeses showed a similar pattern of hardness development during ripening as cheese hardness decreased slightly during the first 4-6 weeks, followed by a steady increase afterwards. Springiness and cohesiveness of all cheeses tended to reduce during ripening. The similar texture of the cheeses in this study might be related to a similar degree of their proteolysis. In addition, it appeared that, differences in fatty acid composition of these cheeses did not affect texture of the cheeses.

No significant difference was observed in concentration of organic acids released during ripening of the cheeses. In comparison to acetic and citric acids, lactic acid was presented in a higher concentration in all cheeses and tended to increase throughout the ripening time. In contrast, concentration of citric acid in all cheeses was inconsistent during ripening. A similar ratio of S/M might be attributed to the comparable organic acid production in all cheeses. Absence of an effect of the modified diets on organic acid production in the cheeses might partly have

contributed to similarities in flavour in the cheese samples. Differences among cheeses in term of their fatty acid composition did not significantly affect cheese flavour. Cheese colour and other cheese sensory attributes such as aroma, firmness, and overall acceptances were also unaffected by supplementation of cottonseed oil and condensed tannins in the diet.

As the final conclusion, the study showed that the supplementation of cow diets with cottonseed oil, condensed tannins, or in combination appeared as a possible strategy for methane mitigation since the consequences of inclusion of these supplements were minimal and could have been alleviated by some processing parameters. It remains to be seen whether the diets had a real impact on minimising methane emission, which was not one of the objectives of this study.

7.2 FUTURE RESEARCH DIRECTION

Supplementations of lipid and cottonseed oil have been reported could reduce methane emission from ruminants. However, these supplementations may alter the yield and technological properties of the obtained milk, which consequently may influence the acceptance of this methane mitigating strategy by the dairy producers. Overall, this study demonstrated that supplementation of cottonseed oil (800 g/d), *Acacia mearnsii*-condensed tannins (400 g/d), or their combination had minimal impact on technological properties of milk of cows fed these supplemented diets. Increase in the amount of unsaturated fatty acids in milk related with the presence of cottonseed oil in cow's diet did not large enough to alter coagulation properties of acid- or rennet-induced milk gels. Future studies are required to examine the effect of this supplementation on properties of other dairy products, such as yoghurt and

butter. Cottonseed oil and condensed tannins supplementations also had minimal impact on properties and consumer acceptance of the Cheddar cheese made from the obtained milk. The presence of cottonseed oil in cow diet increased the amount of unsaturated fatty acid in milk and the Cheddar cheese. Thus, there is a need to examine benefits of these dairy products for human health. In addition, increase in the amount of unsaturated fatty acid in milk has been reported to increase the size of milk fat globule (MFG) size. Further studies are needed to examine the effect of cottonseed oil supplementation on MFG size and its effect on dairy products. Even though our study showed that the obtained Cheddar cheese have comparable properties with data in literature, the relatively high water holding capacity (WHC) value of milk gels in the current study might prompt us to eliminate the micro fluidisation step during cheese manufacturing process in the future studies. Moreover, there is a need to scale-up Cheddar cheese production to ensure the acceptance of these supplemented diets by the dairy producers.

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