The Effect of Salt Reduction, Culture and Process Modifications on the Physicochemical and Microbiological Characteristics of Cheddar-type Cheeses

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بسم الله الرحمن الرحیم

Dedication

It is my honour to dedicate this thesis to my honourable father, *Dr Ahmad Sheibani*, my adorable mother, *Mrs Shahla Majlesi*, my brother, *Dr Mohammad Sheibani*, and my sister, *Mrs Ghazaleh Sheibani*. Without their endless love, support and prayers, my success would be impossible.

تقدیم به پدرم و مادرم

دکتر احمد شیبانی و مهندس شهلا مجلسی

Abstract

The World Health Organization has identified hypertension as one of the leading causes of death in the world, and consumers are subsequently seeking ways to decrease their daily salt dietary intake. This trend is increasing demand for low-salt foods. This thesis contributes to the literature on low-salt cheese production by investigating how salt reduction affects cheese quality. Salt is one of the key ingredients used in cheese manufacturing to perform several functions, including antimicrobial functions, moisture control, formation of texture and structure, management of pH and acidity, and flavour enhancement. Thus, the objective of this study was to produce cheese using a low amount of salt and investigate the effects of salt reduction on the cheese's microbiological, textural, sensory and physicochemical properties, and salt release.

This project was divided into two phases. In phase one, hard-type cheeses were prepared at various salt levels (control = 2.5% w/w, Treatment 1 = 2% w/w, Treatment 2 = 1.5% w/w and Treatment 3 = 1% w/w) using a high proteolytic culture consisting of *Lactobacillus helveticus* and *Streptococcus thermophilus,* followed by storage for eight weeks at 9°C. The chemical, physical, microbiological and sensory characteristics were examined during storage. Salt reduction significantly ($p \leq 0.05$) affected the moisture and salt-in-moisture (S/M) contents, sodium and ash contents, and starter culture cell growth; however, the fat and protein contents and pH values were unaffected. Primary proteolysis (measured as water-soluble nitrogen [WSN]) and intermediate proteolysis (measured as trichloroacetic acid-soluble nitrogen [TCA-SN]) increased between Weeks 0 and 8 in all experimental cheeses in the same salt treatment. Advanced proteolysis (measured as phosphotungstic acid-soluble nitrogen [PTA-SN]) and total free amino acids [TFAA]) in cheeses salted at 2.5% (control) and 2.0% (Treatment 1) were significantly lower than other cheeses (Treatments 2 and 3) during storage. All proteolysis parameters (WSN, TCA-SN, PTA-SN and TFAA) increased significantly during storage due to the use of *L. helveticus*, which has high proteolytic enzyme activity. Higher Angiotensin-I-converting enzyme (ACE)–inhibitory activities were found for Treatments 2 and 3 at the end of Weeks 6 and 8 of storage. The microstructure and texture profile differed significantly during the storage for all treatments, with no

differences in the sensory attributes after eight weeks of storage of cheeses made with 1.5% and 2% salt, compared to the control.

In the second phase of the project, low-salt (1.5% salt) Cheddar cheeses were prepared using a conventional Cheddar cheese starter culture (*Lactococcus lactis subsp. Lactis* and *Lactococcus lactis subsp. cremoris*). The effect of change in pH at whey drainage (6.2, 5.9 and 5.6), casein-to-fat (C/F) ratio (0.6, 0.7 and 0.8) and rennet concentration (0.1 and 0.3 mL/L) on proteolysis, texture, microstructure and salt release were investigated during storage for 180 days at 9 ± 0.5°C. At the same pH, cheeses made with a C/F ratio of 0.6 had significantly higher moisture and ash content than did cheeses with other C/F ratios. The pH of all samples decreased significantly between Days 0 and 120, and stayed constant thereafter. The total lactic acid bacteria growth in cheeses made with 0.3 mL/L rennet concentration was higher at the same C/F ratio and pH 6.2. Similarly, at the same pH, C/F ratio and storage time, the WSN content in cheeses made with 0.3 mL/L rennet was higher than that among cheeses with 0.1mL/L rennet. Both the TFAA and TCA-SN contents increased significantly during storage. The percentages of the extra-hydrophobic peptides (as measured by reverse-phase high-performance liquid chromatography) at Day 0 in cheeses made with pH 5.6 were higher than that among cheeses made at a higher pH value.

In general, the hardness and adhesiveness of all experimental cheeses tended to increase during storage from Days 0 to 60. The springiness and cohesiveness of all experimental cheeses decreased significantly over storage. Cheeses made with a 0.8 C/F ratio had a higher gumminess than did the 0.7 and 0.6 ratios. As elucidated with Environmental Electron Microscopy, the structure became denser, more compact and more homogenous with small gaps at the end of storage (Day 180) due to the production of peptides and amino acids. Cheeses made with the lowest C/F ratio (0.6) showed a higher release of salt (less retention of Na^+ ion in cheese; 50 to 80 mg/100g) than did the other cheeses. No significant differences were noted among the sensory attributes and overall acceptability of the cheese with the highest level of salt release (C/F ratio 0.6, rennet concentration 0.1 mL/L, and pH 6.2).

This study's results demonstrate that the reduction of salt (NaCl) in hard cheese, such as Cheddar cheese, without any salt substitution is possible by modifying the cheese

making process. By using a C/F ratio of 0.6, rennet concentration of 0.1 mL/L and draining pH of 6.2, low-salt cheese (1.5%) can be created without a significant loss in overall acceptability, as confirmed by sensory evaluation.

Certificate

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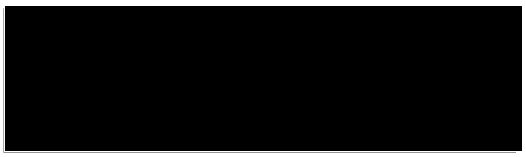
This is to certify that the thesis entitled *The Effect of Salt Reduction, Culture and Process Modifications on the Physicochemical and Microbiological Characteristics of Cheddar-type Cheeses* submitted by Ali Sheibani in partial fulfilment of the requirement for the award of the Doctor of Philosophy in Food Technology at Victoria University is a record of bona fide research work carried out by him 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.



Dr Vijay K Mishra

Declaration

I, Ali Sheibani, declare that this thesis entitled *The Effect of Salt Reduction, Culture and Process Modifications on the Physicochemical and Microbiological Characteristics of Cheddar-type Cheeses* is no more than 100,000 words in length, including quotations 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.

Ali Sheibani

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List of Publications

Book Chapter

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Journal Articles

Sheibani, Ali, Ayyash, MM, Shah, NP & Mishra, VK 2015, 'The effects of salt reduction on characteristics of hard type cheese made using high proteolytic starter culture', *International Food Research Journal*, vol. 22, no. 6, pp. 2452–2459.

List of Oral and Poster Presentations

Oral Presentation

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List of Abbreviations

ACE	Angiotensin-I-converting Enzyme
Ala	Alanine
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
Arg	Arginine
Asp	Aspartic Acid
a_w	Water Activity
$CaCl_2$	Calcium Chloride
CCP	Colloidal Calcium Phosphate
C/F	Casein-to-fat
CFU	Colony Forming Unit
D_{eff}	Diffusion Coefficient
DM	Dry Matter
ENaC	Epithelial Sodium Channel
ES	Expressible Serum
ESEM	Environmental Scanning Electron Microscopy
FDM	Fat in Dry Matter
Glu	Glutamic Acid
Gly	Glycine
HA	Hippuric Acid
HCl	Hydrochloric Acid
HHL	Hippuryl-histidyl-leucine
His	Histidine
HPLC	High-performance Liquid Chromatography
Ile	Isoleucine
KCl	Potassium Chloride
LAB	Lactic Acid Bacteria
Leu	Leucine
Lys	Lysine
M17	Medium for the Growth and Enumeration of Lactic Streptococci

Met	Methionine
$MgCl_2$	Magnesium Chloride
Mmol	Milli-mole
MRS	de Man, Rogosa and Sharpe
NSLAB	Non-starter Lactic Acid Bacteria
Phe	Phenylalanine
Pro	Proline
PTA-SN	Phosphotungstic Acid-soluble Nitrogen
RDI	Recommended Daily Intake
RPHPLC	Reverse-phase High-performance Liquid Chromatography
SE	Standard Error
Ser	Serine
SLAB	Starter Lactic Acid Bacteria
S/M	Salt-in-moisture
SP	Serum Protein
T1	Treatment 1
T2	Treatment 2
T3	Treatment 3
TCA	Trichloroacetic Acid
TCA-SN	Trichloroacetic Acid-soluble Nitrogen
TFA	Trifluoroacetic Acid
TFAA	Total Free Amino Acids
Thr	Threonine
Tyr	Tyrosine
UK	United Kingdom
Urea-PAGE	Urea-polyacrylamide Gel Electrophoresis
US	United States
Val	Valine
v/v	Volume per Volume
v/w	Volume per Weight
WHO	World Health Organization
WSE	Water-soluble Extract
WSN	Water-soluble Nitrogen

Chapter 1: Introduction

1.1 Background

Salt (sodium chloride, NaCl) is currently one of the most commonly used food additives. Besides providing a salty taste and source of dietary sodium, salt contributes significantly to the flavour, texture and preservation of foods, including cheese (Guinee 2004a; Guinee & Fox 2004; Reddy & Marth 1991). Cheese is a salt-containing product that contributes to individuals' daily intake of sodium currently being scrutinised by the scientific community due to its adverse health effects. The intake of sodium from cheese can vary based on different food habits and the types of cheese consumed. For example, salt intake from cheeses has been reported as 9.2% in France, 7.8% in the United Kingdom (UK), 5% in Australia and 8.2% in the United States (US) (Anderson et al. 2010; Meneton et al. 2009; NHMRC 2003). The amount of salt in cheese varies markedly with the type of cheese, ranging from around 0.5 to 0.7% (w/w) in acid curd types, such as cottage and Emmental cheese, to around 4 to 6% (w/w) in pickled cheeses, such as Domiati and feta.

Salt is a major source of the essential dietary sodium required by the human body. It has been reported that sodium ions are important for regulation of blood pressure, water transport in and out of cells, tissue osmolality, and transmission of nerve cell impulses (Otten et al. 2006). However, in the modern Western diet, the dietary intake of sodium is generally two to three times the recommended level of ~6 g NaCl per day. Salt is linked to the development of hypertension, osteoporosis and kidney stones (Buemi et al. 2002; Heaney 2006; Kotchen 2005; Massey 2005). Therefore, health authorities such as the World Health Organization (WHO) (2007) have recommended reducing the levels of salt in food products, including cheese. This has initiated a worldwide attempt by dairy scientists to reduce the salt content in Cheddar cheese in order to reduce sodium intake (WHO, 2007). Cheddar cheese is one of the most popular cheeses in Western countries. It is the most heavily consumed cheese in the UK, and second most consumed cheese in the US, behind mozzarella, with an average annual consumption of 4.5 kg per capita (Nolan 2009).

However, salt reduction negatively affects the quality and safety of cheeses that are normally ripened, such as Cheddar cheese. Reducing the salt content in Cheddar cheese is known to reduce sensory acceptance due to poor flavour development, and to have a reduced shelf-life. Therefore, salt reduction in cheese is an interesting challenge for researchers. Several attempts have been made to reduce the salt content using different techniques, including simple salt reduction and partial salt replacement using other salt replacers, such as potassium chloride (KCl), magnesium chloride ($MgCl_2$) and calcium chloride ($CaCl_2$) in various cheeses, including Cheddar, with a view to avoid or minimise the adverse effects on cheese quality (Guinee & O'Kennedy 2007). Reducing the salt concentration in Cheddar cheese causes higher proteolysis and microbial growth, which increases bitterness and lowers shelf-life. The rate of proteolysis in Cheddar-type cheeses is inversely related to salt concentration (Kelly et al. 1996), the higher the salt concentration, the lower the proteolysis rate. Salt is needed to control proteolysis and the growth of the starter cultures that are responsible for achieving the ideal quality of Cheddar-type cheeses.

Replacing sodium chloride with other salts (such as KCl, $CaCl_2$ and $MgCl_2$) is another alternative to reduce salt content during cheese manufacturing. Some studies have investigated the effect of NaCl replacement with KCl on the characteristics of Cheddar cheese (Grummer et al. 2012, 2013; Reddy & Marth 1993a, 1993b) and identified ratios of salt combinations that produce acceptable cheese quality. The main concern regarding using salt replacers is the formation of undesirable aftertastes, such as bitterness or a metallic and sharp taste (Lawless et al. 2003; Reddy & Marth 1991). In previous studies, flavour enhancers were used to mask this problem (Grummer et al. 2013).

Limited information is available in the open literature regarding how changing the composition of milk during cheese making and the manufacturing process would improve the quality of salt-reduced Cheddar-type cheese. Changing factors such as the casein-to-fat (C/F) ratio, starter culture, rennet concentration and drainage pH will affect salt-reduced cheese quality by influencing the final composition of the cheese and proteolysis occurring during storage and ripening. Reducing the salt in Cheddar cheeses increases the proteolysis and organic acid production by starter culture, as observed

recently by Murtaza et al. (2012) and Murtaza et al. (2014). These changes depend on the amount of casein present in milk in relation to the other constituents, particularly fat; the coagulants and their concentration; the type of starter cultures; and the pH at the time of drainage of the curd. It is possible to achieve quality salt-reduced Cheddar-type cheese if the role of each of these factors is carefully assessed.

1.2 Aim of this Research

The overall aim of this project was to reduce the salt concentration in Cheddar-type cheeses in order to help reduce dietary salt intake to achieve better health, while still producing cheese of acceptable quality. The focus was on Cheddar-type cheeses, which contain a relatively higher amount of salt (up to 3%). The effect of the C/F ratio, rennet concentration and pH at drainage of curd were investigated by measuring the cheese composition, proteolysis, structure and texture, and the effect of cheese metrics on the release of salt.

1.3 Structure of the Thesis

This thesis is structured as follows. Chapter 2 provides a current literature review on salt and its application in the cheese making process, the effects of salt on the quality of cheese, and the relationship of salt to health. In addition, this chapter discusses the different methods of salt reduction.

Chapter 3 presents the results of a study of the effects of salt reduction on hard-type cheese characteristics during storage. Four batches of hard-type cheese were prepared and salted at four different levels, control (2.5% w/w), Treatment 1 (2% w/w), Treatment 2 (1.5% w/w) and Treatment 3 (1% w/w), and stored at 9°C for eight weeks. Samples were collected after zero, two, four, six and eight weeks of storage, and subjected to the following analyses: chemical composition, proteolysis, organic acid profile, mineral (Na^+) content, Angiotensin-I-converting enzyme (ACE)–inhibition activity, texture profile, microstructure and sensory evaluation.

Chapter 4 details the effects of change in the C/F ratio, rennet concentration and pH at drainage on the proteolysis of the salt-reduced Cheddar cheese. The choice of the extent of salt reduction, 1.5% w/w salt (based on curd weight), was based on the results of Chapter 3.

Chapter 5 reports the results of the effects of change in the C/F ratio, rennet concentration and pH at drainage on texture parameters, such as hardness, cohesiveness and adhesiveness, and on the microstructure of the salt-reduced Cheddar cheese.

Chapter 6 discusses the effects of change in the C/F ratio, rennet concentration and pH at drainage on the diffusion and release of salt from the salt-reduced Cheddar cheese, as well as the sensory attributes. All salt-reduced cheeses in Chapters 4, 5 and 6 were ripened at 9 ± 0.5°C for 180 days, and samples were collected on Days 0, 60, 120 and 180 of storage.

Chapter 7 provides an overall conclusion for the study, based on the results presented in Chapters 3 to 6, and offers recommendations for future research.

Chapter 2: Literature Review[1]

2.1 Introduction

'*Natron*' was the first name given to salt by the Egyptians, meaning the 'divine salt'. The Latin name '*salarium*' was used to refer to the amount of salt given to a worker as payment for a job. Since historical times, salt has played an important role in human life, with the Romans, Ancient Egyptians and Middle Eastern people pioneers in using salt as a food preservative (Forbes 1965). While salt plays an essential role in food preservation, processing and food flavour (Silva et al. 2003), it is also known as one of the risk factors for osteoporosis, kidney stones and hypertension (Buemi et al. 2002; Heaney 2006; Kotchen 2005; Massey 2005). Thus, reduction in sodium intake is strongly recommended, and food industries are seeking to reduce salt in processed foods. As high as 75% of dietary salt comes from processed foods (Appel & Anderson 2010), with dairy products contributing 10% of the recommended daily intake (RDI) of salt, of which about 90% comes from cheese alone (Appel & Anderson 2010; Meneton et al. 2009). For example, the daily sodium intake from cheese was reported as 5% in Australia (NHMRC 2013) compared to 7.8% in the UK, 9.2% in France, and 8.2% in the US (Anderson et al. 2010; Meneton et al. 2009;). Health authorities recommended reducing average salt consumption for adults to < 5 g per day (Flock & Kris-Etherton 2011). The recommended sodium intake among Australian children and adolescents is 140–280 milligrams a day for infants, 460–1730mg/day for 1–3 year olds, 600–2300mg/day for 4–7 year olds, and 920–2300mg/day for children aged 8 years and over and for adolescents (NHMRC 2003). It shows that the daily intake of salt in the Australian diet is over 3 to 5 g per day (NHMRC 2003) and must be reduced to match health recommendations.

[1] A major section of this literature review has been accepted as a book chapter: Sheibani, A, Mishra, V, Stojanovska, L & Ayyash, M 2013, 'Salt in cheese: health issues, reduction, replacement and release' (pp. 397–418) in *Handbook on cheese: production, chemistry and sensory properties*, Nova Science Publishers, New York.

Hence, cheese is an ideal dairy product to target for salt reduction in order to reduce dietary intake to comply with the World Health Organization's (WHO) (2007) recommendations.

Cheddar cheese is one of the key cheese types consumed in the world. In Australia, cheese is a major class of manufactured dairy product, with a projected production of 344,000 tonnes in 2015 (Dairy Australia 2015). Per capita consumption of cheese in Australia is projected to be 13.5 kg per year, of which about 55% can be accounted for by Cheddar cheese consumption. Thus, Cheddar cheese has the largest production of all cheeses in Australia, comprising about 52% of production in the same year. However, production trends show an increasing share of non-Cheddar-type cheeses in the last few years, which is attributed to changes in the types of consumers and their behaviour. Due to Australian's significant consumption of cheese, the industry is seeking ways to reduce salt levels in cheeses, without sacrificing safety and quality.

This literature review discusses issues associated with the dietary consumption of high levels of salt. It also explores the role of salt in cheese making in order to examine the challenges encountered when aiming to reduce or replace salt during this process.

2.2 Salt and Health

Sodium plays a vital role in human physiology by regulating the fluid balance in the body, and being involved in electrical signalling in the nervous system (Campbell et al. 1993). Sodium is an important cation that is needed to maintain extracellular volume and serum osmolality, with a role in the active transport of molecules across cell membranes. Thus, salt is required to maintain human health. The Australian National Health and Medical Research Council (NHMRC) (2013) outlined the RDI for sodium as presented in Table 1. Similar guidelines are present in other countries (Otten et al. 2006).

Table 1: RDI of Sodium (mg/day) and Salt (g/day) for Different Life Stages and Genders[1]

Age (years)	Sodium mg/day		NaCl g/day	
Children and adolescents	Recommended	Upper level	Recommended	Upper level
1–3	200–400	1,000	0.51–1.02	2.56
4–8	300–600	1,400	0.76–1.53	3.59
9–13	400–800	2,000	1.02–2.05	5.13
14–18	460–920	2,300	1.18–2.36	5.89
Adults (+18)				
Men	460–920	2,300	1.18–2.36	5.89
Women	460–920	2,300	1.18–2.36	5.89
Pregnant women				
14–18	460–920	2,300	1.18–2.36	5.89
19–30	460–920	2,300	1.18–2.36	5.89
31–50	460–920	2,300	1.18–2.36	5.89

[1] Nutrient Reference Values for Australia and New Zealand (NHMRC 2005, 2013).

Table 1 indicates that the RDI of NaCl is the same for both men and women (1.18 to 2.36 g per day) and that lower intakes are recommended for children below the age of 14 years. For adults, an upper level of 2,300 mg/day (100 mmol/day) was set on the basis of population studies for low levels of hypertension. The actual daily intake of sodium is much higher than the RDI, as evidenced by a nutritional survey of adults in the UK. This survey indicated that the daily intakes of sodium among men and women were nearly five times that of the RDI, at 11 g in men and 8.1 g in women (FDA 2014; Hoare et al. 2003). Intake appears to be in excess of 100 to 200 mmol (1,800 to 3,600 mg) per day (Elliott & Brown 2006; NHMRC 2013; WHO 2007). Excessive consumption of sodium contributes to the health issues discussed below.

2.2.1 Hypertension

According to the seventh Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure, healthy individuals have a blood pressure of approximately 120/80 mmHg. Individuals with blood pressure ≥ 140/90 mmHg are classified as hypertensive, while between 120/80 and 140/90 mmHg are pre-hypertension (Chobanian et al. 2003). Epidemiological studies, treatment trials and animal studies have provided ample evidence of the positive correlation between sodium intake and hypertension, with excessive intake highly correlated with hypertension (He & MacGregor 2007; Kotchen 2005; Penner et al. 2007).

Clinically, this leads to damage to blood vessels through stroke or myocardial ischemia. Uncontrolled elevation of blood pressure (malignant hypertension) also leads to renal failure, heart failure and blindness (O'Shaughnessy & Karet 2006). Eating excess salt stimulates the secretion of too much Ouabain (pronounced 'wah-bane'), a naturally occurring hormone secreted by the adrenal gland, and affecting two proteins (alpha-2 sodium pump and sodium–calcium exchanger) that are responsible for regulating the amount of sodium and calcium in the muscle cells of the arteries (Blaustein et al. 2004). It has also been reported that systolic blood pressure showed a reduction of 4.6 mmHg ($p < 0.001$) corresponding to salt intakes of 1,500 mg/day (65 mmol/day), compared to 2,500 mg/day (107 mmol/day) in people on a control diet (Henney et al. 2010; NHMRC 2013).

2.2.2 Cardiovascular Diseases

Cardiovascular diseases (such as heart attack, heart failure and stroke) are a major cause of death in the world, comprising 30% of total death incidences in 2005, and projected to increase to 47% by 2015 (WHO 2005). Hypertension is a major risk factor for cardiovascular diseases (Alderman 2006; He & MacGregor 2007; Penner et al. 2007) because a rise in blood pressure causes damage to the arteries, which leads to heart failure. Initially, hypertension may cause a slight reduction in the amount of blood reaching the heart, thereby reducing oxygen and nutrient uptake over time, and later causing clogging of the arteries and triggering a heart attack (He & MacGregor 2007; Strazzullo et al. 2009).

2.2.3 Chronic Kidney Disease

Kidneys play the key function of removing metabolic wastes from the blood. Increased salt intake causes progressive loss of normal function, leading to the onset of chronic kidney disease (Massey and Whiting, 1995; Lin et al. 2003; Massey 2005). Excessive salt raises the amount of sodium in the bloodstream and disturbs the sodium and potassium balance, thereby reducing the ability of kidneys to remove wastes from the blood. This also results in higher blood pressure due to the extra fluid in the delicate blood vessels leading to the kidneys (Go et al. 2004; Hall et al. 1999; Schiffrin et al. 2007).

2.2.4 Other Diseases

Epidemiological studies have shown a significantly positive correlation between salt intake and stomach cancer (He & MacGregor 2007; Joossens et al. 1996). Salt increases the growth and activity of *Helicobacter pylori* and thus contributes to the development of cancer (Wang et al. 2013). Salt may also act as an inflammatory agent of the stomach lining, which can expose it to carcinogens (Lambert & Hainaut 2007; Shikata et al. 2006).

Bone remodelling is inversely correlated with sodium intake, and there is direct correlation between sodium intake and urinary calcium excretion in healthy elderly men and women (Massey & Whiting 1996). High intake of sodium leads to a higher exertion of calcium (~0.69 mmol of calcium for each 100 mmol increase in sodium intake) (Heaney 2006; Massey & Whiting 1996). This can lead to bone loss and possibly osteopenia and osteoporosis in individuals with high salt and low calcium intakes.

2.3 Salt in Food

Salt acts as an essential ingredient in food, enhancing flavour, texture and colour. In addition, it is well known for its function as a food preservative. This is the reason that salt can be found in almost every food item (Albarracín et al. 2011). Salt's preservative effects result from lowering water activity, which reduces the growth of many pathogenic microbes (Durack et al. 2008). Salt can cause microbial cells to undergo osmotic shock, resulting in the loss of water from the cell and thereby causing cell death or suppressing growth (Davidson 2001).

Salt has a noticeable effect on the texture of food products. For example, when making bread, salt controls the rate of yeast fermentation and gluten formation, which both affect the bread's texture. Salt also improves the coagulation of proteins, which occurs in cheese and other foods, such as sausage (Albarracín et al. 2011). Saltiness is regarded one of the four major flavours, and salt also affects other flavours, including sweetness and bitterness, in moderate amounts, salt intensifies the sweetness and reduces the bitterness of foods (Taylor & Roberts 2004). Cheese is one of the most popular salt-containing foods widely consumed all over the world.

2.4 Cheese Manufacturing and Salting Methods

Fundamentally, the cheese making process is a dehydration process, wherein between six- to 12-fold concentrations of fat and proteins are achieved. This process can be described generically as the acidification of milk; coagulation of proteins; and removal of whey by cutting, cooking, stirring, pressing, salting and shaping (moulding and

pressing) (Fox et al. 2004b). The fresh curd can then be ripened, as in the case of Cheddar cheese. Figure 1 describes the common steps used during cheese manufacture.

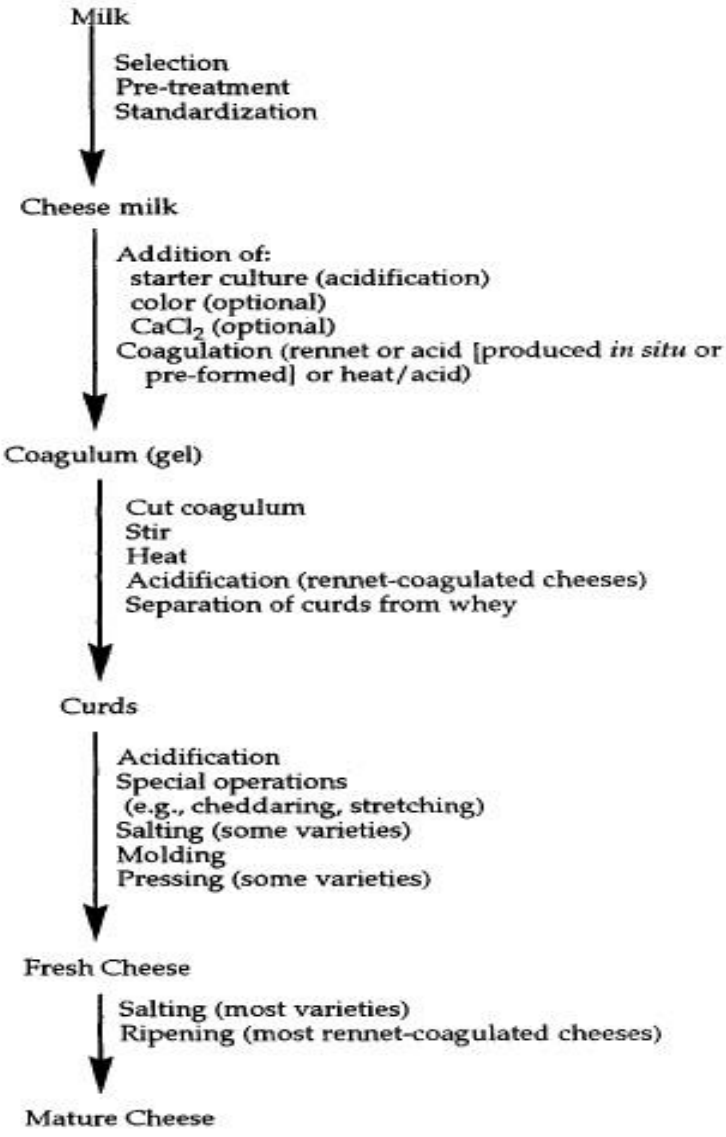


Figure 1: The General Procedure of Cheese Making (Fox et al. 2000a)

Broadly speaking, the steps of cheese manufacturing are as follows:

1. standardising milk by adding cream or skim milk, removing fat and adding milk powder or evaporated milk as needed, followed by pasteurisation of the milk
2. converting standardised milk to cheese curd by coagulating the casein to form a gel that entraps fat. Coagulation is achieved via:
    a. limited proteolysis by selected proteinases, such as rennet
    b. acidification of milk to casein's isoelectric point (pH 4.6)

c. acidification of milk to a pH value greater than 4.6 (~5.2) in combination with heating to around 90°C.

3. cutting the coagulum into small pieces to facilitate the whey syneresis. Coagulated milk is quite stable as long as inert conditions are maintained; however, if cut or broken, it rapidly undergoes syneresis, expelling whey (Fox et al. 2000a)
4. salting the curd to promote further removal of whey, and controlling the curd's moisture content with appropriate acidification, heating and stirring.

The salting of cheese is practised using three key methods:

1. dry salting, the direct addition of dry salt to cheese curd, followed by mixing at the end of manufacture (such as for Cheddar and cottage cheeses)
2. surface dry salting, rubbing dry salt into the surface of the moulded curds (such as for blue-type cheeses)
3. brine salting, soaking moulded cheese in a brine solution (such as for feta, edam and gouda).

After salting, the curd is ripened based on the required condition for each type of cheese. The unique characteristics of the individual cheeses develop during ripening as a result of a complex set of biochemical reactions. The changes that occur during ripening (and hence the flavour, aroma and texture of the cheese) are highly influenced by milk formulation (such as a change in the C/F ratio) and the manufacturing process (the coagulant, added salt, pH at whey drainage, and starter culture). The biochemical changes that occur during ripening are mainly caused by the coagulant, indigenous milk enzymes (plasmin), starter bacteria and their enzymes, and secondary microorganisms and their enzymes (Fox et al. 2000a).

2.5 Effect of Salt on Cheese Quality

Salting is an important step during the manufacturing of all major cheese groups. Salt in cheese plays two major roles: (i) it preserves the cheese and (ii) it contributes to the quality of the cheese (flavour and texture). Figure 2 illustrates the general function and effects of salt in cheese.

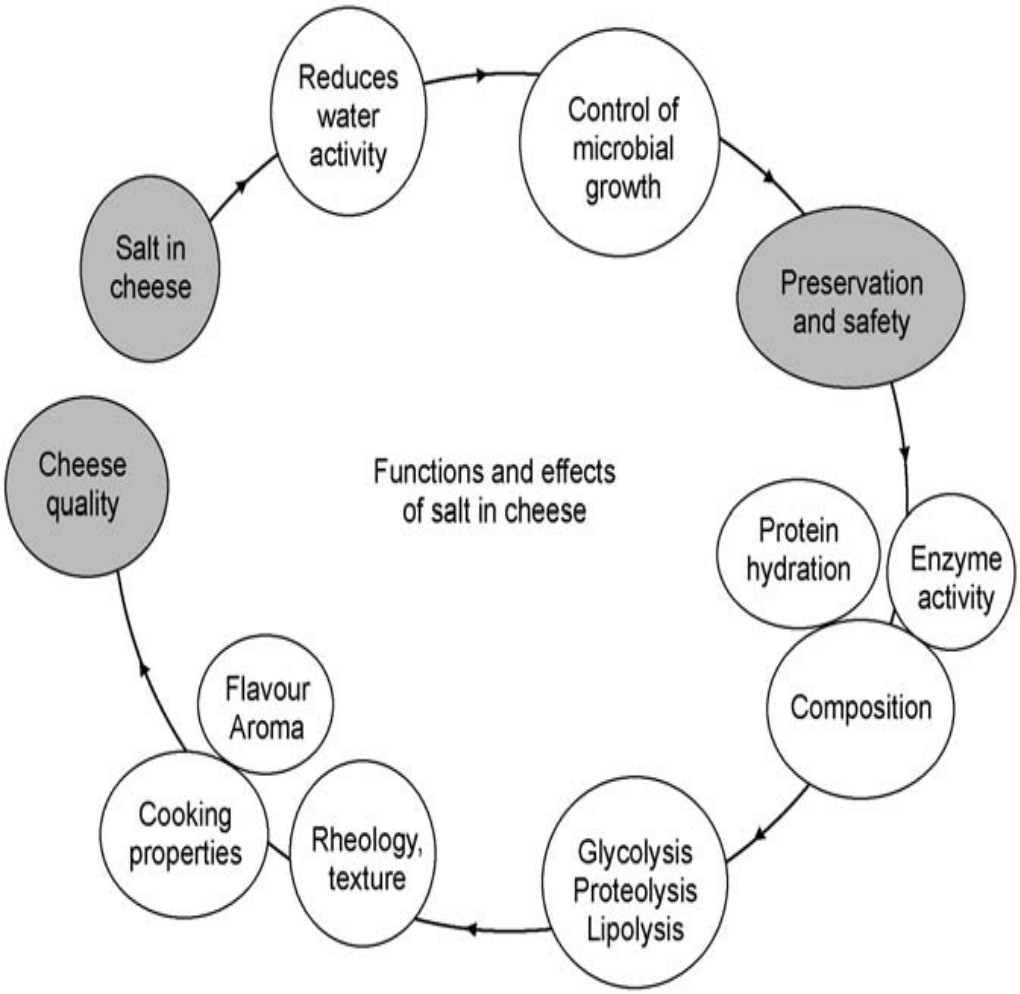


Figure 2: Demonstration of General Function and Effects of Salt in Cheese (Guinee & O'Kennedy 2007)

Salt contributes to the preservation, safety and overall quality of cheeses via its effects on water activity, microbial growth, protein hydration and enzymatic activities, which in turn influence biochemical changes, such as proteolysis (Guinee & O'Kennedy 2007). The quality of cheese depends on the amount of salt added to the curd. Lack of salt in cheese creates a soft and unacceptable texture (Fox et al. 2000a). In addition, the flavour of salt-free cheese is insipid and watery, even to somebody not addicted to salt; thus, addition of a small amount of salt (NaCI, 0·8% w/w) is required to overcome this insipid taste (Fox et al. 2004b).

2.5.1 Preservation Role

Both starter and non-starter bacteria in cheese affect the final quality of cheese. Generally, salt controls the growth of starter lactic acid bacteria (SLAB) and non-starter lactic acid bacteria (NSLAB) and their metabolic products, and prevents undesirable microbial growth and consequently off-flavour (Rowney et al. 2004). Although salt does not act directly as an antimicrobial agent, it has the ability to reduce the water activity (a_w) in foods (Albarracín et al. 2011) due to the ability of salt to associate with water molecules (Fennema 1996). In addition, salt can cause microbial cells to undergo osmotic shock, which causes loss of water from the cell and thereby causes cell death (Davidson 2001). The addition of salt inhibits the excessive growth of SLAB, leading to moderate proteolytic activity in cheese and better cheese quality (Guinee & Fox 2004). Table 2 presents some of the common starter culture bacteria used in cheese making process, and the minimum a_w in which these bacteria survive. Some SLAB, such as *Lactobacillus helveticus*, are highly proteolytic and an increase in a_w creates an appropriate environment for their cell growth. This stimulates greater acid production in cheese, which significantly affects cheese quality (Parente & Cogan 2004).

Table 2: Starter Culture Bacteria Associated with Cheeses and Other Fermented Products (Kongo 2013)

Species/subspecies	Main uses/other comments	Minimum a_w
Lactococcus	Mesophilic starter used for many cheese types.	0.95–0.96
Lc. lactis subsp. lactis	Used in Cheddar, gouda, edam, sour cream and lactic butter.	
Lc. lactis subsp. lactis biovar diacetylactis		
Lc. lactis subsp. cremoris		
Streptococcus	Thermophilic starter used for yogurt and many cheese types,	0.95–0.98
St. thermophilus	particularly hard and semi-hard high-cook cheeses.	
Lactobacillus		0.94–0.96
Lb. acidophilus	Probiotic adjunct culture used in cheese and yogurt.	
Lb. delbrueckii subsp. bulgaricus	Thermophilic starter for yogurt and many cheese types, particularly hard and semi-hard high-cook cheeses.	
Lb. delbrueckii subsp. lactis	Used in fermented milks and high-cook cheese.	
Lb. helveticus	Thermophilic starter for fermented milks and many cheese types, particularly hard and semi-hard high-cook cheeses.	
Lb. casei	Cheese-ripening adjunct culture.	
Lb. plantarum		
Lb. rhamnosus		
Leuconostoc	Mesophilic culture used for edam, gouda, fresh cheese, lactic	0.95–0.96
Ln. mesenteroides subsp. cremoris	butter and sour cream.	
Brevibacterium	Used in smear surface-ripened cheeses, such as camembert, stilton and	0.96–0.98
Brev. linens	limburger, and as a cheese-ripening adjunct culture.	
Propionibacterium	Used in Gruyère and Emmental cheeses.	0.96–0.97
Prop. acidipropionici		
Prop. freudenreichii subsp. shermanii		

Although lactic acid production can be separated from cell growth, it is likely that acid production at low salt levels is accompanied by high cell numbers, which tends to lead to bitterness (Guinee & O'Kennedy 2007) due to the production of low molecular weight peptides.

The a_w of foods depends on moisture content and the concentration of low molecular mass solutes in the moisture phase (Russell & Gould 2003). The a_w of cheeses ranges from 0.99 in Quarg to 0.91 in Roquefort cheese (Table 3). Increasing the salt-in-moisture (S/M) content provides lower a_w, which minimises spoilage and prevents the growth of pathogens (Guinee 2004a).

Table 3: Approximate NaCl, Na, S/M and a_w Levels of Selected Cheeses (Fox et al. 2004b; Guinee & O'Kennedy 2007; McSweeney 2007)

Cheese type	NaCl (%, w/w[1])	Na (%, w/w)	S/M (%, w/w)	a_w
Quarg	0.15	0.06	0.19	0.99
Emmental	0.70	0.27	1.80	0.97
Appenzeller	1.30	0.51	3.60	0.96
Low-moisture mozzarella	1.40	0.55	3.10	0.94
Cheddar	2.00	0.95	5.80	0.95
Limburger	2.00	0.79	4.40	0.97
Gouda	2.30	0.90	5.70	0.95
Danish blue	3.30	1.29	7.70	0.95
Roquefort	4.10	1.61	10.1	0.91
Romano-type	4.10	1.61	13.8	0.94
Feta	4.50	1.76	7.10	0.94
Domiati	6.00	2.35	10.9	0.94
Processed cheese products	0.70–1.62	1.00-1.50	------	0.98

[1] w/w = weight per weight of curd.

Table 4 shows the typical values of the minimum a_w required for the growth of various pathogenic microorganisms in foods, and the a_w of selected cheese varieties. The a_w of most cheese varieties is not low enough to prevent the growth of yeasts, moulds and many bacteria (including pathogens); however, with low pH and low temperature, salt is quite effective in controlling microbial growth in cheese (Guinee & O'Kennedy 2007).

Table 4: Typical a_w of Some Cheeses and Minimum a_w Required for Growth of some Pathogens and Non-Pathogens (Russell & Gould 2003)

Cheese	Typical a_w	Pathogen & Non-Pathogen	Minimum a_w
Quarg	0.99	*Shigella spp*	0.96
Cottage cheese	0.99	*Yersinia enterocolitica*	0.96
Camembert	0.98	*Pseudomonas spp*	0.95
Emmental	0.97	*Escherichia coli*	0.95
Gorgonzola	0.97	*Clostridium botulinum*	0.94
Edam	0.96	*Salmonella spp*	0.94
Cheddar	0.95	*Listeria monocytogenes*	0.92
Gouda	0.95	*Micrococcus spp*	0.87
Parmesan	0.92	*Staphylococcus aureus (aerobic)*	0.86
		Most yeasts and moulds	0.80
		Osmophilic yeasts and moulds	0.55

In dry-salted cheeses, such as Cheddar cheese, acid production (decrease in pH) is inhibited by salt; thus, more residual lactose remains in the curd, which is metabolised by NSLAB that are normally more salt resistant than SLAB in the early stages of ripening (Guinee & Fox 2004). The major consequences of residual lactose metabolism by NSLAB are:

1. production of a racemic mixture of lactose at the end of ripening by conversion of L-lactate to D-lactate
2. L-lactate being metabolised to propionate, acetate and carbon dioxide (CO_2), which are responsible for eye formation and contribute to typical flavour in Swiss-type cheeses
3. some lactate may be oxidised to acetate by *Pediococcus* in Cheddar and Dutch-type cheeses (Fox et al. 1990).

2.5.2 Role in Physicochemical Characteristics of Cheese

2.5.2.1 Casein Hydration or Water-holding Capacity

Salt has an effect on the hydration of casein and consequently on the water-binding capacity of the casein matrix (Guinee & O'Kennedy 2007). Hydration or water-holding capacity is defined as the number of grams of water associated with or occluded by 1 g of dry protein (Fox & Mulvihill 1983). Guo and Kindstedt (1995) and Guo et al. (1997) measured casein hydration in mozzarella cheese indirectly by determining the level of

serum protein (SP) and level of expressible serum (ES) by centrifugation of the cheese at 12,500 g at 25°C. A higher level of SP indicates higher casein hydration and water-holding capacity. Brine-salted mozzarella (1.4% w/w NaCl) cheeses showed a drop in ES from about 16 to 1 g/100 g cheese over the first 10 days of ripening, indicating an increase in the water-binding capacity of the protein. Compared to high-salt (1.36%) cheeses, the level of ES for the unsalted mozzarella cheeses (0.13% w/w NaCl) changed only marginally over the same period, from about 19 to 14 g/100 g cheese. Paulsan et al. (1998) studied the casein hydration for fat-free mozzarella cheeses for which the curd was dry-salted to different levels (0, 0.5 or 1.0%, w/w) prior to stretching in hot water containing 0, 5 or 10% w/w NaC1. Over 24 days of storage, the level of ES in the cheeses from curds stretched in hot water decreased as the level of dry salting prior to stretching was increased from 0.5% to 1.0% w/w. An increase in casein hydration leads to a more viscous and less elastic texture, and changes the elastic fracture behaviour to plastic fracture behaviour in cheese (O'Callaghan & Guinee 2004). Excessive casein hydration results in structure of cheese with poor texture (Fox & McSweeney 1996).

Murtaza et al. (2014) reported that excessive casein hydration in salt-reduced Cheddar cheeses caused a decrease in the hardness of the cheeses during ripening. Additionally, production of a large amount of lactic acid by a starter culture in low-salt cheeses led to decreases of the pH of cheese, thereby amplifying moisture expulsion along with soluble calcium from curd. This resulted in breakdown of the casein–casein linkage in the cheese matrix, and subsequently caused higher hydration of casein.

There is a difference in the hydration behaviour between different types of caseins as affected by an increase in S/M. Mistry and Kasperson (1998) reported that an increase of S/M from 2.7 to 4.5% caused a decrease in hydration of α-casein from 70.97 to 64.72% and β-casein from 20.72 to 17.73%.

2.5.2.2 Effect on Proteolysis

Salt concentration has a direct effect on the activity of the proteolytic enzymes (coagulant, indigenous milk enzymes and lactic acid bacteria [LAB] enzymes) in cheeses (Fox & Guinee 2013). An increase in salt concentration in cheese decreases the a_w, which reduces the enzyme activity because free water, as a solvent, is essential for

enzymatic reactions (Fox & McSweeney 1996; Grummer & Schoenfuss 2011; Upadhyay et al. 2004). In a recent study, proteolysis in Cheddar cheeses was shown to be high (25%) at low salt levels, compared to 18% at a salt level of 2.5% (Murtaza et al. 2014).

Proteolysis is driven by enzymes (rennet) added to the cheese milk and enzymes produced by starter cultures during ripening (Fox et al. 2000a; Fox et al. 2004b). SLAB and NSLAB possess extensive proteolytic systems (Beresford & Williams 2004; Upadhyay et al. 2004). Higher activity of starter microbial proteinase in low-salt cheese leads to development of a bitter taste (Beresford & Williams 2004) due to increased production of low molecular weight peptides.

The residual coagulant is mainly responsible for the degradation of casein to peptides during maturation. Peptides are degraded further by the action of bacterial peptidases to free amino acids, which contribute to flavour development. Degradation of αs_1-casein and β-casein has an inverse relationship with salt concentration. Salt inhibits proteolysis of sodium caseinate, αs_1-casein and β-casein at different levels. However, even in the absence of salt, the proteolysis of β-casein is low. This may be due to the presence of milk salts, which are sufficient to cause inhibition. In addition, protein–protein interactions may contribute to the low level of proteolysis. The C-terminal region of β-casein is very hydrophobic, and intermolecular hydrophobic interactions may cause the chymosin-susceptible bonds to become inaccessible (Fox et al. 2000a; Phelan et al. 1973; Upadhyay et al. 2004).

A low concentration of salt (below 2%) stimulates residual plasmin (milk proteinase) activity in cheese, but is inhibited by higher concentrations. There is an inverse trend between salt concentration and plasmin activity (Fox et al. 2000a; Kilcast & Angus 2007; Upadhyay et al. 2004).

2.5.3 Effect on Starter and Non-starter Culture Activity

Salt significantly influences the growth of mesophilic (such as *L. lactis ssp.* strains *cremoris* and *lactis*) and thermophilic SLAB. Salt tolerance varies among starter culture strains. For example, *Lactococcus lactis ssp. lactis* is more salt tolerant than

Lactococcus lactis ssp. cremoris (Fox et al. 2000b) and this will affect the sensory quality of cheese, the higher the salt concentration, the lower the growth of bacteria, and vice versa. Salt affects the bacterial population density, lactose use and bacterial ability to reduce curd pH during cheese manufacture (Guinee & O'Kennedy 2007). The activity of starter culture bacteria is stimulated at ~2% (w/w) S/M and extremely inhibited at 5% (w/w) S/M. Reducing salt causes uncontrollable lactic acid production due to the high number of bacteria, leading to an undesired sour taste (Fox et al. 2000b).

In dry-salted cheeses such as Cheddar, inhibition of acid production (decrease in pH) by the addition of salt leads to higher residual lactose in the curd, thereby encouraging the growth of NSLAB that are normally more salt resistant than SLAB during the early stages of ripening (Guinee & Fox 2004). In addition, reduction of salt can trigger growth of secondary microflora (such as *Lactobacillus*, *Pediococcus* and *Micrococcus*) that are indigenous to milk and have survived pasteurisation or entered after pasteurisation, and other pathogenic bacteria in cheese (Fernandes 2008).

2.5.4 Effect on Sensory Attributes

Reducing salt in cheese affects colour change (whiteness) during ripening by increasing the oxidation of lipids. Decrease in salt concentration results in less lipid oxidation, which reduces the change of colour in cheese (Kaya 2002). Salt reduction triggers more aggregation of proteins in the cheese matrix, and consequently causes more pockets of free serum. The edges of these pockets provide a surface at which light can be scattered, giving the cheese an opaque appearance. Salting the cheese causes the absorption of free serum into the matrix, and gives a homogeneous matrix with less light scattering surfaces. Therefore, the salted cheese becomes translucent (Paulsan et al. 1998).

Reducing salt in cheese accelerates protein hydration, which has a major influence on the structure, physical properties and quality of cheese. A soft, weak and pasty body is associated with low salt concentration due to excessive proteolysis. At high salt levels, the cheese texture becomes excessively firm as a result of lower proteolysis and a lower degree of casein hydration.

Salt reduction induces expulsion of colloidal calcium phosphate (CCP) from the curd, leading to a soft structure of cheese (Lucey et al. 2003; Roefs et al. 1985). Reduction of Ca^{2+} ions causes weakness in the casein–casein network throughout the cheese matrix, leading to greater hydration of proteins during storage, and subsequently less hardness (Gunasekaran & Ak 2003; O'Callaghan & Guinee 2004; Vélez-Ruiz 2009).

High acidity (low pH) as a result of salt reduction strengthens the protein bonds by decreasing negative charges on casein molecules. Therefore, less water is absorbed by proteins, resulting in 'longness' (a tendency to fracture only after relatively large deformation) of cheese texture, and greater cohesiveness (Gunasekaran & Ak 2003; O'Callaghan & Guinee 2004).

Salt makes a direct positive contribution to cheese flavour, as most consumers appreciate a salty taste in foods. Salt-free cheese has a rather insipid taste, which can be masked by addition of 0.8% salt (Fox et al. 2000a). Defects such as bitterness are also common at low salt concentrations. It is likely that flavour defects encountered at low salt concentrations arise from excessive or unbalanced enzyme activity. For example, bitterness can occur in Dutch-type cheeses if excessive proteolysis of β-casein by chymosin occurs, which releases bitter C-terminal peptides, such as β-casein fl93-209. Reducing salt increases the acidic taste in cheese, causing an undesired flavour. This occurs in response to a higher a_w in cheese, which increases LAB and NSLAB activity, and thus enhances acid production (Chamba & Irlinger 2004; Fox et al. 2000b).

2.5.5 Effect on Perception of Saltiness and Salt Release from Cheese

Although reducing salt is a serious concern for the public health and food industries, the problem of decreased perception of saltiness in sodium-reduced food products makes development and acceptance of reduced salt products difficult. Perception of saltiness is one of the main factors influencing the acceptability of a food product. Reducing or replacing sodium in cheese decreases its acceptability due to the poor saltiness perceived by consumers. This perception depends on the speed and amount of salt released from the cheese into the mouth during chewing. Increased contact between salt and the tongue papillae may overcome the perception of low salt taste. This depends on

the characteristics of the cheese matrices controlling the diffusion of salt within and from the matrix to the saliva.

The sodium ion (Na^+) is central to perceptions of saltiness (Rawson & Li 2007). Saltiness is perceived mainly due to the sodium ion in collaboration with other elements inside the mouth cavity. One of the mechanisms for sodium detection in the mouth is through the epithelial sodium channel (ENaC), which acts as a salt taste detector by supplying a specific pathway for sodium absorption into taste cells (Morris et al. 1985). Figure 3 illustrates the process of saltiness detection by ENaC. The epithelium is represented as a lipid bilayer (round circles), the area above the lipid bilayer (oral cavity) is the outside of the taste receptor cell, and the area below the lipid bilayer is the interior of the taste receptor cell. The channel itself is comprised of three protein units (α, β and γ) that are negatively charged because of the presence of amiloride, a potassium-sparing diuretic. This channel forms a tunnel through the taste receptor cell that allows Na^+ ions outside the cell to move inside. Once sodium is inside the taste receptor cell, it causes some biochemical reactions that result in calcium ions (Ca^{2+}) entering the cell. The calcium ion is a trigger for the release of neurotransmitters that signal a salty taste to the brain (Henney et al. 2010).

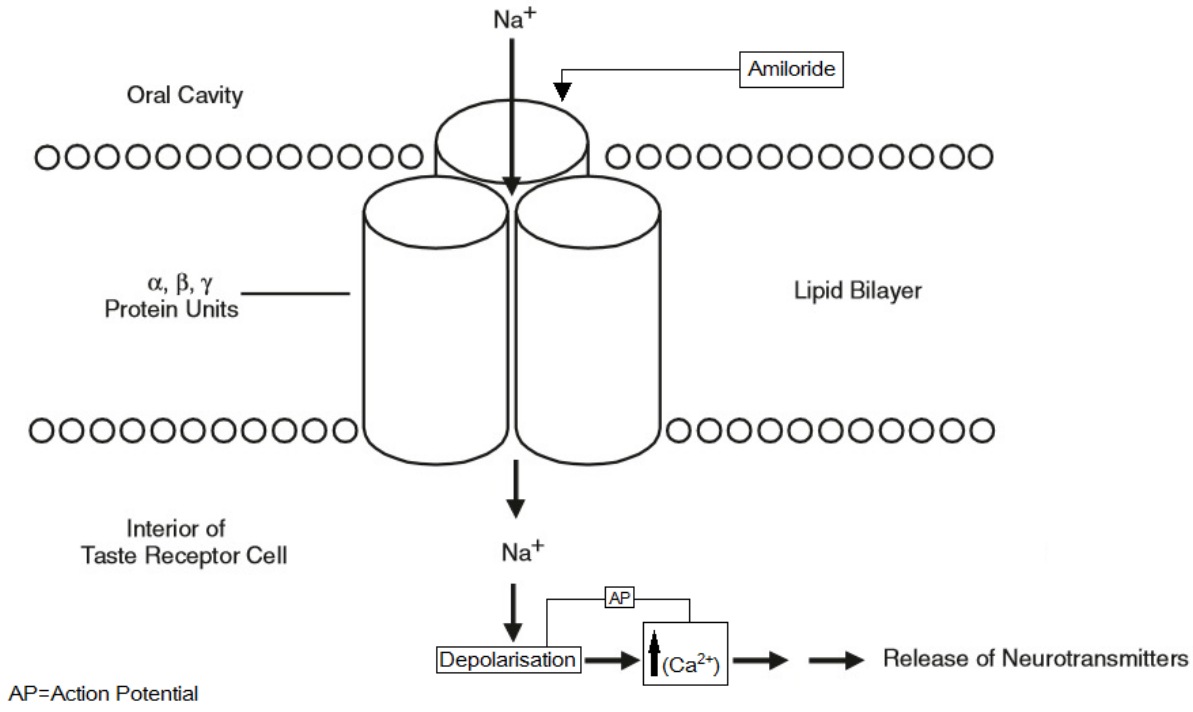


Figure 3: Schematic of the ENaC Responsible for Salty Taste (Henney et al. 2010; Taylor & Roberts 2004)

The perception of saltiness in salt-reduced food products has triggered research into the mechanism of release of NaCl from different food matrices (Taylor & Roberts 2004), including studies investigating the mechanism of salt release from different imitation cheese matrices (Castelli & Du Vale 2013; Floury et al. 2009b). These studies considered and adjusted factors such as dry matter (DM), fat in dry matter (FDM), different salt contents and pH. However, the effects of other important factors, such as coagulant, pH at different stages and ripening time on release of salt from the cheese matrix, require further investigation.

Morris et al. (1985) examined the diffusion of salt in Cheddar cheese salted with different salt levels (0, 2.8 and 5.6 % NaCl), followed by storage at 8°C for 24 weeks. They concluded that establishing a salt equilibrium in Cheddar cheese is a very slow process, and that inadequate distribution of salt would lead to an improper salt equilibrium throughout the cheese during the ripening period. Moreover, the random positioning of salted curd pieces formed in cheese may decelerate this salt equilibrium process throughout the cheese, leading to reduced diffusion within the matrix, and affecting the release of salt during mastication (Morris et al. 1985).

Phan et al. (2008) examined the perception of saltiness when eating model cheeses of different textures. Cheeses were made with two levels of mixing speed (to mix ingredients during the manufacturing process) and two different fat concentrations (40% and 50%). The fat/water ratio, salt concentration in saliva, saltiness perception and masticatory parameters for each product were assessed. Products made with a higher mixing speed (2,000 rpm) showed more salt release in the mouth during mastication. After 20 seconds of mastication, the release of salt in the saliva from cheese containing 50% FDM was ~0.075 g/100g of saliva, compared to 40% FDM, which was ~0.068 g/100g of saliva. The longer the chewing time, the higher the perception of saltiness due to increased deformation of the matrix and increased water content in the mouth cavity because of increased salivation. Fat content showed a positive effect on sodium release in cheeses with higher water content. This may be due to increased formation of salt containing water phase into the fat emulsion at and during in-mouth breakdown of cheese (Phan et al. 2008).

Floury et al. (2009b) studied model cheeses made with different compositions (DM, FDM and salt [0.5 and 1.5 g/100 g]) and investigated the effect of different factors (rennet concentration, fat content and pH) on the release of salt in saliva. An increase in pH at the renneting stage from 6.2 to 6.5 caused an increase in the diffusion coefficient (D) from 2.81 to 3.34 × 10^{-10} m^2 s^{-1}. This might have been due to the decrease in the negative charge of the casein micelles, leading to less interaction with the Na^+ ion. In model cheeses with a lower salt concentration (0.5 g/100 g), the D value significantly decreased (2.81 × 10^{-10} m^2 s^{-1}) due to the tightened microstructure of the cheese as a result of more negative charge in the casein micelles. The composition and geometrical arrangement of the casein network in the matrix was affected by reducing the initial salt content (from 1.5 to 0.5 g/100g), which significantly increased the hardness and cohesiveness and decreased the adhesiveness. Reducing salt causes a decrease in the apparent mean diameter of the protein particles, which decreases the effective pore width of the matrix (Geurts et al. 1974). At low protein content, the diffusion of salt is higher due to rougher and softer microstructure (Floury et al. 2009b).

De Loubens et al. (2011) investigated the effect of mastication on salt release from model cheeses. The breakdown of texture increased the release of salt, while a higher fat content in cheese induced higher breakability, thereby leading to higher release. Lawrence et al. (2012) studied the effects of composition and texture on the release of salt from a solid lipoprotein model. Using milk, they prepared lipoprotein matrices (two levels of DM content [370 and 440 g/kg], two fat content levels [20 and 40% w/w DM], three salt content levels [0.5, 1 and 1.5%] and two pH at renneting levels (6.2 and 6.5]). They then analysed these matrices for texture and sensory profiles. Changes in DM, salt and fat content influenced taste perceptions and texture. The amount of sodium released showed a positive correlation with the moisture and fat content. The increase in moisture and fat content (40%) triggered a higher release of sodium in cheeses containing 1.5% salt. The higher the fat content, the higher the brittleness, and consequently the higher the salt release.

Modifying the cheese processing steps, such as changing the pH at different stages, may affect the salt release from the final cheese product. Changes in the enzymatic activity during ripening, as affected by reduction in salt concentration in cheese, may change the protein structure in the cheese matrix, thereby affecting S/M levels. These modifications

can increase the salt release from cheese into the mouth, thereby increasing the sensation of a salty taste.

2.6 Mechanism of Salt Diffusion in Cheese

Diffusion of NaCl into cheese and migration of water out of cheese is an impeded diffusion process (Geurts et al. 1974; Geurts et al. 1980). There are several components impeding the movements of salt ions and water throughout the cheese matrix. Protein and fat act as a barrier for salt and water movements through the matrix (Guinee 2004a; Guinee & Fox 2004). The outward flux of water (migration of water out of cheese is higher compared with low in-flux of Na^+ and Cl^+) reduces the inward Na^+ and Cl^- flux. Salt and water will encounter a tortuous path created by structural differences due mainly to fat globules and protein mass (Guinee 2004a; Guinee & Fox 2004). Hence, the Na^+ diffusion in cheese moisture is significantly slower than that in pure water (Guinee 2004a; Guinee & Fox 2004).

The movement of Na^+ and Cl^- ions throughout the cheese matrices is impeded by: (i) fat globules and protein aggregates, (ii) the number and size of pores, and (iii) the higher viscosity of cheese moisture (~1.27 times more than pure water at 12.5°C) due to dissolved substances (Fox et al. 2000a).

Salt movement in cheese can be described by Equation 1:

Equation 1:

$$\frac{\partial C}{\partial t} = \nabla [D \times \nabla(C)]$$

where *C* represents the Na or Cl concentration (mol kg^{-1}), *t* is the time (seconds) and *D* is the effective diffusion coefficient (m^2 s^{-1}).

Guinee and Fox (1983) and Simal et al. (2001) reported apparent diffusion coefficients for NaCl in cheese (either brine salted or dry salted) to be approximately 0.23 cm^2/day, and varied from about 0.1 to 0.45 cm^2 per day depending on the cheese composition,

brining conditions and temperature. These values were significantly lower than the 1.0 cm^2 per day in pure water at 12.5°C recorded by the same authors (Guinee & Fox 1983).

During dry salting, salt slowly dissolves in the surface moisture of the curd, causing a flow of whey from the centre to the surface, which dissolves the remaining salt crystals. A large surface area to volume ratio of the curd results in very rapid uptake of salt (10 to 20 min for milled Cheddar curd) during dry salting (Simal et al. 2001; Turhan & Gunasekaran 1999).

2.7 Methods of Salt Reduction in Cheese

2.7.1 Salt Reduction without Substitution

The strategies used to reduce salt in cheese are as follows:

1. gradually reducing the salt in cheese over a period (reduction by stealth), so that it cannot be detected by consumers (Beeren 2013; Liem et al. 2011)
2. reducing the salt concentration in cheese to the limit that does not adversely affect quality and taste (Reddy & Marth 1991)
3. adding flavour enhancers to compensate for the loss of saltiness (Duran-Meras et al. 1993)
4. adjusting the cheese texture to improve the salt distribution inside the cheese in order to enhance salt release (Noort et al. 2010).

Salt reduction without substitution is the preferred technique among cheese manufacturers; however, several concerns need to be addressed. The increase in bitterness during storage and excessive microbial growth are the main issues with low-salt cheeses. Schroeder et al. (1988) investigated the effect of reducing sodium chloride from 1.44 to 0% on the sensory, microbiological and chemical properties of Cheddar cheese. Cheese quality was analysed over seven months of storage. Proteolysis (soluble tryptophan and tyrosine), growth of LAB and a_w (~0.96 to 0.98) increased in the low NaCl cheeses. At the end of storage, the increase in soluble tryptophan from ~75 to 90 mg/100 g and tyrosine from ~210 to 260 mg/100 g resulted from excessive proteolysis in the cheese containing the lowest salt concentration (0.07%, 0.37% and 0.73%). The

overall acceptability of Cheddar cheeses salted between 1.12 and 1.44% was substantially higher than cheeses salted at 0.73% (Schroeder et al. 1988).

Kelly et al. (1996) reported that the rate of proteolysis in Cheddar-type cheese made with salt levels between 0 to 3.3% w/w had an inverse relationship with salt concentration. Water-soluble nitrogen (WSN) production was less in cheeses with high S/M values, while the level of 5% (w/v) phosphotungstic acid-soluble nitrogen (PTA-SN) was higher. S/M influenced the rate of hydrolysis of the caseins. The effect of coagulant on breakdown of the bonds Leu_{192}–Tyr_{193} in β-casein and Leu_{101}–Lys_{102} in α_{s1}-casein was sensitive to NaCl concentration. The production of short peptides (particularly β-CN f193-209) increased in cheeses with low S/M levels, as indicated by reverse-phase high-performance liquid chromatography (HPLC). The physicochemical properties of feta cheese brined at 8, 15 and 18% NaCl were investigated by Prasad and Alvarez (1999) during a storage period of 63 days. The feta cheeses brined in higher salt concentration (15% and 18%) became harder during storage (Prasad & Alvarez 1999).

Kaya (2002) monitored the changes in hardness and colour of Gaziantep cheese kept in brines with different salt concentrations (5, 10, 15, 20 and 25% NaCl), followed by storage for two weeks. Cheeses brined in 20 and 25% salt solutions showed higher hardness (45 to 55 N) than did the cheeses with less salt content (~3 to 11 N). Kilic and Isin (2004) examined the texture of Dil cheese brined at two levels of salt concentrations (3 and 6% [control]) during storage for three months. At higher salt concentration (6%), the Dil cheeses showed harder texture (~7 N/g cheese) than did cheeses with lower salt concentrations (~4 N/g cheese).

Murtaza et al. (2012) examined the effect of NaCl reduction on the production of organic acids in buffalo milk Cheddar cheeses salted at 0, 1 and 2% (NaCl, w/w) during storage for 120 days at 4 and 12ºC. An increased salt level decreased the production of organic acids (lactic from ~20,000 ppm to ~14,000 ppm, acetic from ~550 ppm to ~380 ppm, and citric from ~1,100 ppm to 800 ppm) due to the limited activity of starter bacteria during storage. The texture of buffalo milk Cheddar cheeses made with 2.5, 2.0, 1.5, 1.0 and 0.5% (w/w of the curd) salt concentration and ripened at 6 to 8°C for 180 days showed a significant decrease in hardness (from ~190 to ~110 N), toughness (from ~180 to ~130 kPa) and crumbliness (from ~3.5 to ~2.5) due to an increase in proteolysis

(from ~19 to ~25%) at the end of ripening. Cheese samples with salt concentration < 2% were shown to be less acceptable during sensory analysis (Murtaza et al. 2014).

Ganesan et al. (2014) found that the perception of sensory attributes (salty, sour, umami, bitter and brothy flavours) significantly increased with the increased salt level (from 0.75 to 1.80% w/w). In Cheddar cheeses, salty and buttery attributes were perceived more by the panellists as the salt concentration increased at three months, whereas bitter, brothy and umami attributes were perceived less at higher salt levels. However, at six months, cheeses containing the highest salt level (1.50 and 1.80%) showed increased salty, sour, bitter, buttery, lactone/fatty acid and umami attributes (Ganesan et al. 2014). Consumer preference for low-sodium Cheddar and mozzarella cheeses made with 0.7% and 0.9% salt was low (Ganesan et al. 2014).

2.7.2 Salt Replacements

Salts such as KCl, $CaCl_2$ and $MgCl_2$ have been used to replace NaCl or enhance salt taste in a number of cheeses, such as Cheddar and mozzarella (Van Der Klaauw & Smith 1995). Replacing salt from 650 mg/100 g cheese with 283 mg of KCl and 72 mg NaCl has been found to provide satisfactory sensory quality in Gouda cheese (Martens et al. 1976).

Reddy and Marth (1993a) salted a Cheddar cheese with different NaCl/KCl mixtures (2:1, 1:1, 1:2 and 3:4) to achieve final salt concentrations of approximately 1.75%. The sensory properties were similar to the control cheese made with only NaCl (1.75%). However, cheeses made with equal amounts of KCl and NaCl (1:1) were preferred over the other cheeses. Katsiari et al. (1997, 2000b, 2000c) reported that substitution of NaCl with KCl up to 50% (1NaCl:1KCl) in feta cheese during 180 days of storage did not adversely affect the cheese characteristics, which were similar to the control (4% NaCl). Cheeses salted with 3NaCl:1KCl received higher acceptability scores than did cheeses made with other salt mixtures. The compositional, physicochemical, sensory and textural properties showed no significant difference between cheeses made with the 1NaCl:1KCl mixture and the control cheese (Katsiari et al. 1997; Katsiari et al. 2000c). A 50% reduction in salt content by substituting KCl for NaCl (1NaCl:1KCl) had no

effect on lipolysis, proteolysis or the sensory attributes of Greek Kefalograviera cheese (Katsiari et al. 1998; Katsiari et al. 2001a; Katsiari et al. 2001b).

Laborda and Rubiolo (1999) investigated the proteolysis of Fynbo (a semi-hard Danish cheese) made using a brine solution containing NaCl and KCl salt mixture. Twelve cheeses were salted in a brine of 190g NaCl/L and 12 other cheeses were salted in a solution of 100g NaCl/L and 100g KCl/L for 10 h at 12°C. The Fynbo cheeses salted with the NaCl/KCl mixture were similar in terms of saltiness and firmness with the control made only with NaCl; however, a slight bitterness was noticed in cheeses made with the NaCl/KCl mixture (Zorrilla & Rubiolo 1999).

In another study, the substitution of $CaCl_2$, KCl and $MgCl_2$ for NaCl had no effect on the proteolysis and acidity of Turkish white cheeses stored (for 12 weeks) in different brines containing 14% 1NaCl:1$CaCl_2$, 1NaCl:1KCl, 1NaCl:1$MgCl_2$, 1NaCl:0.33$CaCl_2$ and 0.33KCl:0.33$MgCl_2$ for the same storage period (Guven & Karaca 2001).

Other studies found that the chemical composition, microbial growth and proteolysis of halloumi cheeses (18% salt) made with salt replacers (NaCl:KCl) showed a similar trend to the control (only NaCl) in the same storage period. Cheeses were brined in four different brine solutions including only NaCl, 3NaCl:1KCl, 1NaCl:1KCl and 1NaCl:3KCl, and stored at 4°C. Proteolysis increased during storage in all cheeses, regardless of the salt content. Cheeses made with the 1NaCl:1KCl mixture showed greater similarity to the control than did the other treatments. The hardness of all halloumi cheeses was similar to the control for the same storage period (Ayyash & Shah 2010, 2011a).

A similar study was conducted on low-moisture mozzarella cheese brined in four different brine solutions (4% salt) including only NaCl, 3NaCl:1KCl, 1NaCl:1KCl and 1NaCl:3KCl, and stored at 4°C. For the same storage period, the proteolysis values of cheeses made with a higher KCl ratio were significantly higher than those made with a higher NaCl. Cheeses salted with 1NaCl:1KCl and 1NaCl:3KCl showed higher meltability than did the control (only NaCl). The pH values of cheeses salted with 1NaCl:1KCl and 1NaCl:3KCl were higher than the control cheese (Ayyash & Shah 2011b, 2011c).

Grummer et al. (2012) investigated the effect of different salt replacers (KCl, $MgCl_2$ and $CaCl_2$) on the chemical, physical and sensory attributes of Cheddar cheese. Reduced-sodium cheeses were made by mixtures of NaCl and KCl, $MgCl_2$ or $CaCl_2$ and stored at 4 to 5°C for six months. During the early stages of storage, the a_w of the reduced-sodium cheeses did not show a significant difference to the control (only 1.6% NaCl). The treatments containing 1NaCl:2$CaCl_2$ showed the highest hardness values (8.49 g) compared to the other cheeses. The treatments with $MgCl_2$ and modified KCl showed a softer texture (5.58 and 4.53 g, respectively) compared to those made with $CaCl_2$ and sea salt. The treatments containing $CaCl_2$ and $MgCl_2$ produced a considerable off-flavour in the cheeses (bitterness, metallic, unclean and soapy). The cheeses made with reduced-sodium sea salt were firmer, more brittle and less sticky than those containing NaCl. The cheeses made with 1NaCl:1KCl and 1NaCl:1modified KCl were similar to the cheeses made with only NaCl in most aspects. Therefore, KCl was suggested to replace a portion (almost half) of the NaCl in Cheddar cheese.

In another study, Grummer et al. (2013) prepared Cheddar cheese using two different types of potassium chloride (KCl1 [premier potassium chloride 8799] and KCl2 [modified potassium chloride 14510]) in combination with selected flavour enhancers (Table 5) and reported that the cheeses were liked by the consumer panel, and that the scores of the reduced-sodium cheese with two different KCl sources were no different to those of the full sodium cheese (2% w/w). However, the use of flavour enhancers had some negative effects on the cheese's sensory attributes. For example, a brothy flavour and aroma and umami flavour were increased in the cheese containing disodium 5' inosinate (disodium 3, 4-dihydroxy-5-methyl phosphate), leading to lower consumer acceptance.

Table 5: Flavour Enhancers (g of Enhancer/100 g of Cheese) Used in Reduced-sodium Cheddar Cheeses Containing KCl (Grummer et al. 2013).

Flavour enhancer	Product description	Concentration (g/100 g)	Flavour description
Sav Nat Fl Enhancer 0188404	HVP[1]/YE[2]	0.131	Umami/brothy, salty
Sav Salt Reducer/Enhancer 0188807	YE	0.131	Umami/brothy, Cheddar
Sav Salt Reducer/Enhancer 0189154	YE and natural flavour	0.131	Unclean, salty
Sav MSG Replacer 0187641	HVP/YE/IMP[3]/GMP[4]	0.075	Process cheese, low bitter
50/50 Disodium Inosinate/Disodium Guanylate	IMP/GMP blend	0.225	Beefy, umami/brothy, unclean
DM Choice Natural Flavour 'Potassium Blocker Type'	Natural flavour	0.146	Clean, nondescript
Natural Salt Replacer #2 (#319678)	Salts, sugar, natural flavours, and whey	0.150	Buttery, astringent
Natural Salt Replacer #3 (#319679)	YE, natural flavour	0.150	Cheddar, salty
Natural Cheddar-Type Flavour #1411344-Powder	Natural flavour	0.249	Unclean, musty
Natural Masking Flavour #1411308-Powder	Natural flavour	0.200	Process cheese, bland
Natural and Artificial Masking Flavour #1411662-Powder	Natural and artificial flavour	0.200	Fruity, fruit punch
Natural Cheddar Flavour WONF #1411086-Spray Dry	Natural flavour	0.498	Unclean, metallic
Natural Cheese Flavour WONF #2406-Powder	Natural flavour	0.249	Umami/brothy, astringent, less salty
AJITIDE IMP Disodium 5' Inosinate	IMP	0.233	Beefy, umami/brothy
Flavorshure C-Salt CC98	Choline chloride	0.100	Buttery
CJTIDE Disodium 5' Guanylate	GMP	0.233	Buttery

[1] HVP = hydrolysed vegetable protein
[2] YE = yeast extract
[3] IMP = disodium 5'inosinate
[4] GMP = disodium 5'guanylate.

To summarise, although salt replacers may contribute to a salty taste, they may also induce undesirable aftertastes, such as a bitter, metallic and sharp taste; hence, their application in cheese manufacturing must be carefully considered (Lawless et al. 2003; Reddy & Marth 1991). To mask or avoid these defects, flavour enhancers have been suggested for use as sodium replacers in cheese (Grummer et al. 2013). Replacing NaCl with other salts and flavour enhancers has demonstrated acceptable results without adverse effects on cheese characteristics, compared with simple salt reduction. Moreover, out of several salts investigated, KCl was found to be the most acceptable for partial NaCl replacement. In addition, KCl is not associated with the development of hypertension or cardiovascular diseases (Buemi et al. 2002; Geleijnse et al. 2007).

2.8 Chapter Summary

Although reducing salt is a serious concern for public health and food industries, the problem of acceptance of sodium-reduced food products by consumer's remains to be solved to derive health benefits. As sodium ions (Na^+) are central to the perception of saltiness (Rawson & Li 2007), reducing or replacing sodium in food decreases the food's acceptability due to a low salty taste perceived by consumers, depending on the speed and amount of salt released from the food into the mouth during mastication. Therefore, accelerating the contact of salt with the tongue papillae will decrease the perception of low saltiness. The release of salt from cheese depends on the characteristics of the matrices controlling the diffusion of salt.

Modifying the cheese matrix via manipulating the composition of cheese milk (such as the C/F ratio) and the cheese processing steps (such as changes in rennet concentration and pH at different stages) may significantly affect the salt release from the final cheese product. These modifications may contribute to increasing the amount of salt released from the cheese into the mouth during mastication, and thus increase the perception of a salty taste.

A successful reduction in the sodium content of cheese is only possible via research on the effect of the composition of milk in terms of protein and fat content, in order to understand the influence of these factors on the overall quality of Cheddar-type cheeses. Additionally, variation in the process of making Cheddar cheese (such as the pH at drainage and variation of proteinase [rennet] activity) requires further investigation.

Chapter 3: The Effects of Salt Reduction on Hard-type Cheese Characteristics Made Using High Proteolytic Starter Culture During Storage[2]

3.1 Introduction

Salt has been used in food preparation since ancient time and is being used in modern food manufacturing processes due to its sensory and preservation properties. In cheese, salt contributes to flavour (Guinee 2004a), accelerates whey removal from the milled curd and controls the growth of undesirable bacteria (Emmons & Modler 2010). However, excessive intake of salt has been associated with high blood pressure, kidney stones and osteoporosis (Turk et al. 2009). Therefore, health authorities in the US have recommended a limitation on the dietary intake of sodium to about 2.5 to 3 g per day (McCarron et al. 2009). The WHO has also recommended reducing average salt consumption for adults to < 5 g per day (Flock & Kris-Etherton 2011). The daily intake of salt in the Australian diet is over 3 to 5 g per day (NHMRC 2003) and must be reduced to match these recommendations.

Cheeses contribute about 4% of individuals' daily sodium intake in the UK (Ash & Wilbey 2010), 9.2% in France (Meneton et al. 2009) and 5% in Australia (NHMRC 2003). Thus, reducing the salt in cheese will assist in meeting the recommended safe daily dietary intake of sodium. This is particularly significant because cheese is one of the most popular dairy products, and its consumption is likely to increase (Fox & McSweeney 2004). It has been estimated that, by consuming cheeses with medium salt content (2% w/w), the dietary sodium intake would decrease to approximately 20% (He & MacGregor 2010). Reduction or substitution of salt can induce subtle and significant changes in the quality of cheese (Guinee 2004b). Lack of flavour, loss of desired texture and excessive growth of microorganism in cheese are known quality defects associated

[2] A major part of this chapter has been published as a journal article: Sheibani, A, Ayyash, MM, Shah, NP & Mishra, VK 2015, 'The effects of salt reduction on characteristics of hard type cheese made using high proteolytic starter culture', *International Food Research Journal*, vol. 22, no. 6, pp. 2452–2459.

with salt reduction (Guinee & Fox 2004), mainly due to effects on the biochemistry of the ripening processes.

However, replacing sodium chloride (NaCl) with other salts, such as KCl and $MgCl_2$, also leads to noticeable bitterness and a metallic taste (Grummer et al. 2012). McMahon et al. (2009) reported that the moisture content of feta cheeses decreased when brine concentration increased at 3°C. The hardness of fat-reduced Cheddar cheese showed a decrease due to a reduction of salt in moisture (S/M) and increase in moisture (Mistry & Kasperson 1998). In other studies, partial substitution of NaCl with KCl did not have a significant effect on the proteolysis of feta cheese (Merćep et al. 2010) or Minas cheese, a semi-soft white Brazilian cheese (Gomes et al. 2011). In addition, no significant difference in the textural characteristics of halloumi cheese was found upon partial substitution of salt with KCl (Ayyash et al. 2011).

One of the approaches to offset potential textural and flavour issues is to improve the metabolic activity of starter cultures, with adjunct cultures having significant proteolytic activity (Børsting et al. 2012; Sato et al. 2012). The rennet and adjunct starter culture can contribute to the formation of both total peptides and soluble peptides. The peptidase and proteinase enzymes from the adjunct starter culture are capable of hydrolysing proteins effectively, thus releasing more intermediate and smaller size peptides. These soluble nitrogenous compounds can contribute directly to cheese flavour. For example, using *Lactobacillus helveticus*, a strong proteolytic culture, results in higher production of amino acids [Aspartic Acid (Asp), Glutamic Acid (Glu), Serine (Ser), Histidine (His), Glycine (Gly), Threonine (Thr), Arginine (Arg), Alanine (Ala), Tyrosine (Tyr), Valine (Val), Methionine (Met), Phenylalanine (Phe), Isoleucine (Ile), Leucine (Leu), Lysine (Lys) and Proline (Pro)] and non-protein nitrogen, which influences the flavour in cheese (Nateghi 2012). In contrast, excessive production of amino acids and hydrolysis of protein result in a poor and unacceptable structure of cheese. Reduction of salt accelerates an increase in peptidase and proteinase enzymes from the starter culture, as well as an increase in protein hydration, which adversely influences the texture and flavour of cheese (Guinee & Fox 2004).

The objective of the current study was to investigate the effect of salt reduction on the chemical composition, starter culture growth, organic acid production, proteolysis, anti-hypertensive properties, microstructure and textural profile of Cheddar cheese made with a starter culture preparation containing the high proteolytic starter culture (*L. helveticus*) in combination with *S. thermophilus*, during storage at 4.5°C for eight weeks.

L. helveticus was selected because of its high proteolytic activity, proven health benefits and ability to survive through the gastrointestinal tract (McIntosh et al. 1999; Ong & Shah 2008a). *L. helveticus* was expected to (i) produce more free amino acids, which contribute to flavour development and affect the texture, and (ii) produce peptides that contribute to health benefits, as these peptides are known to inhibit the Angiotensin-I-converting Enzyme (ACE) that is involved in causing high blood pressure.

3.2 Materials and Methods

3.2.1 Cheese Making

In this study, cheese was manufactured according to Schroeder et al. (1988), with some modifications. Full cream fresh cow's milk (3.7% fat, C/F ratio ~0.70, pH 6.8) was purchased from a local dairy plant and pasteurised (pasteurising unit: Unipulse Pty Ltd, Victoria, Australia) at 72°C for 15 seconds, then cooled to 32°C and divided into four 50 kg lots. Each lot was transferred into a 50 litre cheese vat (Unipulse Pty Ltd, Victoria, Australia) and, after addition of a 10% calcium chloride solution (2.5 mL per litre of milk), a freeze-dried high proteolytic culture consisting of *L. helveticus* and *S. thermophilus* (2.5% [w/v]; TCC-20 Chr. Hansen, Bayswater, Victoria, Australia) was added. After 35 min of ripening, when the pH of the milk dropped by 0.3 units (from 6.5 to 6.2), 5 mL of diluted single-strength rennet (CHY-MAX® 200IMCU/mL, Chr. Hansen) was added with rigorous mixing for two min. The milk coagulated in 35 min (coagulum tested by cutting with a spatula) and the curd was cut into 1 cm^3 cubes and allowed to stand for 10 min, followed by stirring and gradually raising the temperature to 38°C (~50 min at pH ~6.2 to 6.0). The whey was drained and curd was banked, followed by cutting into slabs to ease whey drainage. Curd slabs were given a period of ~50 min to reach the pH of 5.3.

Four lots of 5.2 kg curd collected from each vat were milled and salted with different amounts of NaCl (2.5% w/w for control, 2% w/w for Treatment 1 [T1], 1.5% w/w for Treatment 2 [T2] and 1% w/w for Treatment 3 [T3]). The salted curds were pressed at a pressure of 2.41 kilopascals, overnight at room temperature. The pressed curd was cut into 250 g pieces and vacuum packed in oxygen barrier bags (Collinsons Pty Ltd, Fawkner, Australia) using Multivacs vacuum packaging equipment (Multivac Sepp Haggenmuller, Wolfertschwenden, Germany). The packaged cheeses were labelled and stored at 4.5°C for eight weeks. Samples were collected fortnightly for analyses. This experiment was repeated independently three times.

3.2.2 Chemical Composition

Moisture was determined via the oven-drying method using acid-washed sand (Sigma, St Louis, Missouri, US) at 102°C, fat was determined via the Babcock method, protein was determined via the Kjeldahl method, and ash was determined via the muffle furnace method, according to the Association of Official Analytical Chemists (AOAC) methods (AOAC International 1995). For pH measurement, 20 g of grated cheese was macerated with 20 mL distilled water, and the pH of the resulting slurry was measured by a digital pH meter (MeterLab, Pacific Laboratory Products, Blackburn, Victoria, Australia) after calibration. All analyses were undertaken in triplicate.

3.2.3 Determination of Sodium and Calcium Content of Cheese

A multi-type inductively coupled plasma atomic emission spectrometer (ICPE-9000; Shimadzu Scientific Instruments [Oceania] Pty Ltd, Rydalmere, New South Wales, Australia) was employed to determine the sodium, calcium and potassium content in cheeses according to Ayyash and Shah (2010), while cheese samples were prepared according to Cortez et al. (2008). Briefly, grated cheeses (5 g) from the shredded samples were digested in a mixture of HNO_3 (69%) and $HClO_4$ (70%) (ratio 5:1) (Merck Pty Ltd, Victoria, Australia) on a hot plate until the digests were clear. The clear digests were filtered through a 0.45 µm filter (Millex) and analysed using ICPE-9000, which consisted of an ASC-6100 autosampler, a HVG-ICP hydride generator, a HFS-2 hydrofluoric acid sample injection system, an NCB-1200 low-temperature thermostatic

chamber, and the software package ICPE-9000. In order to calculate the Na^{+} and Ca^{+2} concentrations in the samples, a standard curve consisting of the three elements was prepared at 1, 10, 20, 30 and 40 mg/L in MiliQ water (Merck Millipore, Bayswater, Victoria, Australia).

3.2.4 Microbiological Analysis of Cheese

The *Streptococcus thermophilus* and *Lactobacillus helveticus* in the cheese were enumerated via the pour-plating method, as described by Tharmaraj and Shah (2003). Eleven grams of grated cheese and 99 mL of sterile distilled water were blended for two min in a stomacher-400 laboratory blender (Seward Medical, London, UK). Serial dilutions were made in sterilised solutions of 0.1% peptone and water (Sigma). *L. helveticus* and *S. thermophilus* were grown on de Man, Rogosa and Sharpe (MRS) agar and M17 agar (Merck Pty Ltd, Victoria, Australia), respectively. Inoculated (1mL of selected dilutions) plates of MRS agar were incubated anaerobically (using anaerobic jars, Becton Dickinson Microbiology Systems, Sparks, Maryland, US) and M17 agar aerobically at 37°C for 48 h.

3.2.5 Organic Acid Analysis of Cheese

HPLC was employed to determine the lactic, citric and acetic acids, according to Kaminarides et al. (2007), with some modifications. Briefly, 5 g of the grated cheese sample was blended with 25 mL of 0.009 N sulphuric acid and 70 μL of 15.5 N nitric acid, and homogenised with an Ultraturrax homogeniser (Jonke & Kunkel KG, Staufen i Breisgau, Germany) at 10,000 rpm. The resultant slurry was incubated in a 50°C water bath for one hour, followed by centrifugation at 4,000 × g for 20 min at 4°C. Then, 1.5 mL of the soluble portion from the middle based between the fat (upper layer) and casein (sediment) was taken for further centrifugation at 14,000 × g for 10 min, using a bench-top centrifuge (Sorvall RT7, Newtown, Connecticut, US). The supernatant was filtered through a 0.45 μm filter (Millex, Millipore, Bedford, Massachusetts, US) and approximately 1 mL was transferred to HPLC vials for analysis. A 20 μL sample was injected into an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad Laboratory, Richmond, California, US). Sulphuric acid (0.009 N), filtered through a 0.45 μm membrane filter (Millex, Millipore, Bedford, Massachusetts, US) was used as a mobile

phase at a flow rate of 0.6 mL/min. An ultraviolet-visible detector was set at 220 nm with a total HPLC running time of 15 min. The reverse-phase HPLC (RPHPLC) analysis was performed in a HPLC system consisting of a Varian 9012 solvent delivery unit, Varian 9100 autosampler, Varian 9050 variable wavelength ultraviolet-visible tuneable absorbance detector, and 730 data module (Varian Inc., Palo Alto, California, US). All the results were expressed as mg per 100 g cheese, based on wet matter.

3.2.6 Urea-polyacrylamide Gel Electrophoresis (Urea-PAGE)

Preparation of cheese samples and urea-PAGE analysis were undertaken according to Katsiari et al. (2000a), with some modifications. Briefly, 1 g of cheese sample was homogenised with 10 mL of treatment buffer (6 M urea, 0.1 M β-mercaptoethanol, and 0.5% bromophenol blue [0.05%, w/v, in 50% ethanol]) for two min at 10,000 rpm, using a tissue-homogeniser (Polytron, Kinematica AG, Lucerne, Switzerland). The slurry was held at 40°C for 15 min, and then centrifuged at 3,000 × g for 30 min at 4°C. The fat layer (upper layer) was removed and 0.5 mL of the supernatant was mixed with 3.5 mL treatment buffer. Then, 1 mL from this mixture was placed in a 1.5 mL Eppendorf tube and centrifuged at 3,000 × g for 15 min. A 20 μL of whole casein solution (2 mg/mL; Sigma-Aldrich, St Louis, Missouri, US) was mixed with 40 μL treatment buffer. From each sample and prepared whole casein solution, 12 μL was loaded onto ready-gel Tris-HCl gel (12% resolving gel, 4% stacking gel, 10-well, 30 μL, 16 × 16 cm) (Bio-Rad Laboratories Pty Ltd, Gladesville, New South Wales, Australia).

The gels were transferred to a buffer tank (10 g Tris and 29.9 g glycine w/v in 2-L distilled water) equipped with Bio-Rad Protean R-II xi cell powered by a Power Pac 300 (Bio-Rad Laboratories Pty Ltd, Gladesville, New South Wales, Australia) and run for 15 min at 15 mA, and then for two h at 40 mA. The gels were then washed with Milli-Q water three times for 15 min (five min each wash) and fixed in RAPID stain solution (G-Biosciences, St Louis, Missouri, US) for 60 min, followed by gentle shaking until the background became clear and protein bands became visible. Afterward, the gels were washed two to three times for 10 min with deionised water. The gel images were recorded using a Fujifilm intelligent dark box II with Fujifilm LAS-1000 litre V1.3 software (Fujifilm Australia Pty Ltd, New South Wales, Australia).

3.2.7 Proteolysis Assessment by WSN-SN, TCA-SN and Total Free Amino Acids

The water-soluble extract (WSE) of each cheese sample was prepared according to Kuchroo and Fox (1982). Briefly, a mixture of cheese and distilled water (1:2) was kept in a 40°C water bath for 60 min, followed by centrifugation (Sorvall T6000D, Thermo Scientific) at 4000 × g for 30 min. The resultant slurry was filtered through a 0.45-μm filter (Millipore Corp., Bedford, Massachusetts). The total nitrogen in the filtrate extract (3 mL) was determined by the Kjeldahl method (AOAC International 1995).

Trichloroacetic acid-soluble nitrogen (TCA-SN) was determined in 9 mL filtrate obtained after 5 mL of WSE was mixed with 5 mL of 24% TCA (Sigma) and left overnight, followed by centrifugation (Sorvall T6000D, Thermo Scientific) at 4000 × g for 20 min. The total nitrogen in the filtrate was determined via the Kjeldahl method (AOAC International 1995). The extent of secondary proteolysis (5% PTA-SN) was assayed in 9 mL filtrate obtained after 5 mL of WSE was mixed with 5 mL of 10% PTA (Sigma) and left overnight, followed by centrifugation (Sorvall T6000D, Thermo Scientific) at 4000 × g for 20 min.

3.2.8 Measurement of Total Free Amino Acids

The concentrations of the total free amino acids (TFAA) in the WSE of Cheddar cheese were measured using the Cd-ninhydrin method according to Folkertsma and Fox (1992). The Cd-ninhydrin reagent was prepared as follows: 0.8 g of ninhydrin (Sigma-Aldrich, St Louis, Missouri, US) was dissolved in a mixture of 10 mL of glacial acetic acid (100%) (Sigma-Aldrich, St Louis, Missouri, US) and 80 mL ethanol (99.5%; Merck Pty Ltd, Victoria, Australia), followed by the addition of 1 g of $CdCl_2$ (Sigma-Aldrich, St Louis, Missouri, US) already dissolved in 1 mL of Milli-Q water. Then, 100 μL of WSE was placed in a glass test tube, diluted with 1 mL of Milli-Q water and mixed with 2 mL of Cd-ninhydrin reagent. The mixture was heated at 84°C for five min, and cooled to room temperature. Then, the absorbance at 507 nm was measured using a spectrometer (Shimadzu Scientific Instruments [Oceania] Pty Ltd, Rydalmere, New South Wales, Australia). Analyses were undertaken in triplicates.

3.2.9 Peptide Profile of WSE by RPHPLC

The peptide profile of Cheddar cheeses during storage was examined by HPLC according to Cliffe et al. (1993), with some changes. Briefly, 80 mg of freeze-dried WSE was mixed with 2 mL of 0.1% trifluoroacetic acid (Solvent A), followed by centrifugation at 3,000 × g for 10 min, using a bench-top centrifuge (Sorvall RT7, Newtown, Connecticut, US). The supernatant was filtered through a 0.45-μm filter (Millipore Corp., Bedford, Massachusetts, US). A 50 μL amount of the supernatant was injected into the reverse-phase column (C18, 250 mm × 4.6 mm, 5 μm; Grace Vydac, Hesperia, California, US). The details of RPHPLC were provided in Section 3.3.5. Separation was conducted at room temperature at a flow rate of 0.8 mL/min using two solvents as mobile phases (Solvents A and B). Solvent B was 60% acetonitrile (Merck Pty Ltd, Victoria, Australia) containing 0.1% trifluoroacetic acid. A linear gradient was applied from 0 to 100% eluent B over 100 min, and the ultraviolet-visible detector was set at 215 nm.

3.2.10 ACE Inhibitor Activity in WSE

The ACE-inhibitory activity was measured according to Cushman and Cheung (1971) and Janitha et al. (2002) using a HPLC method. Angiotensin-converting enzyme (peptidyl-dipeptidase A, ACE peptidyl-dipeptide hydrolase) and Hippuryl-histidyl-leucine (HHL) were purchased from Sigma-Aldrich (St Louis, Missouri, US) and prepared in Tris buffer (50 mM, pH 8.3) containing 300 mM NaCl. The assay consisted of 50 μL of 3.0 mM HHL, 50 μL of 1.25 milli-units ACE enzyme, and 50 μL of WSE sample. The mixture was placed in a glass tube, incubated for 30 min at 37°C in a water bath without mixing, and then incubated for an additional 30 min after mixing. Glacial acetic acid (150 μL) was added to stop ACE activity. The reaction mixture was kept at -20°C for subsequent HPLC analysis. The hippuric acid (HA) released from HHL by ACE was determined by HPLC. An external standard curve of HA was used to quantify the resultant HA in cheese samples. Ten microliters of the mixture were injected into the HPLC system (as mentioned above). An isocratic system consisting of 12.5% (vol/vol) acetonitrile (Merck Pty Ltd, Victoria, Australia) in Milli-Q water was a mobile phase, and the pH was adjusted to 3.0 using glacial acetic acid. Jupiter Proteo 90A (250 x 10 mm, 10 μm; Phenomenex Australia Pty Ltd, New South Wales, Australia) column

was used for separations, and analytes were detected using an ultraviolet-visible detector at 228 nm. The control reaction mixture contained 50 μL of buffer instead of the assay sample, and was expected to release the maximum amount of HA from the substrate due to uninhibited ACE activity. The percentage inhibition of enzyme activity was calculated as follows:

Equation 2:

$$\%\ \text{of Inhibition} = [\text{HA (control)} - \text{HA}\frac{(\text{Sample})}{(\text{control})}] \times 100$$

3.2.11 Texture Profile Analysis

The texture profile was analysed according to Bryant et al. (1995), with some modifications. Cheese cylinders (30 height × 20 diameters, mm) were cut from the centre of the Cheddar cheese blocks. Specimens were kept at room temperature in small 50 mL containers prior to determining the texture profile. Hardness, cohesiveness, adhesiveness and gumminess were measured using an Instron universal testing machine (model 5564; Instron Ltd, London, UK) based on the criterion described by Pons and Fiszman (1996). The samples were compressed to 30% of their heights using a 500 N load cell with a flat plunger, and the crosshead movement was set to 30 mm per minute. Double compression was achieved and the data were collected using Merline software. Analyses were performed in triplicate.

3.2.12 Cheese Microstructure by Environmental Scanning Electron Microscopy (ESEM)

The microstructure of cheeses was monitored according to Ayyash et al. (2011). Briefly, 0.5 cm^3 cubes of cheese were cut from the centre of the cheese loaves and imaged by FEI quanta ESEM (Philips Electron Optics, Eindhoven, The Netherlands) using ESEM mode. Images were taken at accelerating voltage at 30 kV under vacuum (0.47 kPa) and 1,000 × magnification at 4°C. The specimens were not conductivity coated before imaging.

3.2.13 Sensory Evaluation

Sensory evaluation was conducted after gaining a human ethics application approval from the Victoria University Human Ethics Committee (approval number: HRE13-005). Fourteen panellists were recruited from Victoria University staff and research students to assess the sensory attributes of the experimental cheese samples using a 10-point hedonic test. The panellists were trained in their ability to detect creamy, sour-acid, vinegary, salty and bitter tastes, and to assess overall acceptability. Sensory evaluation was conducted for cheeses at Weeks 0 and 8 of storage. The cheese samples were tempered at room temperature (20 °C) for one hour, cut into pieces and placed on white plates coded with random three-digit numbers. The panellists evaluated four samples per session. Crackers and water were provided between samples to change the taste and rinse the palate and tastebuds. Panellists ranked the attributes using a 10-point scale. For example, for creaminess, zero indicated the absence of a creamy taste and 10 indicated an extremely creamy taste. The salty, sour-acid, vinegary and bitter tastes were all evaluated in the same manner. For overall acceptability, zero indicated that the cheese was not accepted, while 10 indicated that it was highly accepted.

3.2.14 Statistical Analysis

A two-way analysis of variance (ANOVA) was performed to investigate the effect of salting treatment and storage time on the experimental cheese characteristics ($P < 0.05$). Fisher's test (least significant difference) was undertaken to explore the significant difference between means at the same storage period. All data were statistically analysed using SAS 9.0 software (SAS Institute Inc. 2008).

3.3 Results and Discussion

3.3.1 Chemical Composition

Table 6 presents the chemical compositions of the experimental cheeses using different salt levels on Day 0 (immediately after pressing). Salt reduction significantly ($P < 0.05$) affected the moisture and S/M content of the experimental cheeses. The moisture

content of the salt-reduced treatments (T1, T2 and T3) was significantly higher ($P < 0.05$), while the S/M content was lower ($P < 0.05$), than the control. The order of the moisture content was as follows: T3 > T2 > T1 > control, while S/M had the opposite order.

Table 6: Chemical composition, Sodium Content and pH of Experimental Cheeses Made with Four Levels of NaCl at Day 0 of Storage

Salt treatment[1]	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	pH	S/M (%)[2]
C	33.89±1.12[c3]	24.47±0.13[a]	36.67±0.20[a]	1.97±0.18[a]	5.01±0.01[a]	4.40±0.18[a]
T1	36.93±1.11[b]	24.32±0.16[a]	36.47±0.24[a]	1.53±0.18[b]	4.96±0.03[b]	3.07±0.12[b]
T2	38.45±0.23[ab]	24.21±0.19[a]	36.63±0.25[a]	1.37±0.17[c]	4.94±0.02[b]	2.52±0.18[bc]
T3	39.59±0.35[a]	24.61±0.17[a]	36.53±0.21[a]	1.07±0.14[d]	4.81±0.08[c]	1.82±0.15[c]

[1] Salt treatments: C = control (2.5%); T1 = salted at 2%; T2 = salted at 1.5% and T3 = salted at 1%
[2] S/M (%): S/M percentage
[3] Mean value ± standard error (SE) of three trials
[a-c] Means in each column with different letters are significantly different ($P < 0.05$) at same storage period.

Table 7: Sodium and Calcium Content (mg/100 g) of Cheeses Made with Four Levels of NaCl during Eight Weeks of Storage at 4.5°C

	Salt treatment[1]	Storage (weeks)				
		0	2	4	6	8
Sodium	C	594.24±18.4[aA2]	580.41±11.7[aB]	562.86±14.1[aC]	558.76±15.9[aD]	542.22±11.2[aE]
	T1	492.37±12.8[bA]	389.42±6.4[bB]	378.25±11.5[bC]	377.72±11.2[bC]	371.21±10.4[bC]
	T2	386.76±11.1[cA]	365.80±17.2[cB]	345.90±6.92[cC]	339.39±16.9[cD]	329.80±7.2[cE]
	T3	287.81±17.0[dA]	286.75±12.6[dA]	236.74±17.7[dB]	227.22±10.2[dC]	220.56±19.1[dC]
Calcium	C	998.21±10.7[aB]	994.88±11.3[aB]	983.24±18.6[aA]	973.34±11.0[aC]	970.90±9.5[aC]
	T1	991.12±17.8[aB]	986.30±11.7[bA]	963.20±11.0[bC]	954.41±12.0[bD]	945.04±14.5[bE]
	T2	986.24±11.2[bB]	980.60±10.6[bB]	959.42±15.7[bC]	948.31±11.1[cD]	941.88±12.5[bD]
	T3	976.77±15.8[cC]	975.78±15.3[cC]	957.71±16.8[cD]	941.87±17.2[cE]	940.46±17.6[dE]

[1] Salt treatments: C = control (2.5%); T1 = salted at 2%; T2 = salted at 1.5% and T3 = salted at 1%
[2] Mean value ± SE of three trials
[a-d] Means in each column with different letters are significantly different ($P < 0.05$) at same storage period
[A-E] Means in the same row with similar capital letters differed insignificantly.

The lower moisture content and higher S/M percentages in the control cheese were due to the higher salt addition during the salting step of cheese making. These results are in agreement with those of Schroeder et al. (1988), who reduced the salt content in Cheddar cheese and showed that moisture content at higher salt concentration was lower than other treatments with a lower salt content. In the current study, the pH of the control cheese was significantly higher ($P < 0.05$) than the other experimental cheeses with a low salt content (Table 6). This may be attributed to the inhibition of the starter culture occurring during salt addition in the mellowing step (Guinee & Fox 2004). This result agrees with those of Irvine and Price (1961). The ANOVA showed no significant differences ($P > 0.05$) in fat and protein content between the control cheeses and salt-reduced cheeses. Ash content significantly decreased ($P < 0.05$) similar to the salt concentration in cheeses. Salt is usually the major component of the total ash content of cheese (Schroeder et al. 1988). A similar trend was reported by Schroeder et al. (1988) for Cheddar cheese made with different cultures during storage for seven months.

3.3.2 Determination of Sodium and Calcium Content of Cheese

Table 7 presents the sodium and calcium concentrations (mg/100g of cheese) in cheeses salted at four different levels. The sodium content differed significantly ($P < 0.05$) between the experimental cheeses at the same storage period in the same order as the salt addition, that is, control > T1 > T2 > T3. The calcium content of the control and T1 were significantly higher ($P < 0.05$) than those of T2 and T3.

This difference in calcium content might be due to a decrease in pH at the drainage step (~6.2 to 6.0). Sheehan and Guinee (2004) reported that, by decreasing the pH at the time of drainage, the rate of migration of calcium ion from curd to whey would increase. A decrease in pH increases the solubilisation of CCP and thus decreases the amount of CCP crosslinking with casein molecules. This increases the amount of Ca^{2+} ion in cheese moisture, thereby causing greater calcium loss alongside moisture loss (Lucey & Singh 1997; Roefs et al. 1985).

3.3.3 Starter Culture Growth and Production of Organic Acids

Table 8 presents the effect of salt reduction on the growth of starter culture (*L. helveticus* and *S. thermophilus*) and proteolysis of experimental cheeses stored at 4.5°C for eight weeks. The ANOVA showed that salt reduction had a significant effect ($P < 0.05$) on starter culture growth. At the same storage period, *L. helveticus* and *S. thermophilus* growth in T3 were significantly higher ($P < 0.05$) than in the other cheeses. It is well established that a decrease in salt concentration stimulates the growth of bacteria due to providing higher a_w (Parente & Cogan 2004). Schroeder et al. (1988), Mistry and Kasperson (1998) and Rulikowska et al. (2013) also reported a higher growth of starter bacteria in cheeses with lower salt concentration.

It has been reported that microbial growth is inhibited alongside a high salt content increase (Guinee & Fox 2004). It is well established that increase in the salt concentration of cheese causes a reduction in a_w due to a decrease in moisture in the non-fat substance, which results in a drop in the density of the bacteria population (Guinee & O'Kennedy 2007). Rulikowska et al. (2013) also reported that populations of starter culture increased in salt-reduced Cheddar cheeses.

Table 8 shows that, at the same storage period, lactic, acetic and citric acids significantly differed between experimental Cheddar cheeses. It is likely that acid production at low salt levels is accompanied by high cell numbers, which tend to lead to bitterness (Guinee & O'Kennedy 2007). Califano and Bevilacqua (2000) reported that lactic, acetic and citric acids increased during storage of Gouda cheese. As a biochemically dynamic product, cheese undergoes significant changes during ripening (McSweeney & Sousa 2000), which are the consequence of numerous metabolic processes (Farkye 2004; Singh et al. 2003). Lactate and citrate are especially important precursors for a series of reactions leading to the production of organic acids (Forde & Fitzgerald 2000; Parente & Cogan 2004). Lactic and acetic acids increased significantly during storage in all experimental cheeses at the same salt treatment. This may be due to the continuous activity of starter culture in all experimental cheeses (McSweeney & Sousa 2000).

The concentrations of lactic acid were considerably higher than other acids, while citric acid showed a slight drop during ripening (Table 8). This was due to the metabolism of milk's indigenous citric acid (as an energy substance) by starter bacteria to ferment the remaining lactose in the cheese and thus produce lactic acid (Fox et al. 2000b). During cheese making, approximately 98% of the lactose in milk is expelled in whey; therefore, a relatively small amount of lactose (0.3 to 0.6% w/w) remains in the cheese (Fox et al. 1990; Upreti et al. 2006). Using the residual lactose in cheese by starter and non-starter bacteria during ripening is the main reason for the increase of organic acid content in cheese (Fox et al. 2000b). The increase is acetic acid content in cheeses with lower salt concentration (Table 8) is due to a higher metabolism of glucose and galactose (two main constitutive components of lactose) via a fructose-6-phosphate shunt pathway. The result of this fermentation pathway is 3 mol of acetic acid and 2 mol of lactic acid per 2 mol of glucose (Law 1997). Reducing salt stimulates the activity of starter and non-starter bacteria, leading to a higher concentration of organic acids in cheeses with lower salt content (Beeren 2013; Guinee & Fox 2004).

Table 8: Starter Culture Count (log cfu/g) and Organic Acids (mg/100 g) in Cheeses Made with Four Levels of NaCl during Eight Weeks of Storage at 4.5°C

	Salt treatment[1]	Storage (weeks)				
		0	2	4	6	8
L. helveticus[2]	C	8.72±0.01^{bD4}	9.38±0.07^{bB}	9.34±0.05^{bB}	9.10±0.17^{bA}	9.03±0.09^{bC}
	T1	8.67±0.04^{bD}	9.38±0.18^{bB}	9.37±0.01^{bB}	9.32±0.01^{bA}	9.01±0.08^{bC}
	T2	8.71±0.01^{bD}	8.54±0.03^{aE}	9.36±0.01^{aA}	9.32±0.04^{bB}	9.00±0.06^{bC}
	T3	9.21±0.02^{aB}	9.63±0.29^{bA}	9.65±0.01^{aA}	9.36±0.01^{aC}	9.20±0.05^{aB}
S. thermophilus[3]	C	8.69±0.01^{bE}	9.78±0.04^{abB}	9.37±0.11^{bA}	9.25±0.02^{bC}	9.09±0.06^{bD}
	T1	8.61±0.17^{bE}	9.73±0.17^{abB}	9.36±0.02^{bA}	9.27±0.04^{bC}	9.09±0.08^{bC}
	T2	8.66±0.02^{bE}	9.43±0.01^{bB}	9.34±0.01^{bA}	9.25±0.01^{bC}	9.21±0.01^{abD}
	T3	8.67±0.02^{bE}	9.75±0.05^{aB}	9.36±0.01^{aA}	9.29±0.04^{aC}	9.26±0.01^{abC}
Lactic acid	C	1.08±0.05^{aC}	1.16±0.01^{aB}	1.18±0.01^{aB}	1.32±0.05^{aA}	1.47±0.05^{aBA}
	T1	1.13±0.02^{aC}	1.22±0.02^{aBA}	1.38±0.02^{aBC}	1.45±0.02^{aA}	1.65±0.01^{aA}
	T2	1.13±0.02^{aC}	1.22±0.09^{aBA}	1.39±0.08^{aBC}	1.46±0.02^{aA}	1.68±0.02^{aA}
	T3	1.15±0.03^{aC}	1.28±0.01^{aBC}	1.46±0.02^{aBA}	1.53±0.04^{aBAC}	1.68±0.04^{aA}
Acetic acid	C	0.08±0.01^{aC}	0.08±0.02^{aC}	0.10±0.02^{aB}	0.10±0.01^{aB}	0.13±0.03^{aA}
	T1	0.08±0.02^{aC}	0.08±0.03^{aC}	0.11±0.01^{bB}	0.13±0.02^{bBA}	0.15±0.04^{bA}
	T2	0.09±0.01^{bC}	0.09±0.01^{bC}	0.12±0.02^{cB}	0.13±0.02^{bBA}	0.15±0.02^{bA}
	T3	0.09±0.03^{bC}	0.11±0.01^{cB}	0.12±0.03^{cB}	0.15±0.01^{cA}	0.15±0.04^{bA}
Citric acid	C	0.27±0.00^{aB}	0.28±0.00^{aC}	0.28±0.00^{aC}	0.29±0.00^{aA}	0.29±0.00^{aA}
	T1	0.28±0.00^{aC}	0.28±0.00^{aC}	0.28±0.00^{aC}	0.28±0.00^{aC}	0.29±0.00^{aA}
	T2	0.28±0.00^{aC}	0.28±0.00^{aC}	0.28±0.00^{aC}	0.28±0.00^{aC}	0.28±0.00^{aC}
	T3	0.28±0.00^{aC}	0.28±0.00^{aC}	0.28±0.00^{aC}	0.28±0.00^{aC}	0.28±0.00^{aC}

[1] Salt treatments: C = control (2.5%); T1 = salted at 2%; T2 = salted at 1.5% and T3 = salted at 1%
[2] *Lactobacillus helveticus* (cfu/g)
[3] *Streptococcus thermophilus* (cfu/g)
[4] Mean value ± SE of three trials
[a-d] Means in each column with different letters are significantly different ($P < 0.05$) at same storage period
[A-D] Means in the same row with similar capital letters differed insignificantly.

3.3.4 Urea-PAGE

Figure 4 presents the gel electrophoresis of cheese samples salted at different salt levels and stored at 4.5°C for eight weeks. The urea-PAGE patterns for the control and experimental cheeses were similar for all salt treatments at the same storage period. As the storage progressed, new bands appeared on the gel in addition to α-casein and β-casein at each salt treatment. This suggests that proteolysis increased during storage, which concurs with the proteolysis results found in this study (Table 9). From Week 4 of storage onwards, more bands appeared on the gel, demonstrating the progress of proteolysis, especially in the salt-reduced treatments. α-casein was more hydrolysed due to salt reduction; thus, more bands appeared after Week 6 of storage (indicated by the arrows in Figure 4). β-casein was more resistant to hydration, and the addition of salt contributed to eliminating this resistance (Phelan et al. 1973; Sousa et al. 2001). In this study, the reduction of salt caused less hydration of β-casein causing appearance of a condensed and thick band separated from β-casein on the gel during storage.

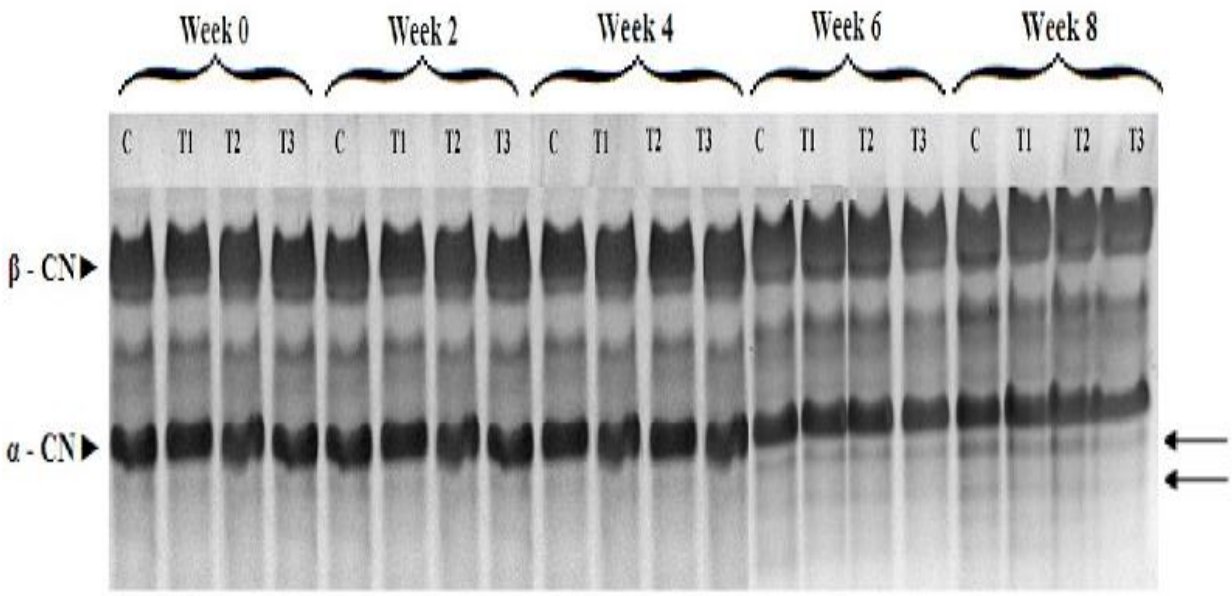


Figure 4: Urea-polyacrylamide Gel Electrograms of Experimental Cheeses Made with Four Levels of NaCl during Eight Weeks of Storage at 4.5°C

3.3.5 Proteolysis Assessment

A significant increase ($P < 0.05$) in WSN, TCA-SN and PTA-SN was observed during storage for the same salt treatment (Table 9), as was also observed by Schroeder et al. (1988) and Murtaza et al. (2014). Table 9 shows the significant ($P < 0.05$) differences in WSN, TCA-SN and PTA-SN content observed between the experimental cheeses at the same storage time. The PTA-SN of cheeses salted at 2.5% (control) and 2.0% (T1) was significantly higher ($P < 0.05$) than the other cheeses (T2 and T3) at the same storage period.

The increase in proteolysis during storage was attributed to the activity of rennet retained in cheeses, and starter culture activity (Sousa et al. 2001; Upadhyay et al. 2004). The slight fluctuation patterns in WSN, TCA-SN and PTA-SN during storage were due to differences in the cheese loaves. However, an overall increase occurred in WSN, TCA-SN and PTA-SN during storage at the same salting treatment due to proteolysis being continued.

This higher PTA-SN in T2 and T3 was attributed to higher production of small peptides and amino acids as a result of greater bacterial proteinase activity occurring in the low-salt cheeses (T2 and T3) compared with the high-salt cheeses (control and T1). It has been reported that a reduction in the salt content of cheese increases the activity of starter and non-starter bacteria due to a higher a_w (Fox et al. 2000a; Parente & Cogan 2004). Therefore, the low salted cheeses (T2 and T3) in this study comprised higher bacteria populations, which caused more proteolytic activity and thus more PTA-SN content. A significant difference in WSN was observed between the experimental cheeses at Week 6 of storage. This may have been due to interior differences between the cheese loaves, and not due to the salt reduction treatments. Geurts (1978) reported that two identical cheese loaves may have differences in parameters.

Table 9: Proteolysis Parameters of Cheeses Made with Four Levels of NaCl during Eight Weeks of Storage at 4.5°C

	Salt treatment[1]	Storage (weeks)				
		0	2	4	6	8
WSN[2]	C	5.16±0.75^{aD}[5]	7.25±0.53^{aC}	9.32±0.19^{aB}	10.90±0.93^{aA}	11.09±0.65^{aE}
	T1	5.23±1.48^{aD}	7.57±0.26^{bC}	9.52±0.48^{bB}	11.57±0.60^{bA}	11.92±0.69^{bE}
	T2	5.75±1.49^{bD}	7.78±0.11^{cC}	9.82±0.48^{cB}	11.91±0.13^{aA}	12.28±0.15^{cE}
	T3	5.86±1.34^{bD}	7.91±0.37^{cC}	9.97±0.27^{cB}	12.45±0.36^{bA}	12.87±0.49^{aA}
TCA-SN[3]	C	3.23±0.42^{aA}	3.25±0.03^{aA}	3.98±0.19^{aA}	4.28±0.11^{aB}	4.66±0.19^{aB}
	T1	3.25±0.40^{aA}	3.29±0.10^{aA}	3.99±0.29^{aA}	4.52±0.33^{aB}	4.75±0.27^{aB}
	T2	3.77±0.48^{bA}	3.84±0.57^{bA}	4.48±0.17^{bB}	4.98±0.34^{bB}	5.34±0.08^{bC}
	T3	3.83±0.56^{bA}	3.99±0.10^{bA}	4.63±0.11^{bB}	5.32±0.55^{cC}	5.46±0.21^{bC}
PTA-SN[4]	C	2.32±0.15^{aC}	2.33±0.40^{aC}	2.60±0.12^{aB}	2.88±0.13^{aA}	2.83±0.62^{abA}
	T1	2.44±0.69^{aC}	2.32±0.35^{aC}	2.88±0.22^{aB}	2.33±0.39^{aC}	2.38±0.27^{aC}
	T2	2.33±0.04^{aC}	2.72±0.22^{bC}	2.92±0.23^{bB}	3.44±0.53^{bB}	3.68±0.16^{bB}
	T3	2.42±0.09^{aC}	2.80±0.36^{bC}	2.97±0.09^{bC}	3.71±0.15^{bB}	3.77±0.23^{bB}

[1] Salt treatments: C = control (2.5%); T1 = salted at 2%; T2 = salted at 1.5% and T3 = salted at 1%
[2] WSN: WSN as a percentage of total nitrogen
[3] TCA-SN: 12% TCA-SN as a percentage of total nitrogen
[4] PTA-SN: 5% PTA-SN as a percentage of total nitrogen
[5] Mean value ± SE of three trials
[a-c] Means in each column with different letters are significantly different ($P < 0.05$) at same storage period
[A-E] Means in the same row with similar capital letters differed insignificantly.

3.3.6 Measurement of TFAA

Figure 5 presents the concentrations of the TFAA of the experimental Cheddar cheeses during storage. The ANOVA showed a significant ($P < 0.05$) difference in TFAA between cheeses at the same storage time.

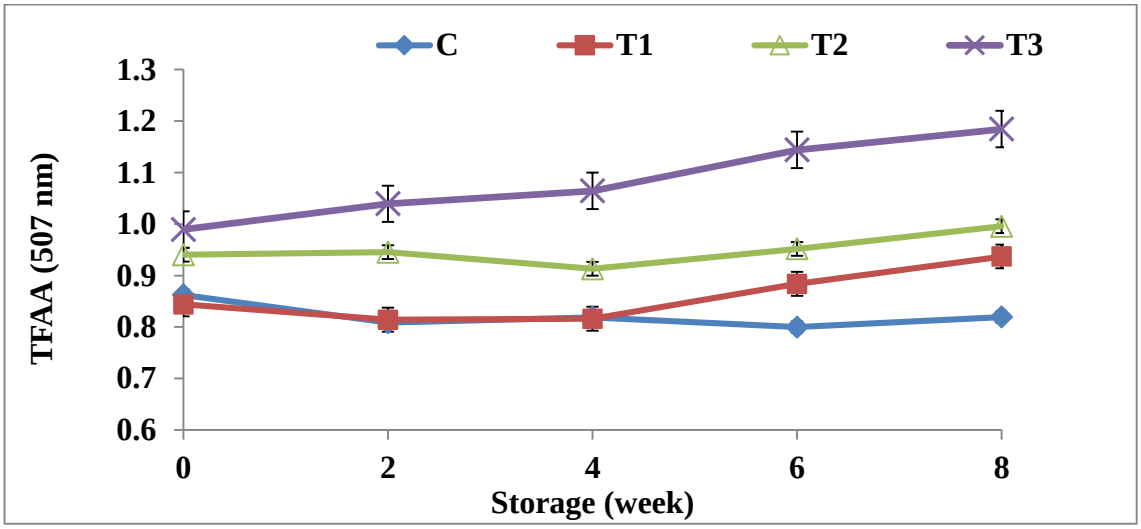


Figure 5: TFAA in Experimental Cheeses Made with Four Levels of Salt during Eight Weeks of Storage at 4.5°C

TFAA is an index of the proteolytic enzymes activity produced by SLAB, mainly those that hydrolyse small peptides to produce free amino acids (McSweeney & Sousa 2000; Upadhyay et al. 2004). Figure 5 shows that the TFAA of cheeses salted with 1.5% and 1% NaCl (T2 and T3, respectively) were higher than the other experimental cheeses at the same storage period due to less inhibition of SLAB bacterial activity at low salt concentration (Guinee & Fox 2004). Upadhyay et al. (2004) reported that a decrease in salt content can directly affect the rate of proteolysis, which increases the amount of free amino acids. For all the experimental cheeses, TFAA increased ($P > 0.05$) during storage for the same salting treatment. During the period of storage, as time increased, so did the TFAA; however, after a longer storage period (ripening time), the difference due to reduced salt became increasingly prominent. This concurs with the increase in proteolysis that occurred in the experimental cheeses during storage (Table 9).

3.3.7 Peptide Profile

Figure 6 presents the peptide profile chromatogram at Week 8 of storage of the Cheddar cheeses salted with four different salt levels. The separation of peptides through the C18 column was based on the hydrophobicity of peptides. At the same salt treatment, hydrophobic peptides (appearing at the last stage) were dominant compared with hydrophilic peptides. It has previously been reported that hydrophilic peptide peaks appear before 14 min of elution time, whereas the peaks appearing after 14 min of elution time are considered hydrophobic (Gomez et al. 1997; Lau et al. 1991).

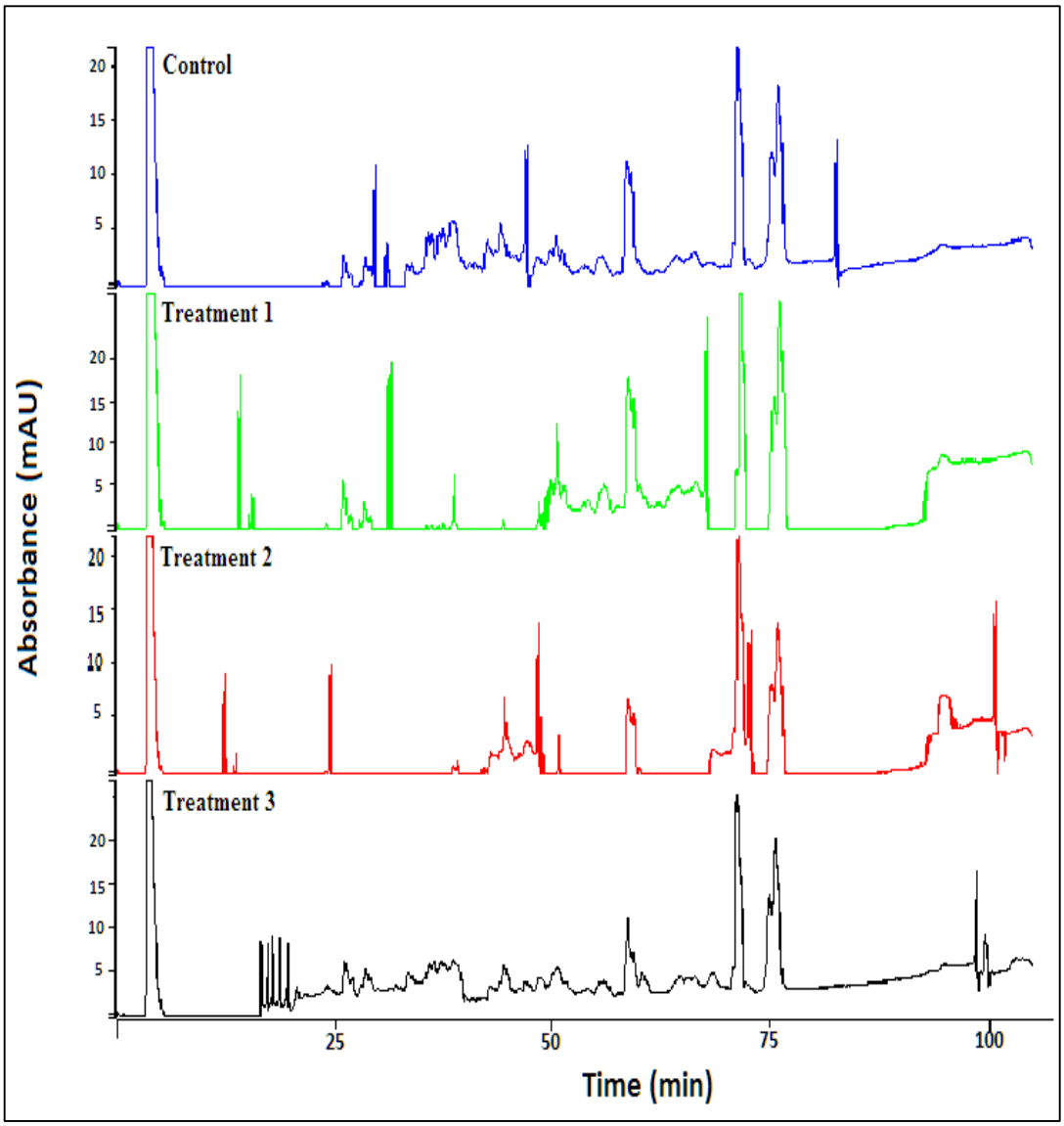


Figure 6: Peptide Profile of Cheddar Cheeses Made with Four Levels of NaCl during Eight Weeks of Storage at 4.5°C

Figure 6 shows that the number of peptides appearing at an earlier time on the chromatogram decreased alongside salt content decrease. Reducing salt triggers enhanced metabolism of resultant peptides due to enzyme activity during proteolysis by non-starter bacteria, which in turn increases the amount of free amino acids (Guinee & Fox 2004; Upadhyay et al. 2004). This explains the cause of the shrinkage of the peptide peak at ~60 min of retention time in cheeses with a lower salt content (Figure 6).

Significant differences were observed in the main hydrophobic peptide peaks (β-casein [f193-209]) among the salt-reduced cheeses. It has previously been reported that reducing salt stimulates the production of bitter hydrophobic peptides, such as β-casein (f193-209) during storage. Hydrophobic peptides are mostly categorised as sources of bitterness (Fox et al. 2000a; Moller et al. 2013). Thus, excessive accumulation of small hydrophobic peptides leads to development of bitterness in cheeses containing lower salt (T2 and T3). The higher the chymosin residues, the higher the proteolysis rate, and thus the higher the peptide development (Farkye et al. 1991; Fox 1989; Fox & Guinee 2013; Fox & McSweeney 1996; Grappin et al. 1985; Sousa et al. 2001; Upadhyay et al. 2004). The amount of chymosin residues and its activity are affected by pH at drainage and moisture content, respectively, the higher the pH value at whey drainage, the higher the amount of chymosin residues in cheese. In addition, an increase in the moisture content of cheese (T1, T2 and T3) encourages increased proteolytic activities of chymosin (Guerreiro et al. 2013; Guinee & Fox 2004; Sousa et al. 2001; Upadhyay et al. 2004), which increases peptide production. An increase in salt increases whey expulsion during the mellowing and moulding steps. This decreases the moisture content in cheese and thus the activity of chymosin. In this study, lowering the salt amount increased the moisture content and thus increased the proteolytic activities of chymosin residues. As such, more bitter peptides (β-casein, f193-209) were produced. Moller et al. (2013) reported higher production of bitter peptides (β-casein, f193-209) in Cheddar cheeses containing higher moisture and lower salt concentration.

3.3.8 ACE-inhibitory Measurement

Figure 7 presents the ACE-inhibitory activity of experimental Cheddar cheeses salted at four different levels and stored at 4.5°C for eight weeks. Experimental cheeses

containing higher salt levels (control and T1) had lower ACE-inhibitory activities compared with T2 and T3 during storage. Following the proteolysis pattern described before, ACE-inhibitory activities increased significantly ($P < 0.05$) with prolonged storage. Due to the residual activity of proteolytic enzymes, ACE-inhibitory activity was highest at Week 8 of storage for the treatment containing the least amount of salt. The increase in ACE-inhibitory activities during storage is in accordance with findings by Ong and Shah (2008b) for Cheddar cheese. It has been reported that small and shorter peptides (such as κ-casein [f96-102] and β-casein [f193-209]) have higher ACE-inhibitory activities than longer peptides. κ-casein (f96-102) has shown ACE-inhibitory activities with an IC_{50} of 9.64 ± 3.67 μgmL^{-1}. In addition, β-casein (f193-209) isolated from casein by extracellular proteinase from *L. helveticus* has been shown to possess ACE-inhibitory activity with IC_{50} of 101 μgmL^{-1} (Ong & Shah 2008b). Proteolysis in cheese is a multi-phase process initiated as a result of chymosin residues' activities on intact casein, especially alpha-caseins. This produces intermediate (large) peptides, which are metabolic substrates for proteolytic enzymes produced by starter and non-starter culture in cheese (Sousa et al. 2001). Consequently, small peptides and free amino acids are produced. It is believed that these small peptides possess more ACE-inhibitory activities than do large (intermediate) peptides (Sieber et al. 2009).

However, while small peptides possess ACE-inhibitory activities (Sieber et al. 2009), they are also associated with bitterness in cheese. This can be amplified by the activity of high proteolytic starter culture (*L. helveticus*) at lower salt concentrations, leading to greater development of small peptides. In a previous study, Cheddar cheeses made using *L. helveticus* and ripened at 4°C showed higher (~30%) ACE-inhibitory activity compared to a control cheese made with *Lactococcus* (Ong & Shah 2008a).

The proteolysis process is affected by several factors, including storage temperature and S/M. The increase of S/M decreases the activity rate of all proteolytic enzymes in cheese. S/M level highly correlates with the amount of salt added during the cheese making process. In this study, lowering the salt amount from 2.5% to 1% during cheese making decreased the S/M in cheese (Table 6), and thereby increased the extent of proteolysis (Table 9). Therefore, ACE-inhibitory activities were higher in cheeses with lower salt content (Figure 7).

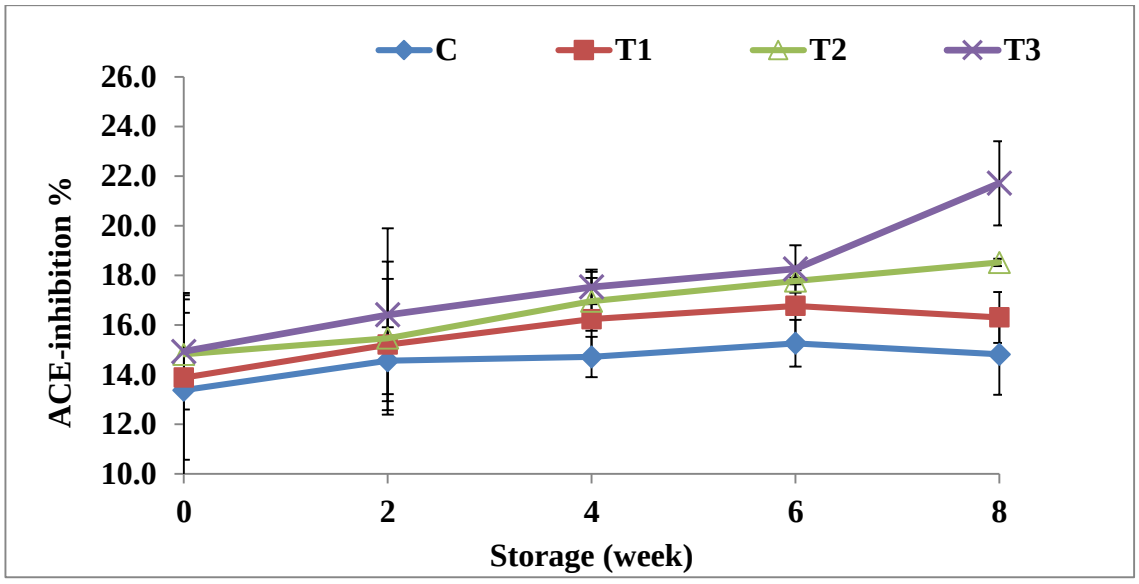


Figure 7: ACE-inhibitory Activity of Cheddar Cheeses Made with Four Levels of NaCl during Eight Weeks of Storage at 4.5°C

3.3.9 Texture Profile Analysis

Table 10 presents the texture profile results measured by an Instron texture analyser for Cheddar cheese salted at four different levels and stored at 4.5°C for eight weeks. The hardness and gumminess of the experimental cheeses decreased ($P < 0.05$), while the adhesiveness increased ($P < 0.05$) during storage at the same salt treatment (Table 10). At the same storage time, the hardness of cheeses salted with 2.5% and 2.0% (T1) was significantly higher ($P < 0.05$) than that of the other treatments (T2 and T3). The cohesiveness of the control cheese was significantly lower ($P < 0.05$) than the other experimental cheeses. Hardness and cohesiveness follow a contradictory trend, an increase in hardness decreases cohesiveness, and vice versa (Fox et al. 2000a; Gunasekaran & Ak 2003).

The gumminess of the control and T1 cheeses was significantly higher ($P < 0.05$) than that for T2 and T3 (Table 10). Gumminess is directly affected by hardness because it results from hardness × cohesiveness (Fox et al. 2000a; Gunasekaran & Ak 2003). Adhesiveness showed a significant difference ($P < 0.05$) between cheeses at the same storage period. The higher ($P < 0.05$) hardness and gumminess of control and T1 was due to a higher salt amount added to these cheeses than in T2 and T3. The increase in

salt content would decrease the amount of moisture content in the cheese, and thereby increase the hardness and gumminess of the cheese (Guinee 2004a).

An increase in proteolysis (Table 9) during storage leads to a soft texture in cheeses (Pollard et al. 2003; Upadhyay et al. 2004). It is recognised that the increased proteolysis and decrease in S/M affect the cheese texture (Guinee 2004a; Mistry & Kasperson 1998), resulting in textural shortness and crumbliness. Textural shortness demonstrates a weaker cohesion force between cheese particles, and the cheese consequently crumbles during mastication and elicits a less creamy sensation (Moller et al. 2013). In addition, the development of texture in cheese has a direct correlation with the amount of casein hydration. A decrease in the salt content may lead to an increase in the expulsion of colloidal calcium phosphate (CCP) from curd as a result of pH drop (Roefs et al. 1985). This leads to greater casein hydration, which creates a soft texture in cheese (Guinee 2004a). Moreover, reducing salt influences cheese pH, which affects starter and enzyme activities, leading to changes in the cheese's textural characteristics (Lawrence et al. 2004).

Table 10: Texture Profile of Cheddar Cheeses Made with Four Levels of NaCl during Eight Weeks of Storage at 4.5°C

	Salt treatment[1]	Storage (weeks)				
		0	2	4	6	8
Hardness	C	19.17±0.47^{aA2}	16.32±0.74^{aB}	15.26±0.14^{aC}	13.14±0.76^{aD}	11.06±0.85^{aE}
	T1	18.11±0.39^{bA}	15.45±0.36^{bB}	13.19±0.36^{bC}	11.78±0.72^{cBC}	10.75±0.22^{bD}
	T2	14.54±0.35^{cA}	11.50±0.67^{cB}	9.84±0.45^{cC}	8.69±0.71^{cD}	8.24±0.62^{cD}
	T3	13.27±0.49^{dA}	10.25±0.21^{cB}	9.05±0.47^{cC}	7.37±0.45^{dD}	6.71±0.34^{dE}
Cohesiveness	C	0.640±0.04^{aA}	0.660±0.03^{aA}	0.680±0.16^{aA}	0.700±0.01^{aB}	0.750±0.02^{aB}
	T1	0.670±0.01^{abA}	0.710±0.01^{bB}	0.730±0.16^{bB}	0.750±0.02^{bB}	0.780±0.02^{bB}
	T2	0.690±0.03^{aA}	0.720±0.00^{bB}	0.760±0.10^{bB}	0.790±0.01^{bB}	0.790±0.02^{bB}
	T3	0.760±0.01^{bA}	0.790±0.02^{bA}	0.840±0.03^{cB}	0.870±0.07^{cB}	0.890±0.03^{cB}
Adhesiveness	C	0.120±0.03^{aA}	0.520±0.10^{aB}	0.570±0.02^{aB}	0.650±0.04^{aC}	0.680±0.19^{aC}
	T1	0.190±0.05^{abA}	0.570±0.19^{abB}	0.540±0.03^{aC}	0.660±0.03^{aD}	0.760±0.12^{bE}
	T2	0.240±0.02^{cA}	0.580±0.07^{abB}	0.630±0.08^{bC}	0.750±0.07^{bD}	0.770±0.09^{bD}
	T3	0.280±0.01^{cA}	0.670±0.08^{bB}	0.650±0.22^{cC}	0.820±0.03^{cD}	0.870±0.20^{cD}
Gumminess	C	12.27±0.37^{aA}	10.77±0.56^{aB}	10.38±0.97^{aB}	9.20±0.47^{aC}	8.30±0.78^{aD}
	T1	12.13±0.21^{aA}	10.97±0.35^{aB}	9.63±0.19^{bC}	8.84±0.39^{bD}	8.39±0.37^{aD}
	T2	10.03±0.25^{bA}	8.28±0.17^{bB}	7.48±0.60^{cC}	6.87±0.05^{cD}	6.51±0.36^{bD}
	T3	10.09±0.57^{bA}	8.10±0.51^{bB}	7.60±0.60^{cC}	6.41±0.24^{cD}	5.97±0.68^{cE}

[1] Salt treatments: C = control (2.5%); T1 = salted at 2%; T2 = salted at 1.5% and T3 = salted at 1%

[2] Mean value ± SE of three trials

$^{a-e}$ Means in each column with different letters are significantly different ($P < 0.05$) at same storage period

$^{A-E}$ Means in the same row with similar capital letters differed insignificantly.

3.3.10 Cheese Microstructure by ESEM

Figures 8 and 9 present the ESEM images of the experimental cheeses at zero and eight weeks of storage, respectively. Figure 8 shows small voids and cavities in all experimental cheeses. These cavities were more obvious in the cheeses with lower salt content (T2 and T3). However, the cavities reduced slightly with prolonged storage, and this was more noticeable in cheeses at the end of the storage period (Figure 9). Increased proteolysis (Table 9) over time produced small soluble peptides in the serum phase of cheese (Fox & McSweeney 1996; Upadhyay et al. 2004), thereby producing a denser and closer structure. Reducing salt and using a high proteolytic starter culture amplified the disappearance of voids and cavities. This resulted from excessive production of soluble peptides in the serum phase of cheese, especially at lower salt concentrations (T1, T2 and T3), leading to suffuse of pores in the cheese matrix. Figure 9 illustrates a more dense and compacted matrix in the cheeses with the least salt concentration (T2 and T3) at the end of the ripening period (Week 8).

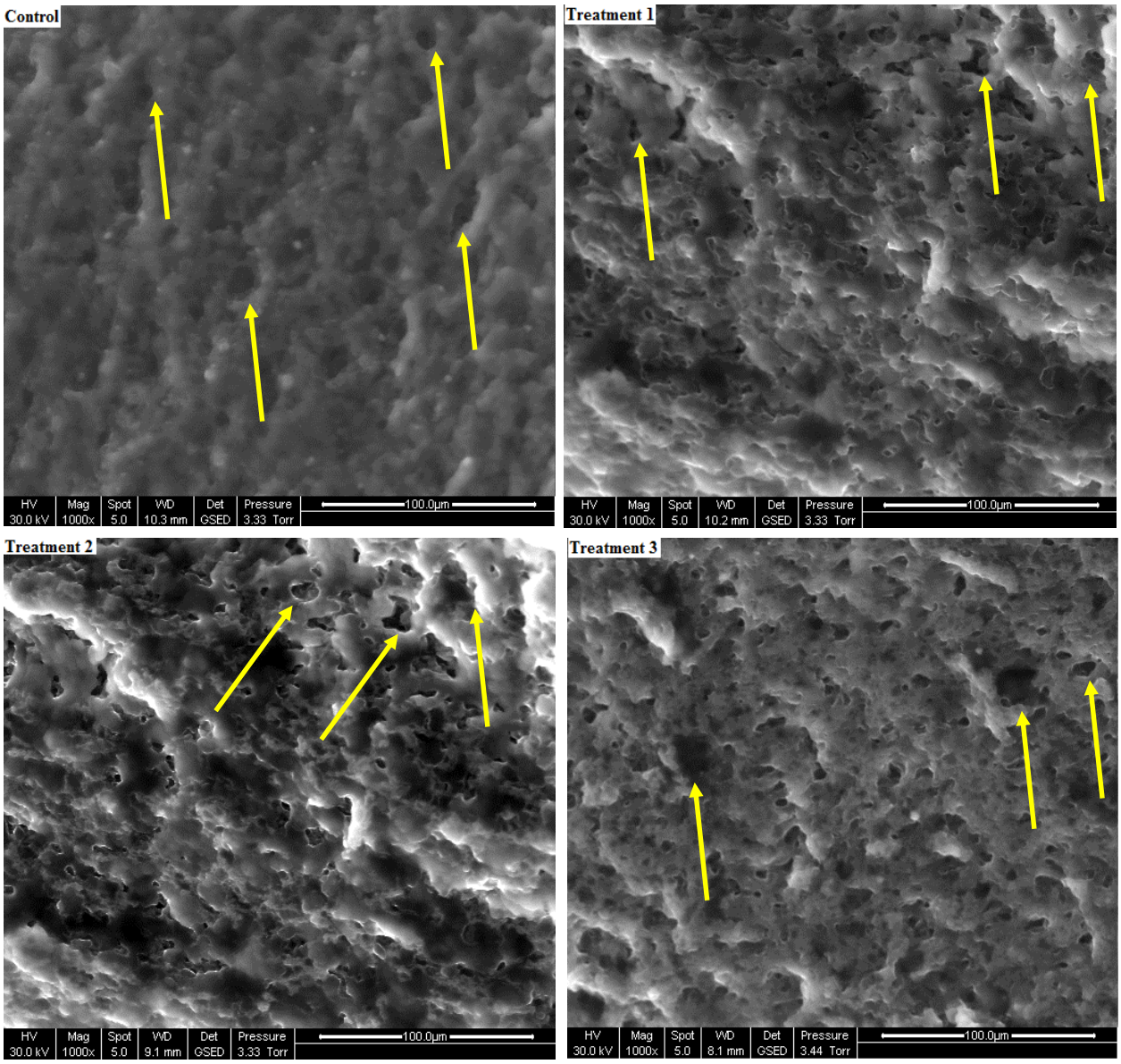


Figure 8: ESEM Images of Cheeses Made with Four Levels of NaCl during Week 0 of Storage at 4.5°C. Arrows are Indicating Cavities and Pores within the matrix.

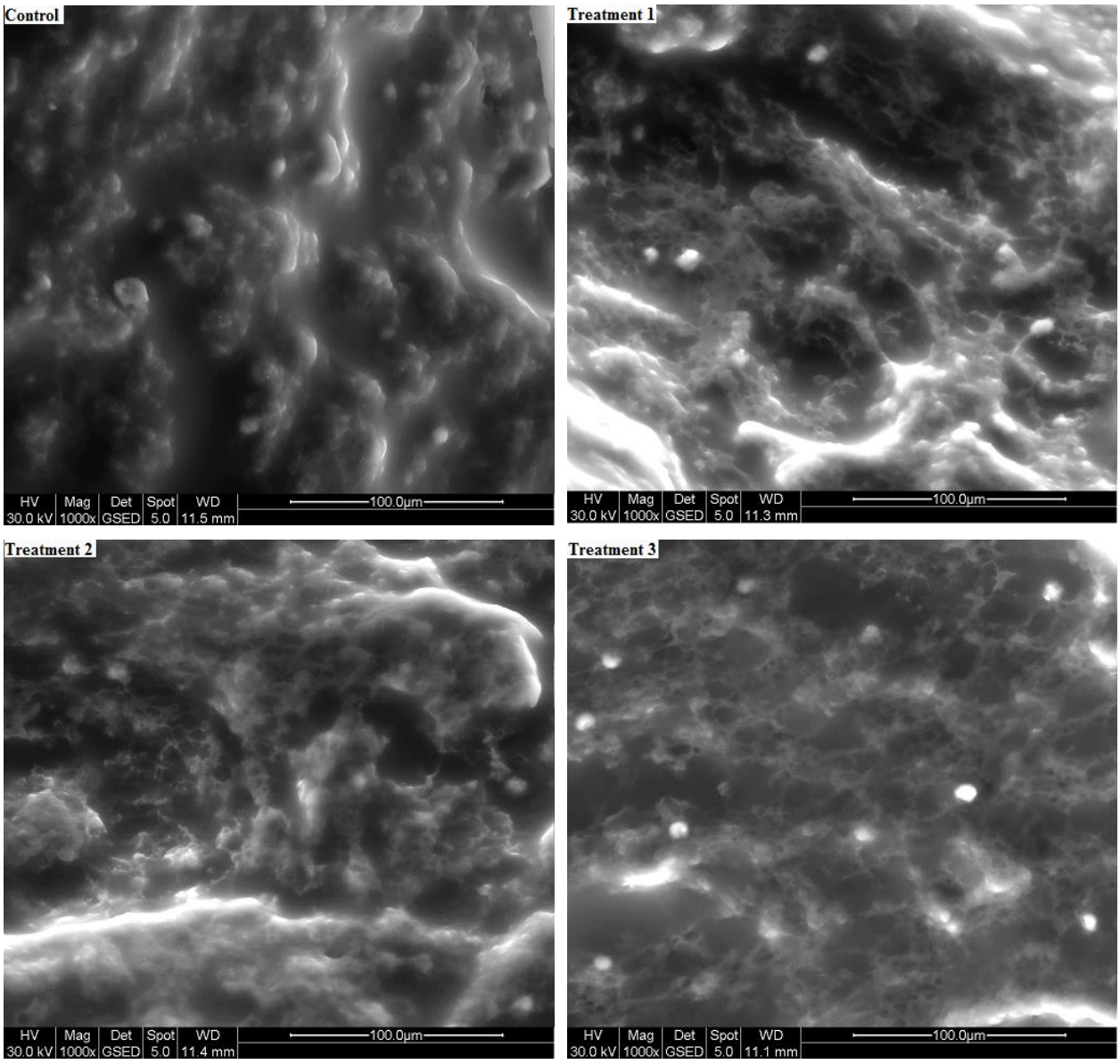


Figure 9: ESEM Images of Cheeses Made with Four Levels of NaCl during Eight Weeks of Storage at 4.5°C

3.3.11 Sensory Evaluation

Table 11 presents the scores for the creamy, sour-acid, salty, bitter, vinegary, and acceptability attributes of the experimental cheese samples (Weeks 0 and 8). At Week 0, there were no significant differences between the experimental cheeses for any sensory attribute, except saltiness. However, significant differences were clearly observed at Week 8. The cheese with 2.5% salt (control) received higher ($P < 0.05$) acceptability scores than did the cheese with low salt content. The lowest salted cheese samples had

lower ($P < 0.05$) acceptability scores than did the other treatments. Besides saltiness, the effect of salt on proteolysis affected the flavour (Upadhyay et al. 2004). The vinegary attribute received higher ($P < 0.05$) scores in T2 and T3, compared to the control and T1, at Week 8. A high salt content in cheeses inhibits bacterial growth (Guinee 2004a), which reduces the production of the acetic acid that contributes to a vinegary sensation (Table 8). The sour-acid, bitter and vinegary taste sensations increased significantly ($P < 0.05$) during storage in all experimental cheese samples. This was due to the production of organic acids (sour-acid and vinegary) and peptides (bitterness) associated with bacteria growth in the low-salt samples (Table 8 and Figure 6, respectively).

Table 11: Sensory Attributes of Experimental Cheeses at Weeks 0 and 8 of Storage at 4.5°C

	Salt treatment[1]	Storage (weeks)	
		0	8
Creaminess	C	6.17±0.58[a2]	5.96±0.55[a]
	T1	6.13±0.40[a]	5.93±0.50[a]
	T2	6.10±0.41[a]	5.93±0.60[a]
	T3	6.19±0.56[a]	5.93±0.54[a]
Sour-acid	C	3.40±0.63[a]	4.75±0.55[a]
	T1	3.59±0.60[a]	5.36±0.59[b]
	T2	3.66±0.68[a]	5.50±0.64[b]
	T3	3.87±0.58[a]	5.63±0.56[b]
Saltiness	C	6.26±0.55[a]	6.53±0.51[a]
	T1	6.22±0.59[a]	5.60±0.49[b]
	T2	5.83±0.52[b]	5.52±0.66[b]
	T3	5.70±0.59[b]	4.84±0.50[c]
Bitterness	C	1.80±0.55[a]	5.29±0.83[a]
	T1	1.94±0.61[a]	5.32±0.75[a]
	T2	2.19±0.55[b]	5.93±0.80[a]
	T3	2.23±0.60[b]	6.25±0.66[b]
Vinegary	C	2.73±0.59[a]	3.86±0.72[b]
	T1	2.73±0.70[a]	3.91±0.61[b]
	T2	3.86±0.63[b]	3.93±0.65[b]
	T3	3.97±0.57[a]	4.39±0.63[c]
Acceptability	C	6.54±0.74[a]	6.05±0.61[a]
	T1	6.44±0.49[a]	6.00±0.64[a]
	T2	6.30±0.59[a]	5.99±0.74[a]
	T3	6.23±0.41[a]	4.39±0.58[c]

[1] Salt treatments: C = control (2.5%); T1 = salted at 2%; T2 = salted at 1.5% and T3 = salted at 1%
[2] Mean value ± SE of three trials
[a-c] Means in each column with different letters are significantly different ($P < 0.05$) at same storage period.

3.4 Conclusion

This study showed that salt reduction significantly influences the chemical composition (moisture, ash and pH), proteolysis, texture profile (hardness, adhesiveness, cohesiveness, springiness and gumminess), microstructure and sensory properties of cheese. The moisture content of the cheese was affected during production and ripening. Salt reduction is a major factor affecting the moisture content in cheese. The protein and fat content of the cheese did not differ significantly from changing the salt concentration. The ash content showed a difference between the experimental cheeses due to the change in salt concentration during the cheese making process.

The starter culture growth, WSN, TCA-SN, PTA-SN and TFAA of cheeses with a lower salt content were higher ($P < 0.05$) than in the control (2.5% salt). The sensory quality of cheeses was also predominately influenced by salt reduction due to its effect on proteolysis. The influence of salt reduction, particularly below 2% salt, was mainly due to the higher proteolytic activity of the starter culture, as indicated by PTA-SN values at the same storage time. The ACE inhibition also increased with a maximum inhibition of 19%, corresponding to the cheese with 1% salt, stored for eight weeks. Although, a direct comparison cannot be made between our findings and existing studies; however, Ong & Shah (2008a) have reported significant increase in organic acids production (~2%), proteolysis parameters (~10%), and ACE inhibition (~30%) of Cheddar cheese upon using *L. helveticus* comparing to regular Cheddar culture at similar storage conditions. These values are shown to be higher than our findings due to longer storage time at a higher temperature. Also, the salt concentration in our study was significantly lower comparing to other studies.

The hardness and gumminess were reduced significantly when the salt concentration was $\leq 1.5\%$ for the entire eight weeks of storage. The differences in the protein matrices due to the proteolysis pattern were reflected in the microstructure of the cheeses, as evidenced by the disappearance of cavities and voids due to the peptides and amino acids filling the cavities in the low-salt cheeses.

Chapter 4: Proteolysis of Reduced-salt Cheddar Cheese as Affected by Changes in the Casein-to-fat Ratio, Rennet Concentration and pH at Drainage

4.1 Introduction

Proteolysis is pivotal to Cheddar cheese quality and depends on various factors, such as the enzymes of milk (plasmin), added coagulants (chymosin) and starter culture accelerating the proteolytic process in Cheddar cheese (Fox & McSweeney 1996). The cheese flavour is directly affected by the amount and type of peptides and free amino acids produced during ripening (Sousa et al. 2001), with peptides produced during proteolysis under certain conditions causing bitterness in Cheddar cheese (Guinee & Fox 2004). Agboola et al. (2004) reported that the bitterness in ovine cheese made with microbial coagulant was high due to the production of hydrophobic peptides. A similar study was undertaken by Børsting et al. (2012) on fat-reduced Cheddar cheese made with different coagulants, in which Cheddar cheeses made with bovine chymosin developed a strong bitter taste. Pollard et al. (2003) suggested that enhancing proteolytic activity in Cheddar cheese decreased firmness and increased crumbliness. The conversion of casein into polypeptides and smaller water-soluble peptides leads to a soft texture.

Salt is added in the Cheddar cheese making process for a variety of reasons, as reviewed thoroughly by Guinne and Fox (1984) and Guinee and Fox (2004b). Salt in cheese influences proteolysis indirectly. Higher salt content results in lower SLAB and NSLAB activity, reduced proteolysis, and consequently lower peptide production, which reduces the sensory quality of cheese (Guinee 2004a). However, salt is associated with some health issues, including hypertension, osteoporosis, kidney stones and cardiovascular diseases (Turk et al. 2009); therefore, reduction in salt content of Cheddar cheese is investigated by changing the formulation and manufacturing steps of cheese making. Reducing salt content adversely affects most characteristics of cheese, as described by several researchers (Fox 1987; Guinee & Fox 2004; Singh & Cadwallader 2008).

Sheibani et al. (2013) reported that quality Cheddar cheese can be manufactured with reduced salt content by controlling and monitoring proteolysis. Therefore, the current study was designed to investigate factors influencing the proteolysis of low-salt Cheddar cheeses. There appears to be a lack of information about how proteolysis is affected by modifying the casein-to-fat (C/F) ratio, rennet concentration and pH at drainage, particularly with low-salt Cheddar cheese. Thus, the objective of this study was to establish the effect of changes in drainage pH, rennet concentration and C/F ratio on the proteolysis of salt-reduced Cheddar cheese (1.5% w/w) during storage at 9 ± 0.5°C for 180 days.

4.2 Materials and Methods

4.2.1 Experimental Design

Using a full factorial design, this study examined the effect of the C/F ratio of the milk (0.6, 0.7 and 0.8), pH at drainage (6.2, 5.9 and 5.6) and rennet concentration (0.1 and 0.3 mL per litre of milk) on proteolysis. Eighteen experimental cheeses (3 C/F ratios × 3 pH levels × 2 rennet concentrations) were made. Table 12 presents the code of each experimental cheese. The experimental design was replicated three times. The mean composition of milk was as follows: protein = 3.2g/100g, fat = 3.8g/100g, carbohydrate = 4.9g/100g, sodium = 41mg/100g, calcium = 117mg/100g and pH = 6.70. Composition of milk used was analysed to assess and monitor any changes. No significant variation in milk composition was found. Skim milk and cream were used to obtain the desired C/F ratio (0.60, 0.70 and 0.80).

Table 12: Experimental Design

Factors			Treatment codes
pH	**C/F ratio**	**Rennet concentration (mL/L)**	
6.2	0.6	0.1	(6.2/0.6/0.1)
		0.3	(6.2/0.6/0.3)
	0.7	0.1	(6.2/0.7/0.1)
		0.3	(6.2/0.7/0.3)
	0.8	0.1	(6.2/0.8/0.1)
		0.3	(6.2/0.8/0.3)
5.9	0.6	0.1	(5.9/0.6/0.1)
		0.3	(5.9/0.6/0.3)
	0.7	0.1	(5.9/0.7/0.1)
		0.3	(5.9/0.7/0.3)
	0.8	0.1	(5.9/0.8/0.1)
		0.3	(5.9/0.8/0.3)
5.6	0.6	0.1	(5.6/0.6/0.1)
		0.3	(5.6/0.6/0.3)
	0.7	0.1	(5.6/0.7/0.1)
		0.3	(5.6/0.7/0.3)
	0.8	0.1	(5.6/0.8/0.1)
		0.3	(5.6/0.8/0.3)

4.2.2 Cheese Preparation

The cheeses were prepared following the protocol of Kosikowski (1977), with some modifications. Pasteurised bovine skim milk and cream were obtained from a local supplier (Parmalat, Victoria, Australia) and the C/F ratio was standardised to 0.6, 0.7 or 0.8, followed by homogenisation (HST Homogeniser HL3, Unipulse Pty Ltd, Victoria, Australia) at 5 x 10^6 and 2.5 x 10^7 Pa at 50°C. Afterwards, the milk was kept overnight at 4°C. The following day, the milk was transferred to temperature-controlled cheese vats (25 L, Unipulse Pty Ltd, Victoria, Australia) and tempered at 32°C for 30 min. After addition of 10% calcium chloride solution (2.5 mL per litre of milk), a commercial freeze-dried Cheddar cheese culture consisting of *Lactococcus lactis lactis* and *Lactococcus lactis cremoris* (0.25% [w/v]; R-704 Chr. Hansen, Bayswater, Victoria, Australia) was added and held for 35 min, before a 0.1 or 0.3 mL/L of diluted single-strength rennet (CHY-MAX® 200IMCU/mL, Chr. Hansen) was added with rigorous mixing for one minute. The milk was coagulated in 35 to 40 min and the curd (pH 6.48) was cut into 1 cm^3 cubes using cheese wire knives. The curd was cooked at 38°C until the pH dropped to 6.2, 5.9 and 5.6. The whey was then drained from the curd

before Cheddaring at 38°C until pH reached 5.3. The milled curds were salted with salt (1.5% w/w, curd basis) and mellowed for 10 min. The salted curds were hooped in 2.5 kg capacity moulds and pressed at a pressure of 2.41 kPa overnight at room temperature. The pressed curd was vacuum packaged in oxygen barrier bags (Collinsons Pty Ltd, Fawkner, Australia) using Multivac vacuum packaging equipment (Multivac Sepp Haggenmuller, Wolfertschwenden, Germany), and then ripened at 9.0 ± 0.5 °C for 180 days. The cheeses were sampled at Days 0 (immediately after pressing), 60, 120 and 180 of storage, and then vacuum packaged again. All analyses were undertaken in duplicates.

4.2.3 Chemical Composition

Moisture was determined via the oven-drying method using acid-washed sand (Sigma, St Louis, Missouri, US) at 102°C, fat was determined via the Babcock method, protein was determined via the Kjeldahl method, and ash was determined via the muffle furnace method following AOAC methods (AOAC International 1995). For pH measurement, 20 g of grated cheese was macerated with 20 mL distilled water, and the pH of the resulting slurry was measured using a digital pH meter (MeterLab, Pacific Laboratory Products, Blackburn, Victoria, Australia) after calibration. The total sodium content of the cheeses was measured using a multi-type inductively coupled plasma atomic emission spectrometer (ICPE-9000; Shimadzu Scientific Instruments [Oceania] Pty Ltd, Rydalmere, New South Wales, Australia), according to Ayyash and Shah (2010).

4.2.4 Enumeration of Total LAB and NSLAB

The total LAB growth in the cheeses was measured using the pour-plating method described by Mistry and Kasperson (1998), with minor modifications. In brief, 11 g of grated cheese and 99 mL sterile distilled water were blended for two min in a stomacher-400 laboratory blender (Seward Medical, London, UK). One millilitre of resultant mixture was used to prepare serial dilutions in sterilised 0.1% peptone. The total LAB was enumerated on MRS agar (Merck Pty Ltd, Bayswater, Victoria, Australia) and incubated anaerobically using anaerobic jars (Becton Dickinson Microbiology Systems, Sparks, Maryland, US) and carbon dioxide (CO_2) generating kits (Oxoid BR0038B, Oxoid Ltd, West Heidelberg, Victoria, Australia) at 37°C for 48

h. NSLAB growth was enumerated using M17 agar (Oxoid) aerobically at 37°C for 48 h (Jordan & Cogan 1993).

4.2.5 Proteolysis Assessment

4.2.5.1 Water-soluble Nitrogen (WSN)

The water soluble extract (WSE) of each cheese sample was prepared according to Kuchroo and Fox (1982). Briefly, a mixture of cheese and distilled water (1:2) was kept in a 40°C water bath for 60 min, followed by centrifugation (Sorvall T6000D, Thermo Scientific) at 4000 × g for 30 min. The resultant slurry was filtered through a 0.45 μm filter (Millipore Corp., Bedford, Massachusetts, US). The total nitrogen in the filtrate extract (3 mL) was determined by the Kjeldahl method (AOAC International 1995).

4.2.5.2 Twelve Per Cent Trichloroacetic Acid-soluble Nitrogen (TCA-SN)

Twelve per cent TCA-SN was determined in 9 mL filtrate obtained after 5 mL of WSE was mixed with 5 mL of 24% TCA (Sigma) and left overnight, followed by centrifugation at 4000 × g for 20 min. The total nitrogen in the filtrate was determined by the Kjeldahl method (AOAC International 1995).

4.2.5.3 Measurement of Total Free Amino Acids (TFAA)

The filtrate collected for total nitrogen determination was used to determine the TFAA concentration by employing the Cd-ninhydrin method according to Folkertsma and Fox (1992).

4.2.5.4 Peptides Profile by RPHPLC

The peptide profile of the experimental cheeses was analysed by RPHPLC using an analytical column (C18, 250 mm × 4.6 mm, 5 μm; Grace Vydac, Hesperia, California, US), as described previously by Ong and Shah (2008b). The peptide fractions [(hydrophilic, hydrophobic (about 20 amino acids) and extra-hydrophobic (over 20 amino acids)] were calculated according to the method of Sadat-Mekmene et al. (2013), based on the elution times. Peptides that appeared between the retention times of 2 to 22

min, 22 to 50 min and 50 to 100 min were considered hydrophilic (A), hydrophobic (B) and extra-hydrophobic (C), respectively. The percentages were calculated according to the following formula:

Equation 3:

$$\%\ of\ each\ fraction = \frac{the\ sum\ of\ area\ counts\ of\ fraction\ peaks}{total\ area\ counts\ of\ all\ peaks\ from\ 0\ to\ 100\ min} \times 100$$

4.2.6 Statistical Analysis

The general linear model was used to examine the effect of drainage pH, C/F ratio and rennet concentration and their interactions at α = 5% for each storage time. Fisher's least significant difference test was conducted to test the significant differences between the means of all 18 experimental cheeses at each storage period ($P < 0.05$). Data analysis was undertaken using SAS software V9.0 (SAS Institute Inc. 2008).

4.3 Results and Discussion

4.3.1 Chemical Composition and pH

Milk standardisation in cheese making is a very important step to control cheese quality. This standardisation enables cheese manufacturers to manage the seasonal variations in milk composition that would otherwise lead to variation in the chemical composition of cheese and subsequently the cheese quality (Walker et al. 2007). The results of this study show that variation in the C/F ratio influenced several of the measured parameters. Table 13 presents the chemical compositions of the experimental Cheddar cheeses made with different drainage pH, C/F ratio and rennet concentrations at Day 0 (right after pressing) of storage. A higher amount of moisture was retained (35 to 37%) in cheeses made with pH 6.2 compared to those made with pH 5.9 and 5.6. An opposite trend was observed for S/M content. A drop in pH at drainage from 6.2 to 5.6 caused a significant drop in moisture content (2.0 to 3.0%), but showed a moderate increase in ash content. Further, at the same pH, cheeses made with a C/F ratio 0.6 had a higher ($P < 0.05$) moisture content compared with 0.7 and 0.8.

At the same drainage pH level, the ash content in cheeses made with 0.6 C/F ratio was lower ($P < 0.05$) than those made with C/F ratio 0.7 and 0.8. An increase in the C/F ratio increased the dry matter (DM) in cheeses, leading to a decrease in moisture content. In addition, the increase in C/F ratio increased the ash content, corresponding to a decrease in moisture content and subsequent increase in S/M. These results align with Fox et al. (2000a), who reported that an increase in fat content (decreased C/F ratio) contributes to increasing the level of moisture in non-fat substances. Fat is occluded in the para-casein network of the cheese, and impedes syneresis. Fat globules trigger less aggregation of the surrounding para-casein network, and subsequently reduce the degree of moisture expulsion. Lawrence et al. (2004) also stated that increasing the fat content (decreasing the C/F ratio) in milk during cheese manufacture reduces the expulsion of whey.

The drainage pH had a significant effect on the moisture content of the experimental Cheddar cheeses. The moisture content decreased when the drainage pH decreased from 6.2 to 5.6. This may be attributed to the high whey repulsion occurring as a result of acidity increase during cooking. An increase in the time between cutting the curd and whey drainage is known to increase acid development, thereby further enhancing whey repulsion from the curd (Tunick et al. 2007). The decrease in drainage pH may have increased the solubilisation of CCP from the casein micelle to whey, and consequently increased the loss from curd, which decreases the total ash content in cheeses (Roefs et al. 1985). In a previous study, the calcium content in mozzarella cheese decreased as a result of decreased pH (from 6.40 to 6.12) at the whey drainage step (Yun et al. 1995).

In the current study, the sodium content of cheeses ranged from 360 to 425 mg/100g (Table 13). The slight differences in the sodium content of the cheeses could be due to changes in the total solid content of the cheeses, which could have affected the S/M content (Fox et al. 2000a; Fox et al. 2004b).

Figure 10 presents the pH of the 18 experimental cheeses during ripening. The pH of all cheeses decreased significantly ($P < 0.05$) from Days 0 to 120 from about 4.9 to 4.6. After 120 days, the pH did not change. The prolonged cooking step used in this investigation may have induced a high initial drop of pH. However, the trend of pH

during ripening may be attributed to the activity of LAB, which reduced by the end of the storage (Parente & Cogan 2004). An increase in the C/F ratio and rennet concentration provided more protein fragments as a substrate for starter culture activity, which was the reason for the significant drop in the pH of cheeses made with a higher rennet concentration (Fox et al. 2004b).

Table 13: Chemical Composition of Experimental Reduced-salt Cheddar Cheeses (1.5% w/w of curd) at Day 0 of Storage

pH	C/F ratio[1]	Rennet Con.	Moisture%	Protein%	Fat%	Ash%	Sodium[2]
6.2	**0.6**	**0.1**	37.26±0.23^{a3}	24.18±0.23^{b}	33.38±0.24^{a}	2.61±0.02^{a}	412.2±5.9^{a}
		0.3	37.11±0.10^{a}	24.11±0.11^{b}	33.63±0.31^{a}	2.40±0.07^{a}	406.7±8.5^{a}
	0.7	**0.1**	36.38±0.20^{b}	24.65±0.14^{b}	30.13±0.60^{b}	2.40±0.03^{a}	379.9±8.3^{b}
		0.3	36.24±0.16^{b}	24.51±0.20^{b}	29.88±0.43^{c}	2.44±0.09^{a}	397.3±6.2^{b}
	0.8	**0.1**	36.19±0.24^{b}	25.78±0.24^{a}	30.05±0.48^{b}	2.76±0.01^{a}	403.4±7.8^{a}
		0.3	36.11±0.35^{b}	25.23±0.32^{a}	29.63±0.83^{c}	2.77±0.03^{a}	406.1±6.2^{a}
5.9	**0.6**	**0.1**	35.11±0.17^{c}	24.02±0.30^{b}	33.88±0.24^{a}	2.65±0.06^{a}	407.8±3.3^{a}
		0.3	34.92±0.12^{d}	24.12±0.13^{b}	33.73±0.24^{a}	2.47±0.08^{a}	373.1±9.1^{b}
	0.7	**0.1**	34.55±0.29^{e}	24.81±0.25^{b}	30.75±0.60^{b}	2.59±0.07^{a}	389.2±6.1^{b}
		0.3	34.36±0.28^{e}	24.99±0.45^{b}	32.13±0.24^{d}	2.62±0.01^{a}	414.0±8.6^{a}
	0.8	**0.1**	33.99±0.12^{e}	25.91±0.38^{a}	29.88±0.43^{c}	2.78±0.03^{a}	408.6±7.1^{a}
		0.3	33.59±0.25^{e}	25.71±0.18^{a}	29.25±0.52^{c}	2.79±0.02^{a}	393.0±4.2^{b}
5.6	**0.6**	**0.1**	34.86±0.19^{d}	24.95±0.29^{b}	33.38±0.24^{a}	2.46±0.05^{a}	404.7±7.6^{a}
		0.3	34.64±0.10^{d}	24.60±0.32^{b}	33.53±0.24^{a}	2.37±0.02^{a}	413.5±4.1^{a}
	0.7	**0.1**	33.89±0.13^{e}	24.79±0.20^{b}	31.75±0.32^{e}	2.58±0.06^{a}	424.5±6.3^{a}
		0.3	33.67±0.09^{e}	24.70±0.24^{b}	31.88±0.52^{e}	2.56±0.06^{a}	390.8±5.4^{b}
	0.8	**0.1**	33.55±0.24^{e}	25.37±0.33^{a}	29.63±0.52^{c}	2.83±0.03^{a}	411.3±4.9^{a}
		0.3	33.10±0.27^{e}	25.39±0.59^{a}	29.50±0.54^{c}	2.84±0.02^{a}	361.2±7.3^{b}

[1] C/F ratio = casein-to-fat ratio

[2] Sodium = sodium content of cheese (mg/100g)

[3] Values are average of four replicates (mean ± SE)

[a-e] Means in each column with different letters are significantly different ($P < 0.05$).

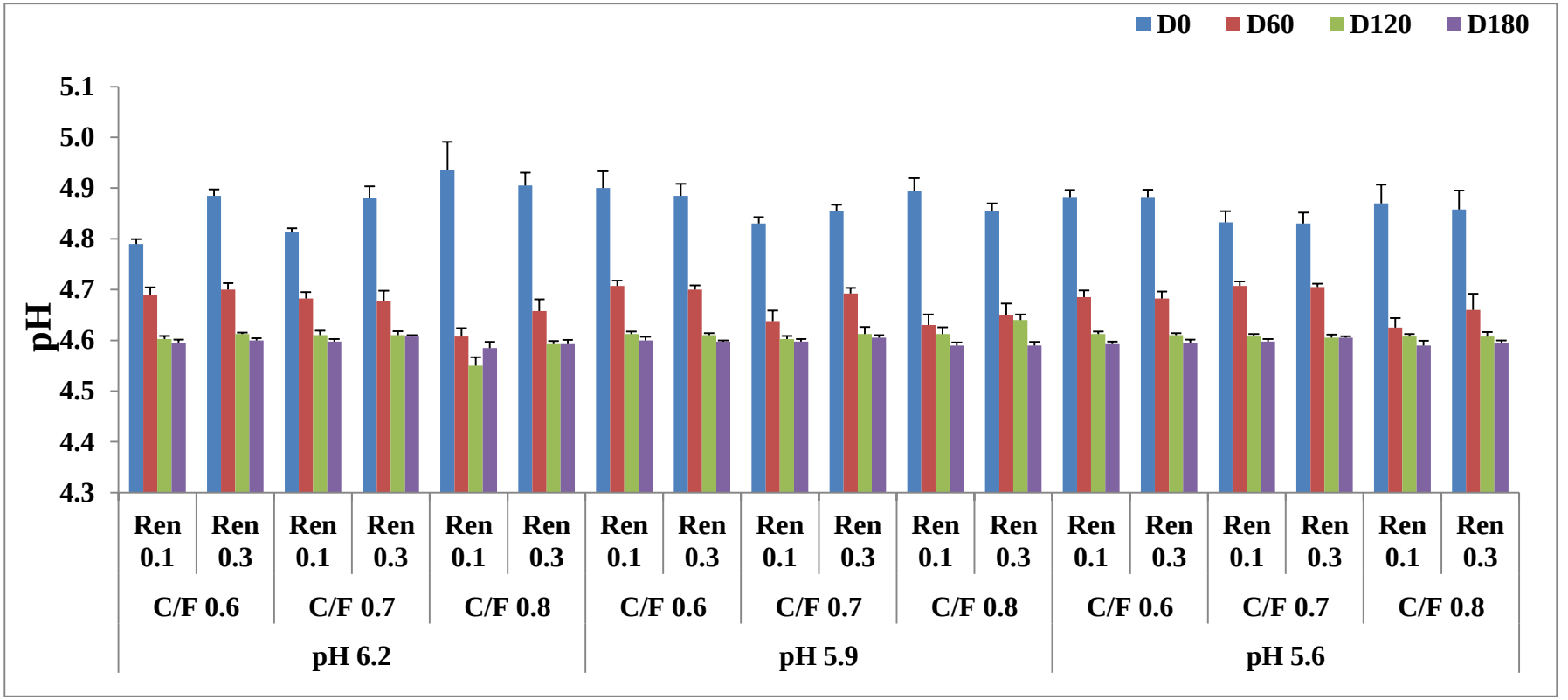


Figure 10: pH of Experimental Cheddar Cheeses Made with Different Treatment during 180 Days of Storage at 9 ± 0.5°C

4.3.2 Enumeration of Total LAB and NSLAB

Figures 11 and 12 present the total LAB and NSLAB growth at Days 0 and 180 of the 18 cheeses, respectively. In general, a higher population of LAB over the storage in the cheeses made with higher amount of fat (C/F ratio 0.6) is in accordance with Fenelon et al. (2000), who reported that the higher starter numbers in the full fat curd was due to comparatively lower syneresis. An increase in the fat content of cheese decreases the protein fraction, which triggers less protein denaturation and less discharge of moisture out of the curd (Fox et al. 2004b; Lawrence et al. 2004). This provides a higher a_w, which supports higher bacterial growth (Parente & Cogan 2004).

Figure 11 shows that the total LAB growth in the cheeses made with 0.3 mL/L rennet was higher ($P < 0.05$) than the cheeses with 0.1 mL/L rennet at the same C/F ratio and pH. At pH 5.9 and 5.6, the differences in total LAB growth in the cheeses made with 0.1 and 0.3 mL/L rennet were insignificant ($P > 0.05$) at the same C/F ratio and storage period. The increase in rennet concentration increased the amount of residual rennet in the curd, causing a higher rate of primary proteolysis (Upadhyay et al. 2004) and leading to production of more peptides used as substrates by LAB. As a result, the bacterial growth in cheeses made with 0.3 mL/L rennet was higher than in the cheeses with 0.1 mL/L rennet. At pH 5.6, regardless of C/F ratio, the total LAB growth in cheeses at Day 0 was significantly higher ($P < 0.05$) than at pH 6.2 and 5.9 (Figure 11). This may be due to prolonged cooking time, which provided an appropriate environment for LAB to grow (Fox et al. 2000b).

The NSLAB growth at Day 180 was significantly higher ($P < 0.05$) than that at Day 0 (Figure 12). At Day 180, the growth of NSLAB in all cheeses with 0.3 rennet concentration was higher (~0.1 to 0.2 Log_{10} $CFU.g^{-1}$). This may be due to a higher level of peptides, resulting from primary proteolysis (Upadhyay et al. 2004). The C/F ratio showed no significant effect on NSLAB.

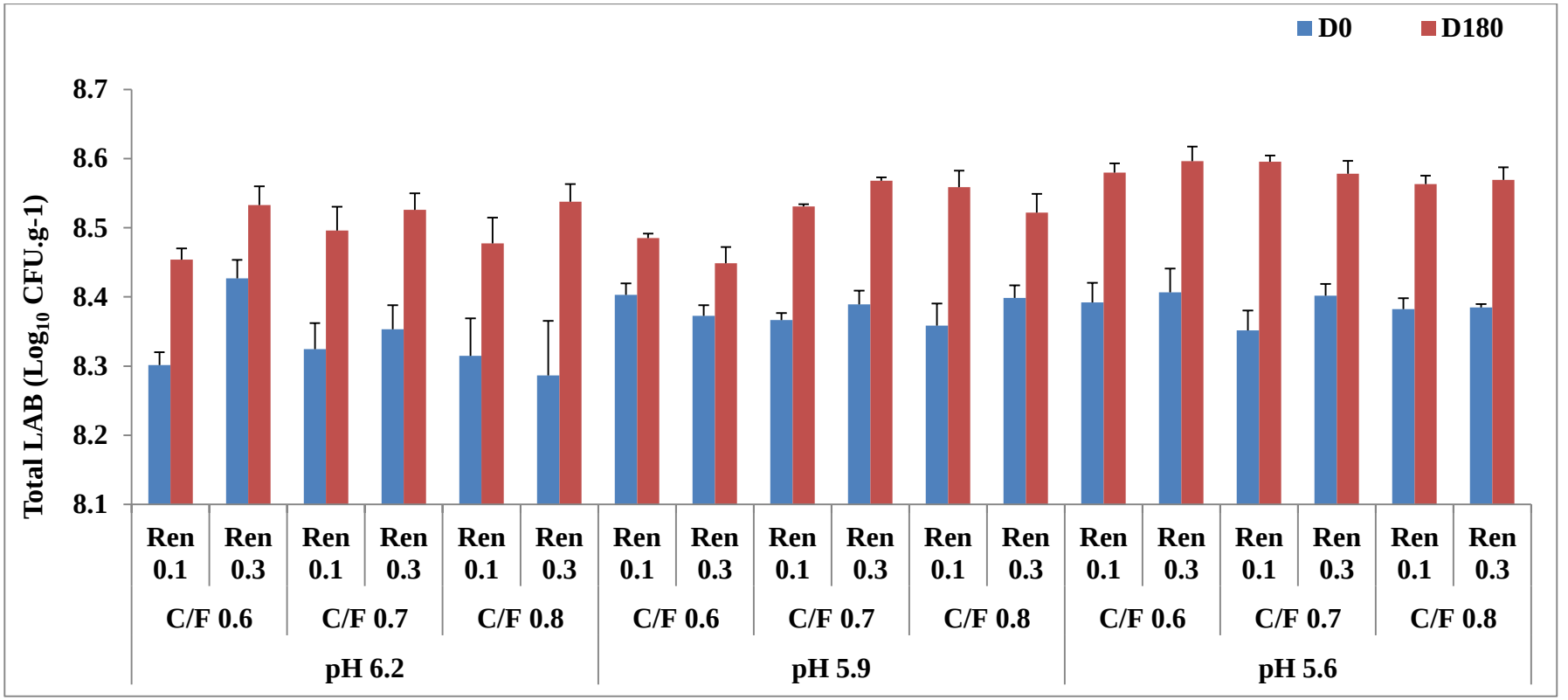


Figure 11: Total LAB of Experimental Cheddar Cheeses Made with Different Treatment at Days 0 and 180 of Storage at 9 ± 0.5°C

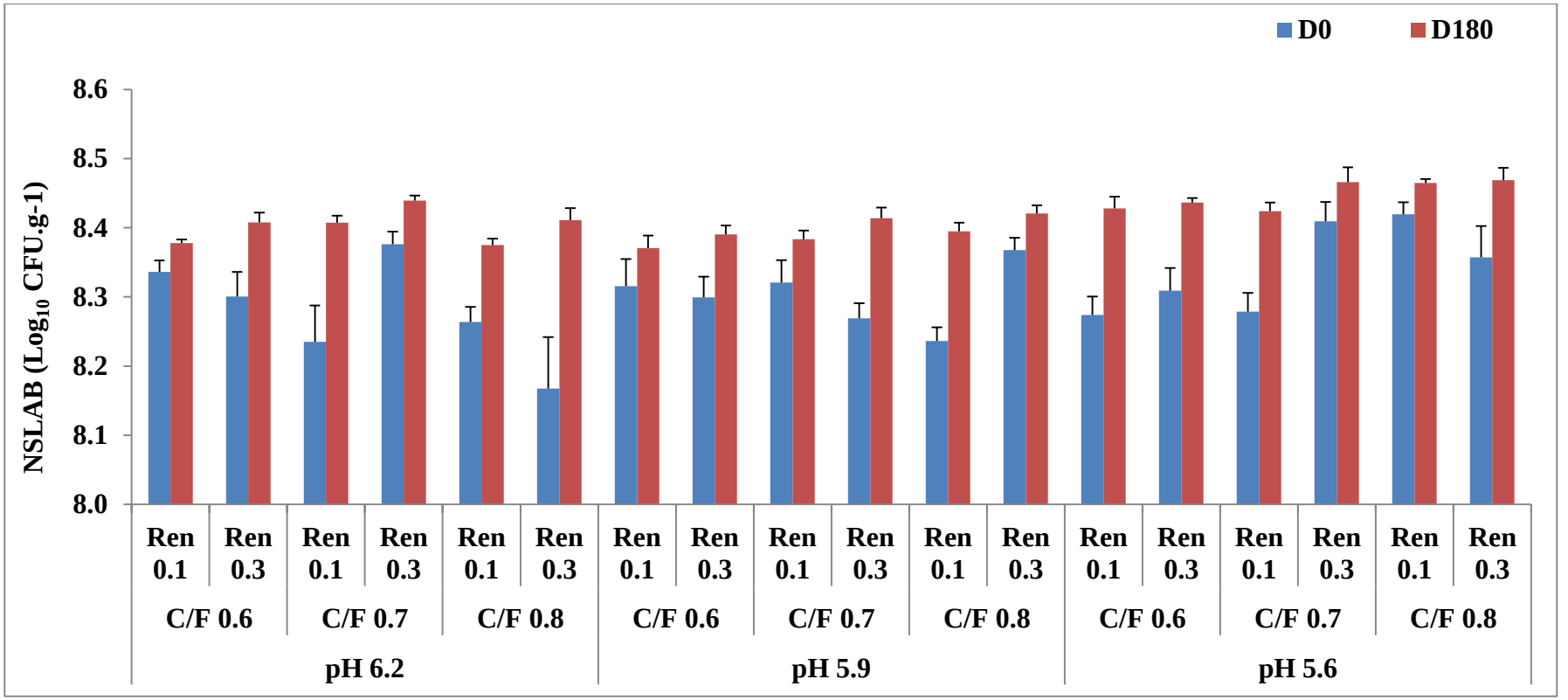


Figure 12: NSLAB of Experimental Cheddar Cheeses Made with Different Treatment at Days 0 and 180 of Storage at 9 ± 0.5°C

4.3.3 Proteolysis Assessment by WSN, TCA-SN, TFAA and Peptide Profile

The effect of change in the C/F ratio, rennet concentration and drainage pH on protein and moisture content affected the proteolysis in the salt-reduced Cheddar cheeses. The experimental cheeses that contained higher protein content had generally higher proteolysis rate (TCA-SN). However, the influence of C/F ratio on WSN was unclear. This may be attributed to WSN dependency on the remaining coagulant in cheese after drainage. Figure 13 presents the WSN content of the cheeses. In all experimental cheeses, WSN content increased significantly ($P < 0.05$) with the storage period (Figure 13). With a same drainage pH, C/F ratio and storage time, WSN content in the cheeses made with 0.3 rennet was higher ($P < 0.05$) than in the cheeses made with 0.1 mL/L rennet, indicating higher primary proteolysis occurring in cheese by coagulant residues (Fox & McSweeney 1996). Rennet and/or chymosin have a significant role in dictating the overall quality of cheese. Rennet activity initiates by cleaving the peptide bond (Phe_{105}–$Meth_{106}$) in κ-casein, which triggers coagulation of milk, the first step in cheese making (Crabbe 2004). Upadhyay et al. (2004) reported that the primary stage of proteolysis occurs due to the hydrolytic action of rennet residues in cheese. Rennet residues primarily start hydrolysis of α_{s1}-casein (Phe_{23}–Phe_{24}) with minor or no lysis action on β-casein (Fox & McSweeney 1996).

Increasing the rennet concentration significantly increased the WSN (Figure 13), and subsequently increased the related proteolysis parameters, such as TCA-SN and TFAA (Figures 14 and 15, respectively). These results are in accordance with the results of Soodam et al. (2015), who reported that pH 4.6 SN/TN increased significantly with increasing Hannilase rennet concentrations in Cheddar cheese. Kubiš et al. (2001) found that the pH 4.6 soluble nitrogen contents of Cheddar-type goat's milk cheeses increased during ripening, and its level was generally proportional to rennet levels. In other words, the acceleration of primary proteolysis stage may have occurred due to the increase in rennet concentration increasing nitrogenous substrates for further proteolysis by enzymes produced by SLAB and NSLAB in Cheddar cheese. In addition to the effect of the pH of whey at drainage, the excessive level of residual rennet left in the curd was higher, which affected the proteolysis.

During storage, several biochemical changes occur in cheese due to several hydrolytic activities, including proteolysis, lipolysis and glycolysis. Several factors influence the rate of hydrolytic activities during storage time (Fox & McSweeney 1996; Sousa et al. 2001). The results of this study have confirmed that storage time significantly affects the proteolysis parameters. WSN, TCA-SN and TFAA increased significantly during storage time, especially in cheeses made with a higher C/F ratio and rennet concentration. These results align with the results of several previous studies (Aston et al. 1983; Belitz & Kaiser 1993; Kaiser et al. 1992; Upadhyay et al. 2004). This trend of increase in WSN, TCA-SN and TFAA may be attributed to the proteolytic agents (such as rennet and bacterial enzymes) in Cheddar cheeses (Fox & McSweeney 1996).

In this study, the WSN content in cheeses made with C/F ratio 0.6 was significantly higher ($P < 0.05$) than that in cheeses made with 0.7 and 0.8 at pH 6.2. This trend disappeared at pH 5.9 and 5.6. This may have been due to the higher moisture content of cheeses made with 0.6 C/F ratio. Enzymatic activity in cheese has a direct relationship with moisture content, whereby an increase in moisture content develops rennet activity, as indicated by WSN (Fox et al. 2004a). It has previously been reported that β-casein is highly resistant to proteolysis in Cheddar cheese. Higher moisture and low salt contents in low-salt cheeses enhances proteolysis of β-casein by reducing the resistance to proteolysis (Phelan et al. 1973).

Drainage pH affects the coagulant retention in curd, which affects the proteolysis of cheeses (Lawrence et al. 1987). Reducing the pH at drainage increases the amount of coagulant remaining in the curd, and then increases the proteolysis rate during ripening (Upadhyay et al. 2004). In the current study, proteolysis increased when the drainage pH decreased from 6.2 to 5.6, as evidenced by the increased WSN, TCA-SN and TFAA values. These results align with those of Yun et al. (1995), who reported that proteolysis in mozzarella cheese increased as influenced by reduced whey pH at drainage. The main reason for this trend was the higher amount of coagulant remaining in the curd during Cheddar cheese processing. According to Fox and McSweeney (1996), proteolysis in cheese is divided into three sequential stages:

1. the primary stage, which is initiated by residual coagulant in cheese
2. the secondary stage, which begins with the action of proteases and peptidases in the starter culture in cheese

3. the advanced stage, which begins with the action of aminopeptidases produced by the starter culture and non-starter culture bacteria in cheese.

Thus, in this study's results, the amount of coagulant remaining in cheese was higher, which led to rapid onset of the primary stage of proteolysis, as observed in the WSN (Figure 13).

Subsequently, proteolytic enzymes produced by SLAB hydrolysed large and intermediate peptides effectively, which was obvious in the TCA-SN (Figure 14). The TCA-SN content in all experimental cheeses increased significantly ($P < 0.05$) during storage. At the same pH and storage period, the TCA-SN content increased significantly ($P < 0.05$) as the C/F ratio was raised from 0.6 to 0.8 (Figure 14). All cheeses with a higher rennet concentration and protein content showed a greater TCA-SN value, suggesting higher secondary proteolysis in cheese due to the function of LAB and other hydrolytic activities (McSweeney & Fox 1997). The increase in protein content in cheeses as a result of the high C/F ratio increased in the TCA-SN content.

This allowed for the aminopeptidases produced by SLAB and NSLAB to hydrolyse small peptides, and then produce a higher amount of TFAA (Figure 15). The TFAA content increased significantly ($P < 0.05$) over the entire storage period. Normally, TFAA indicates advanced proteolytic activity in cheese, which results in greater production of small size peptides and amino acids (McSweeney & Fox 1997). At 120 and 180 days of storage, the TFAA content in all cheeses made with a C/F ratio 0.8 and pH 5.6 was higher than that in cheeses made with pH 5.9 and 6.2 (Figure 15). An increase in rennet concentration caused an increase in the TFAA in all cheeses. The pH and storage had a more pronounced effect on the TFAA compared with the C/F ratio and rennet concentration, since a reduction in pH provided an appropriate environment for the proteolytic enzymes produced by LAB and NSLAB. A higher production of bacterial enzymes contributed to excessive proteolytic activity, and thus higher TFAA (Fox et al. 2000a; McSweeney & Fox 1997).

The ratios of the hydrophilic, hydrophobic and extra-hydrophobic peptides in fractions obtained from all treatment combinations were affected by storage time and rennet concentration ($P < 0.05$). Storage time was correlated negatively with production of hydrophilic peptides (-0.619, $P < 0.001$) and positively with extra-hydrophobic peptides

(0.709, $P < 0.001$) in the extract, indicating that concentration of extra-hydrophobic peptides increased over time compared to hydrophilic peptides (Figure 16). At Day 0, hydrophobic peptides were dominant in all cheeses, followed by hydrophilic. Afterwards, the percentage of extra-hydrophobic peptides increased (~13%) significantly ($P < 0.05$). The sharp increase in extra-hydrophobic peptides during storage was due to additional proteolytic activity of NSLAB as their number increased significantly at the end of the storage period (Figure 12).

The trend in peptide profile was due to the proteolytic activities of rennet residues, proteinase and peptidases of NSLAB in cheeses. The initial low salt concentration in this study improved the activity of the proteolytic enzymes during ripening. At a higher salt concentration, these enzymatic effects were noticeably inhibited, as mentioned by Fenelon et al. (2000). Storage time played a major role in the changes of the peptide profile that occurred in Cheddar cheeses. The hydrophilic peptides decreased significantly from Days 0 to 60 of storage, and then remained constant over storage. This was due to the initial proteolytic activities of the rennet residues in the cheeses, as well as the proteinase and peptidases of NSLAB.

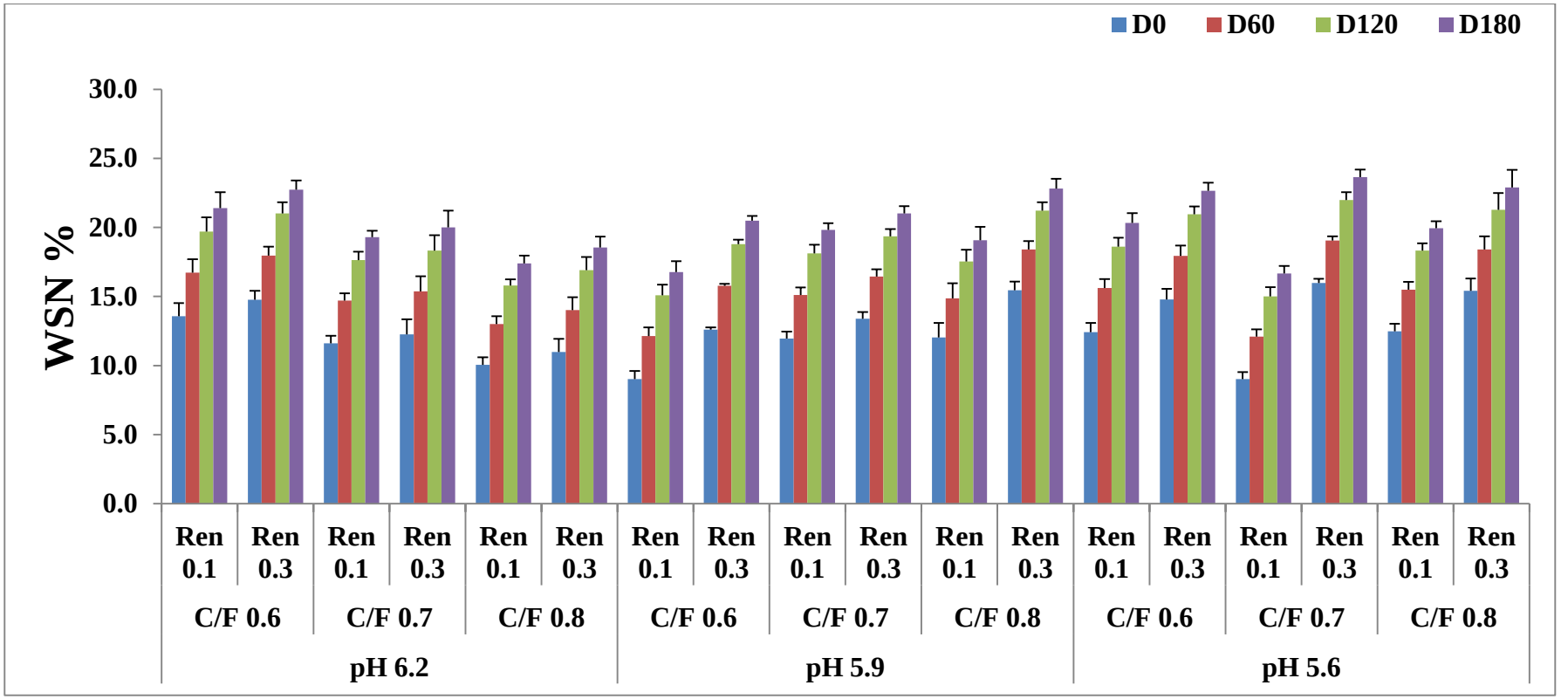


Figure 13: WSN (%) Content of Experimental Cheddar Cheeses Made with Different Treatment during 180 Days of Storage at 9 ± 0.5°C

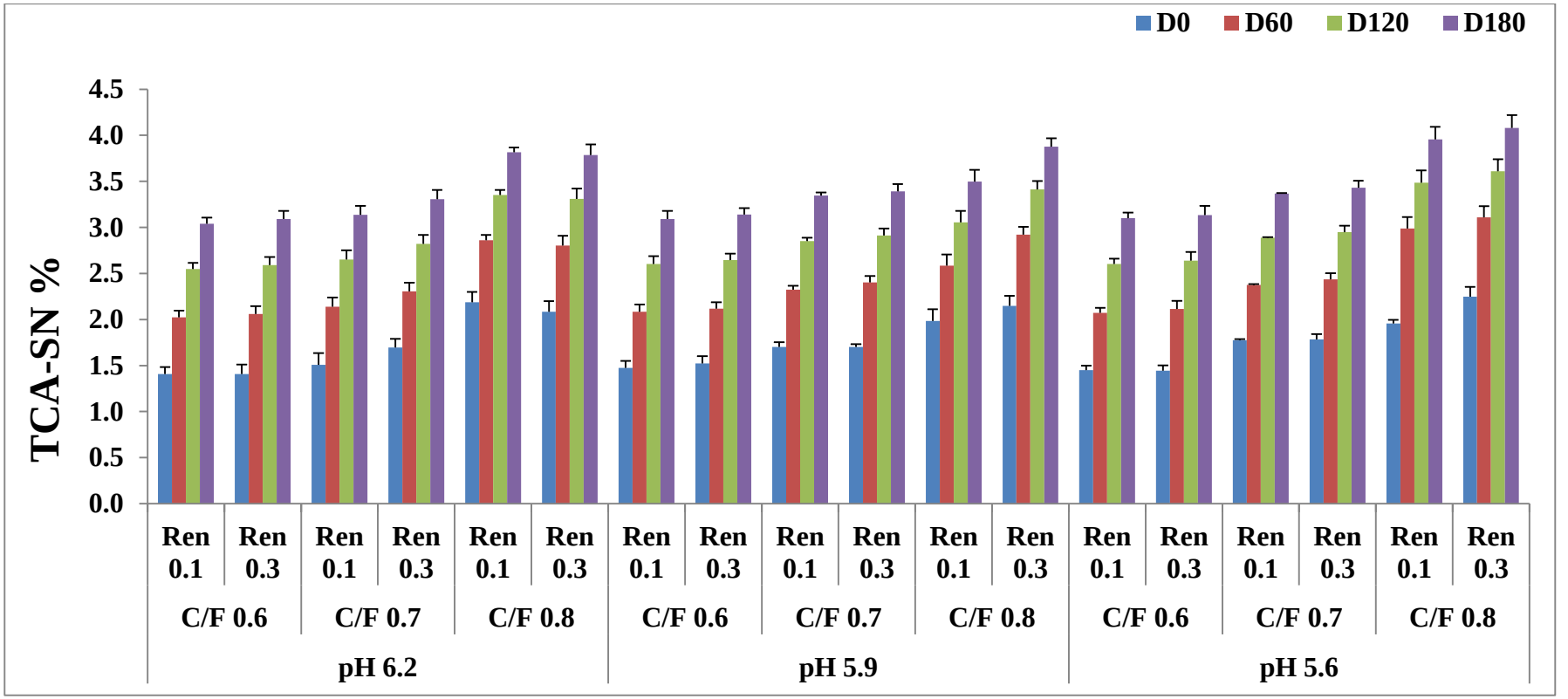


Figure 14: TCA-SN (%) Content of Experimental Cheddar Cheeses Made with Different Treatment during 180 Days of Storage at 9 ± 0.5°C

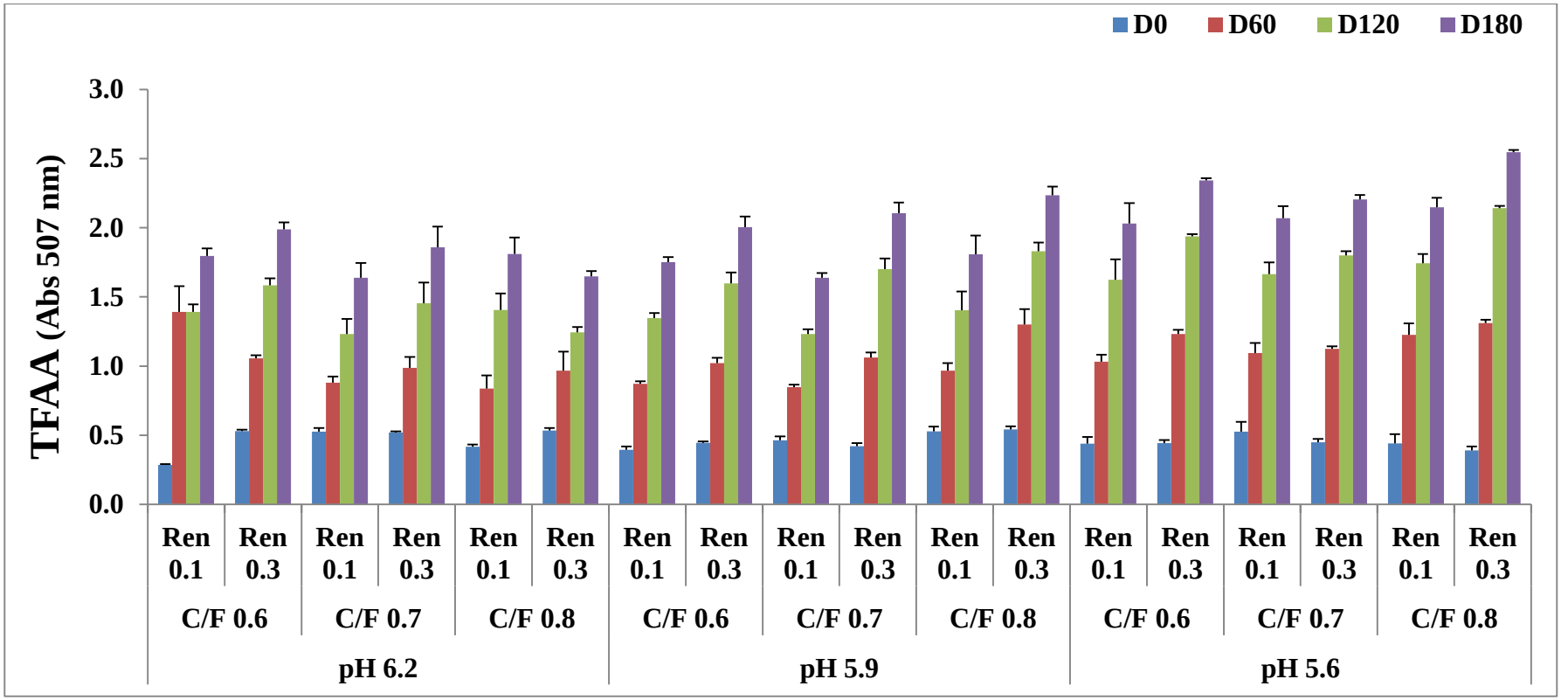
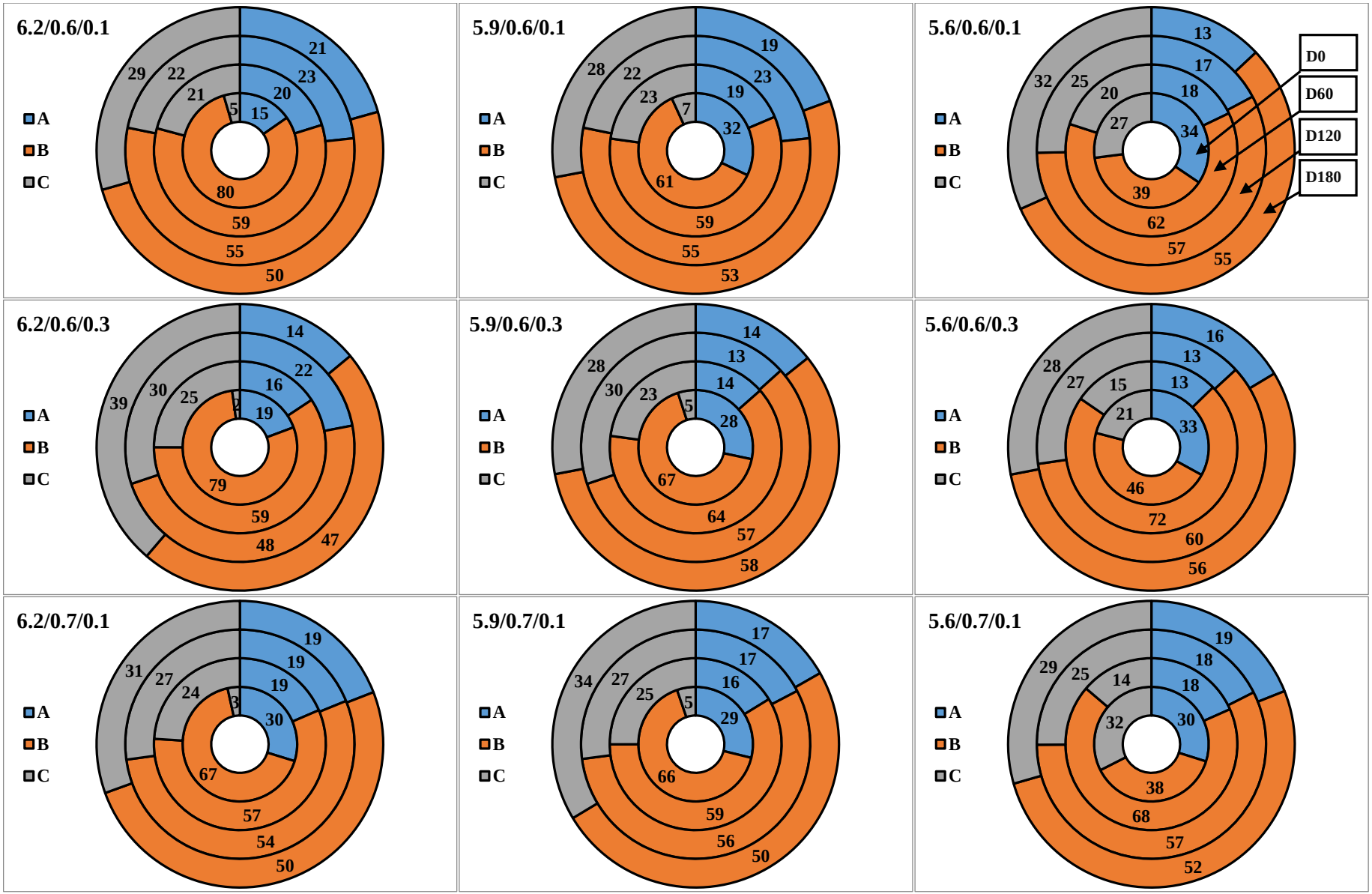


Figure 15: TFAA (Absorbance 507 nm) of Experimental Cheddar Cheeses Made with Different Treatment during 180 Days of Storage at 9 ± 0.5°C


6.2/0.6/0.1
A
B
C
21
23
20
15
5
29
22
21
80
59
55
50
5.9/0.6/0.1
A
B
C
19
23
19
32
7
28
22
23
61
59
55
53
5.6/0.6/0.1
A
B
C
13
17
18
34
27
32
25
20
39
62
57
55
D0
D60
D120
D180
6.2/0.6/0.3
A
B
C
14
22
16
19
2
39
30
25
79
59
48
47
5.9/0.6/0.3
A
B
C
14
13
14
28
5
28
30
23
67
64
57
58
5.6/0.6/0.3
A
B
C
16
13
13
33
21
28
27
15
46
72
60
56
6.2/0.7/0.1
A
B
C
19
19
19
30
3
31
27
24
67
57
54
50
5.9/0.7/0.1
A
B
C
17
17
16
29
5
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27
25
66
59
56
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5.6/0.7/0.1
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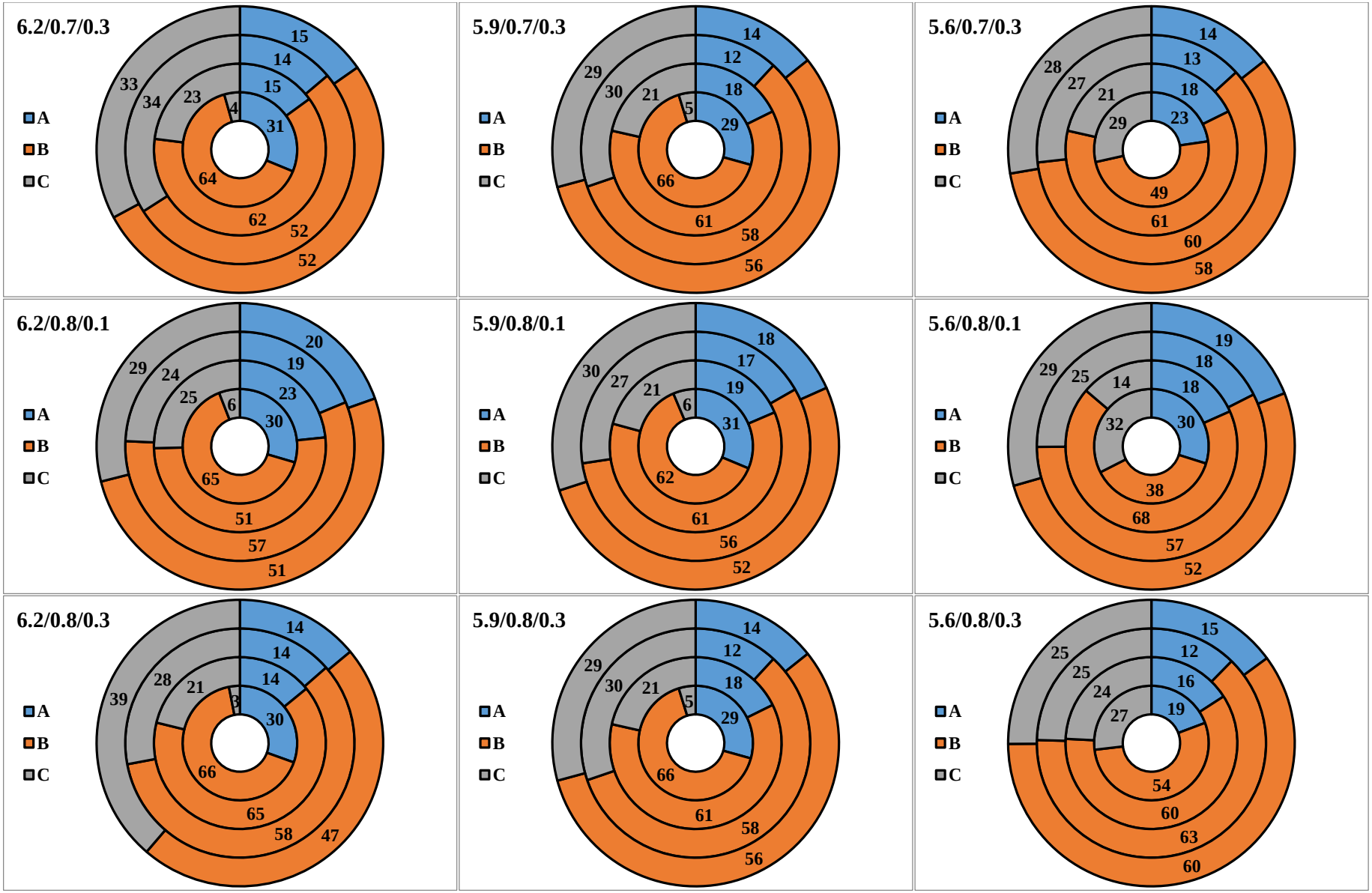


Figure 16: The Change in Percentages of Hydrophilic (A), Hydrophobic (B) and Extra-hydrophobic (C) Peptides of Cheddar Cheeses Made with Different Treatment during Storage at 9 ± 0.5°C

4.4 Conclusion

The moisture and ash content of cheeses made with C/F ratio 0.6 was significantly higher than cheeses made with other ratios at the same drainage pH. An increase in the C/F ratio during the manufacture process caused an increase in the total solid content; therefore, the moisture content decreased. Proteolysis in the salt-reduced Cheddar cheese increased when the C/F ratio and rennet concentration levels were increased for all levels of pH at drainage. In addition, the hydrophilic, hydrophobic and extra-hydrophobic peptide profiles followed a similar trend to standard salt concentration. More hydrophobic peptides were produced as a result of an increase in the overall residual rennet concentration over the storage period, which assisted the activities of the SLAB and NSLAB. In addition to the benefits of low salt intake, a higher quantity of peptides in low-salt Cheddar cheese may have health benefits, a topic that requires further investigation.

A comparison between Cheddar cheeses made in this study and regular Cheddar cheeses proves that increase in C/F ratio and rennet contents along with salt reduction significantly increases the proteolysis. Murtaza et al. (2012) reported that salt reduction in Cheddar cheese (from 2% to 0% NaCl w/w) contributes to higher proteolysis during storage. Proteolysis in this study was similarly affected by salt reduction; however, greater proteolysis was observed due to increased C/F ratio and rennet contents.

Similar finding on effect of varying C/F ratio was reported by Kumar et al. (2011). Feta cheeses made with higher C/F ratio (0.8) showed increase in proteolysis and decrease in pH at the end of storage. These values are greater in our study due to lower salt concentration and different rennet contents.

Some of the key factors such as moisture content, type of starter culture, rennet concentration, and amount of salt are known to be highly effective on proteolysis (Phelan et al. 1973; Fox & McSweeney 1996; Sousa et al. 2001; Upadhyay et al. 2004). Therefore, further investigation on wider range of these factors may contribute to production of salt-reduced Cheddar cheese with higher sensory quality. A successful reduction in salt content of cheese is only possible via understanding the effect of the composition of milk in terms of protein and fat content on the overall quality of Cheddar-type cheeses.

Chapter 5: Texture and Microstructure of Reduced-salt Cheddar Cheese as Affected by Changes in the Casein-to-fatRatio, Rennet Concentration and pH at Drainage[3]

5.1 Introduction

Texture is an important parameter indicating the quality of cheese. This parameter is highly dependent on the proteolysis and breakdown of the protein network, decrease in a_w through water binding by liberated carboxyl and amino groups, and increase in pH (McSweeney & Sousa 2000). The composition of cheese affects the texture profile and microstructure of cheese through the Salt-in-moisture (S/M), fat and solid content (Dimitreli & Thomareis 2007). Variation in the key components of milk, particularly fat, protein and the manufacturing process, can affect cheese quality (Bryant et al. 1995; Ganesan et al. 2014).

Reducing salt in cheese without replacement affects most characteristics of cheese (Sheibani et al. 2013). Kaya (2002) reported that Gaziantep cheeses with a higher concentration of salt (20% and 25% NaCl) showed a higher hardness and lower change in colour (whiteness) compared with the control cheese. Kilic and Isin (2004) examined the texture of Dil cheese brined at two salt concentrations (3 and 6% NaCl) during storage for three months. Dil cheeses brined in a higher salt concentration (6% NaCl) showed a harder texture (~7 N/g cheese) than did cheeses with lower salt (3% NaCl, ~5 N/g cheese) at the end of storage. This may be due to the solubilisation of proteins and filling the serum between the protein fibres. More casein may fill the spaces between the protein fibres, potentially leading to a greater number of protein interactions and strengthening of the protein network.

Replacing salt with replacers such as KCl and $MgCl_2$ may compensate for textural losses in cheese; however, this may also trigger a metallic and bitter aftertaste (Grummer et al. 2012). Lefier et al. (1987) investigated the replacement of salt with

[3] The major part of this chapter has been reviewed by *International Food Research Journal* for publication.

$NaCl/MgCl_2$ mixture in Gruyere cheese. Cheeses made with the $NaCl/MgCl_2$ mixture showed an acceptable taste with a slight bitterness and soft body. Ayyash et al. (2011) investigated the effect of partial substitution of NaCl with KCl on halloumi cheese brined in four different 18% brine solutions (only NaCl, 3NaCl:1KCl, 1NaCl:1KCl and 1NaCl:3KCl) and then stored at 4°C for 56 days. The hardness, cohesiveness, adhesiveness and gumminess of halloumi cheeses kept in the 1NaCl:1KCl brine solution were similar to those stored in the NaCl only.

Thus, reducing salt without salt replacement, alongside manipulating the composition of milk, may affect the concept of saltiness and compensate for the undesirable texture defects in salt-reduced cheeses. The objective of this study was to investigate the effect of changes in drainage pH, rennet concentration and C/F ratio on the texture and microstructure of salt-reduced Cheddar cheese (1.5% salt) during storage at 9 ± 0.5°C for 180 days.

5.2 Materials and Methods

5.2.1 Experimental Design

The experimental design of this part was the same as that discussed in Chapter 4 (Table 12). To ensure that no significant variation was introduced by compositional differences, this study analysed the chemical composition of the milk in each trial, which was found to be insignificant.

5.2.2 Cheese Making

The process of cheese making was undertaken according to the method of Kosikowski (1977), with some modifications, as described in Chapter 4.

5.2.3 Chemical Composition

The compositional analysis was similar to that presented in Chapter 4, and performed according to the AOAC methods (AOAC International 1995).

5.2.4 Texture Profile Analysis

The texture profile was obtained according to the method of Halmos et al. (2003), with some modifications. Cheese cylinders (30 mm height × 20 mm diameters) were cut from the centre of the experimental cheese blocks. The specimens were kept at room temperature using small 50 mL containers prior to determination of the texture profile. Hardness, cohesiveness, adhesiveness, gumminess and springiness were measured using a texture analyser model TA-XT2 (Stable Microsystem, Surrey, UK). The samples were compressed to 30% of their heights using a flat head plunger (TA-40: 4" diameter, aluminium cylinder, 10 mm tall; Stable Microsystem, Surrey, UK) and the crosshead movement was adjusted to 30 mm per minute. Double compression was achieved and the data were collected using Texture Exponent Software (Stable Microsystem Ltd). Analyses were performed in triplicate.

5.2.5 Microstructure of Cheese

ESEM was used to monitor the microstructure of the cheeses, as described by Ayyash et al. (2011). Briefly, 0.5 cm^3 cubes of cheese were cut from the centre of the cheese block and imaged via FEI quanta ESEM (Philips Electron Optics, Eindhoven, The Netherlands) using ESEM mode. Images were taken at an accelerating voltage at 30 kV under vacuum (0.47 kPa) using 1,000 × magnification at 4°C.

5.2.6 Statistical Analysis

Statistical analysis was undertaken similarly to that described in Chapter 4. Data analysis was undertaken using SAS software V9.0 (SAS Institute Inc. 2008).

5.3 Results and Discussion

5.3.1 Texture Profile

5.3.1.1 Hardness of Cheese

Table 14 presents the results of hardness for all the experimental cheeses made with a different pH, casein-to-fat (C/F) ratio and rennet concentration during storage of 180

days. Regardless of pH and rennet, cheeses made with C/F ratio 0.8 had higher ($P < 0.05$) hardness than did cheeses with C/F ratio 0.7 and 0.6. In general, the hardness of all experimental cheeses tended to increase ($P > 0.05$) during storage from Days 0 to 60, and then decreased significantly ($P < 0.05$) until the end of storage (Table 14). Generally, the cheeses made with 0.3 mL/L rennet had lower hardness ($P > 0.05$) than did cheeses with 0.1 mL/L rennet.

Table 14: Hardness of Experimental Salt-reduced Cheddar Cheeses during 180 Days of Storage at 9 ± 0.5°C

pH	C/F ratio[1]	Rennet	Storage (day)			
			0	60	120	180
6.2	0.6	0.1	26.5^{gA2}	54.7^{eB}	49.4^{deC}	34.9^{fD}
		0.3	24.5^{fgA}	25.1^{jA}	24.5^{jA}	23.4^{ghB}
	0.7	0.1	16.1^{iA}	52.8^{deB}	42.6^{eC}	36.1^{efD}
		0.3	12.2^{kA}	27.1^{iB}	26.9^{hiB}	22.9^{ghC}
	0.8	0.1	35.5^{eA}	73.0^{bB}	60.7^{cC}	43.1^{cdefD}
		0.3	19.1^{hA}	49.9^{fB}	32.8^{fgC}	20.8^{iD}
5.9	0.6	0.1	35.6^{eA}	54.6^{eB}	52.8^{dC}	43.0^{deD}
		0.3	24.8^{fgA}	41.0^{gB}	28.2^{iC}	26.9^{gD}
	0.7	0.1	16.6^{iA}	53.2^{deB}	46.4^{deC}	37.4^{eD}
		0.3	12.5^{kA}	27.3^{iB}	26.9^{hiC}	26.6^{gD}
	0.8	0.1	36.5^{bcA}	80.6^{aB}	71.2^{aC}	53.4^{cD}
		0.3	29.2^{fA}	74.7^{bB}	39.6^{gC}	32.8^{fgD}
5.6	0.6	0.1	45.2^{bA}	62.4^{cB}	53.3^{dC}	48.1^{dD}
		0.3	43.7^{cA}	50.1^{efB}	35.8^{fC}	32.0^{fgD}
	0.7	0.1	21.2^{ghA}	59.6^{dB}	43.9^{eC}	43.8^{deD}
		0.3	14.0^{jA}	35.8^{hB}	29.4^{hC}	23.9^{ghD}
	0.8	0.1	53.3^{aA}	81.4^{aB}	70.3^{abC}	64.7^{aD}
		0.3	38.3^{dA}	59.0^{dB}	61.2^{cC}	61.0^{bD}
SEM[3]			2.82	4.16	4.05	4.02

[1] C/F ratio = casein-to-fat ratio
[2] Mean value
[3] SE of the mean
[a-k] Means in each column with different letters are significantly different ($P < 0.05$) at same storage period
[A-D] Means in the same row with different capital letters are significantly different.

Cheese texture is influenced by the chemical composition of cheeses, manufacturing procedure and ripening (Lucey et al. 2003). In this study, cheese hardness was influenced by treatments during the first weeks of ripening. Afterwards, the main effect on the texture profile was a result of the ripening process. The greater hardness of

cheeses made with 0.8 C/F ratio may be attributed to the higher dry matter (DM) and higher protein contents of these cheeses. This agrees with Bryant et al. (1995) and Ong et al. (2013), who reported that reducing the fat content of Cheddar cheese (increase in protein content) enhanced the hardness of cheese. Hennelly et al. (2005) also reported that higher moisture content in cheese leads to a softer cheese body. In this study, cheeses having lower pH showed higher hardness. This may be attributed to demineralisation upon pH drop to 5.9 and 5.6. Hassan and Lucey (2001) reported that Ca^{2+} interaction with casein in Cheddar cheeses decreased from ~64% to ~56% during ripening as a result of pH decrease. The reduction in pH decreases the colloidal calcium phosphate (CCP) in cheese, which decreases the electrostatic repulsion between caseins, resulting in a harder cheese texture (Lucey et al. 2003). In the current study, an increase in rennet concentration negatively affected the hardness of cheese made with 0.3 rennet due to the higher rate of protein breakdown occurring in the cheese as a result of the higher rennet residue remaining in the cheese. This may explain the lower hardness profile of cheeses made with 0.3 mL/L rennet compared with 0.1 mL/L rennet (Table 14).

5.3.1.2 Cohesiveness of Cheese

Table 15 presents the results of cohesiveness of all the experimental cheeses made with a different pH, C/F ratio and rennet concentration during storage of 180 days. Cohesiveness decreased significantly ($P < 0.05$) during 60 days of storage and then showed an increasing trend. In general, from Day 60 onwards, cheeses made with a drainage pH 6.2 showed higher cohesiveness than did those made with drainage pH 5.9 and 5.6. Rennet had a significant effect ($P < 0.05$) on cohesiveness. As can be seen from Table 15, the cohesiveness of cheeses made with different rennet concentrations generally differed significantly ($P < 0.05$), regardless of pH and C/F ratio.

Cohesiveness is defined as the strength of the internal bonds to protect the cheese body from rupture when biting completely through the cheese (Gunasekaran & Ak 2003; O'Callaghan & Guinee 2004). Dropping the pH before drainage increases the demineralisation of curd, which decreases the CCP in casein micelles (Lucey et al. 2003). Within the casein micelles, casein molecules are held together mostly by hydrophobic interactions and CCP crosslinks (Fox & Brodkorb 2008). These CCP links

are dissolved with the decrease in pH; thus, caseins may be liberated throughout the serum phase (Dalgleish & Law 1989; Pyne & McGann 1960). In contrast, a decrease in pH prevents liberated caseins from movement (Dalgleish & Law 1989); thus, no separation of caseins from serum occurs.

Moreover, reducing CCP decreases electrostatic repulsion between caseins, which increases the association between casein molecules in cheese (Lucey & Singh 1997), and thereby increases hardness and decreases cohesiveness. This may explain the decrease in cohesiveness in this study's cheeses with a lower pH value (5.9 and 5.6) during the storage period.

Hardness has an opposite trend to cohesiveness. An increase in hardness creates a more brittle and less cohesive cheese texture (Fox et al. 2000a; Gunasekaran & Ak 2003; Maldonado et al. 2013). In contrast, an increase in protein and decrease in fat content in cheeses as a result of an increase in C/F ratio contribute to a decrease in hardness and thus an increase in cohesiveness (Bryant et al. 1995). In the current study, the increase in cohesiveness after 60 days of ripening was due to excessive hydration of casein due to the initial low salt concentration in the cheeses. It is well established that excessive casein hydration results in a softer cheese texture, and thus greater cohesiveness (Fox et al. 2000a; Fox & McSweeney 1996; Upadhyay et al. 2004).

The increase in cohesiveness in cheeses made with a higher C/F ratio (0.7 and 0.8) and rennet concentration (0.3 mL/L) was lower than that in other cheeses. This was due to the increased pattern of proteolysis resulting from the greater amount of protein and rennet residue in these cheeses (Fox & McSweeney 1996; Upadhyay et al. 2004).

Table 15: Cohesiveness of Experimental Salt-reduced Cheddar Cheeses during 80 Days of Storage at 9 ± 0.5°C

pH	C/F ratio[1]	Rennet	Storage (day)			
			0	60	120	180
6.2	0.6	0.1	0.90^{aA2}	0.63^{aB}	0.68^{aB}	0.77^{aC}
		0.3	0.88^{abA}	0.61^{aB}	0.65^{aB}	0.76^{aC}
	0.7	0.1	0.88^{abA}	0.47^{cB}	0.56^{bC}	0.63^{bD}
		0.3	0.86^{abA}	0.39^{dB}	0.48^{cC}	0.55^{cD}
	0.8	0.1	0.71^{bA}	0.49^{cB}	0.59^{bC}	0.67^{bD}
		0.3	0.67^{cA}	0.34^{dB}	0.44^{cC}	0.58^{cD}
5.9	0.6	0.1	0.67^{cA}	0.42^{cB}	0.62^{aC}	0.75^{aD}
		0.3	0.66^{cA}	0.35^{dB}	0.59^{bC}	0.66^{bD}
	0.7	0.1	0.65^{cA}	0.62^{aB}	0.68^{aAB}	0.73^{aC}
		0.3	0.66^{cA}	0.47^{cB}	0.55^{bC}	0.62^{bD}
	0.8	0.1	0.50^{dA}	0.26^{eB}	0.38^{dC}	0.54^{cD}
		0.3	0.52^{dA}	0.25^{eB}	0.36^{aC}	0.42^{dD}
5.6	0.6	0.1	0.67^{cA}	0.24^{eB}	0.44^{cC}	0.66^{bA}
		0.3	0.66^{cA}	0.23^{eB}	0.44^{cC}	0.56^{cD}
	0.7	0.1	0.73^{bA}	0.34^{dB}	0.44^{cC}	0.53^{cD}
		0.3	0.69^{cA}	0.34^{dB}	0.36^{dB}	0.41^{dC}
	0.8	0.1	0.67^{cA}	0.54^{bB}	0.56^{bBC}	0.59^{cC}
		0.3	0.76^{bA}	0.34^{dB}	0.36^{dBC}	0.38^{eC}
SEM [3]			0.019	0.022	0.027	0.017

[1] C/F ratio = casein-to-fat ratio
[2] Mean value
[3] SE of the mean
[a-d] Means in each column with different letters are significantly different ($P < 0.05$) at same storage period
[A-D] Means in the same row with different capital letters are significantly different.

5.3.1.3 Gumminess of Cheese

Table 16 presents the gumminess of all experimental cheeses made with a different pH, C/F ratio and rennet concentration during storage of 180 days. In general, the cheeses made with 0.8 C/F ratio had a higher gumminess ($P < 0.05$) than did the cheeses with 0.7 and 0.6 C/F ratios, regardless of pH and rennet. The gumminess of all treatments increased significantly ($P < 0.05$) up to Day 60, and then showed a decreasing trend to the end of storage (Table 16).

Table 16: Gumminess of Experimental Salt-reduced Cheddar Cheeses during 180 Days of Storage at 9 ± 0.5°C

pH	C/F ratio[1]	Rennet	Storage (day)			
			0	60	120	180
6.2	0.6	0.1	23.7^{cA2}	25.7^{fB}	15.0^{hiC}	11.9^{fD}
		0.3	23.7^{cA}	37.9^{cB}	16.6^{hC}	13.5^{eD}
	0.7	0.1	19.0^{dA}	36.4^{cdB}	28.3^{eC}	20.5^{cAD}
		0.3	12.9^{fgA}	23.9^{fgB}	11.9^{iC}	10.4^{gD}
	0.8	0.1	34.2^{aA}	55.6^{aB}	43.3^{aC}	28.1^{aD}
		0.3	25.4^{bcA}	33.2^{deB}	31.3^{dC}	25.5^{bA}
5.9	0.6	0.1	23.9^{cA}	34.3^{dB}	22.2^{fAC}	13.8^{eD}
		0.3	16.4^{deA}	24.3^{fgB}	12.0^{iC}	8.1^{hD}
	0.7	0.1	14.6^{defA}	22.1^{gB}	18.8^{gC}	12.5^{fD}
		0.3	10.7^{gA}	15.8^{hB}	12.1^{iC}	6.6^{iD}
	0.8	0.1	26.1^{bA}	47.5^{bB}	35.2^{bC}	25.4^{bD}
		0.3	19.6^{dA}	31.2^{eB}	12.2^{iC}	10.3^{gD}
5.6	0.6	0.1	17.9^{eA}	24.0^{fgB}	12.1^{iC}	10.8^{gD}
		0.3	16.1^{de}	16.6^{hi}	8.0^{j}	6.2^{i}
	0.7	0.1	13.4^{fA}	22.8^{gB}	14.7^{hiC}	8.2^{hD}
		0.3	8.4^{hA}	9.9^{jB}	8.0^{jA}	6.8^{iC}
	0.8	0.1	23.9^{cA}	33.8^{deB}	33.0^{cB}	16.7^{dC}
		0.3	14.6^{defA}	15.2^{hB}	12.0^{iC}	8.5^{hD}
SEM[3]			1.9	2.65	2.47	2.17

[1] C/F ratio = casein-to-fat ratio
[2] Mean value
[3] SE of the mean
[a-j] Means in each column with different letters are significantly different ($P < 0.05$) at same storage period
[A-D] Means in the same row with different capital letters are significantly different.

Gumminess (chewiness), the required energy to disintegrate cheese to a state ready for swallowing, is defined as the product of hardness × cohesiveness, and is subsequently influenced by changes in these two parameters (Fox et al. 2000a; Gunasekaran & Ak 2003). Therefore, in the current study, gumminess correlated positively with hardness. This suggests that gumminess was influenced by pH, C/F ratio and rennet in a similar manner to hardness. These findings align with Irudayaraj et al. (1999), who reported that gumminess follows the same trend as hardness. The loss of CCP and consequent reduction in Ca^{2+} content in cheese as a result of pH drop (more moisture expulsion) directly affect the hardness and gumminess of cheese (Lucey et al. 2003). This occurs even more in salt-reduced cheeses due to less Na^{+} concentration, which is in exchange with Ca^{2+} throughout the cheese matrix. Upon reducing the Ca^{2+} ion, the casein–casein network weakens, leading to greater hydration of proteins during storage, and

subsequently less hardness and gumminess (Gunasekaran & Ak 2003; O'Callaghan & Guinee 2004; Vélez-Ruiz 2009).

5.3.1.4 Adhesiveness of Cheese

Adhesiveness is defined as the stickiness of a sample in the mouth throughout mastication, or the amount of force required to remove the cheese from the palate during eating (Gunasekaran & Ak 2003). Table 17 presents the results of the adhesiveness of all experimental cheeses made with a different pH, C/F ratio and rennet concentration during storage of 180 days. In general, adhesiveness decreased significantly ($P < 0.05$) over the storage period in all experimental cheeses. Generally, adhesiveness showed a decrease in response to pH dropping at drainage from 6.2 to 5.6.

An increase in protein altered the protein matrix, making the cheese matrix more compact and therefore less adhesive. A similar trend was observed by Bryant et al. (1995), who stated that lower values of adhesiveness were observed in low-fat (high-protein) cheeses ripened for up to four months. This can explain the decreased adhesiveness of the current study's cheese made with a higher C/F ratio (0.8). During ripening, in addition to proteolysis, moisture also moves out of the protein matrix, resulting in a more homogeneous matrix that decreases adhesiveness during storage (Irudayaraj et al. 1999).

Table 17: Adhesiveness of Experimental Salt-reduced Cheddar Cheeses during 180 Days of Storage at 9 ± 0.5°C

pH	C/F ratio[1]	Rennet	Storage (day)			
			0	60	120	180
6.2	0.6	0.1	0.14^{aA2}	0.11^{aB}	0.10^{aC}	0.22^{bD}
		0.3	0.13^{bA}	0.08^{dB}	0.07^{dC}	0.18^{dD}
	0.7	0.1	0.13^{bA}	0.10^{bB}	0.05^{fC}	0.13^{gA}
		0.3	0.11^{cA}	0.09^{cB}	0.08^{cC}	0.13^{gD}
	0.8	0.1	0.10^{dA}	0.07^{eB}	0.06^{eC}	0.09^{hD}
		0.3	0.10^{dA}	0.06^{fB}	0.06^{eB}	0.06^{iB}
5.9	0.6	0.1	0.10^{dA}	0.09^{cB}	0.08^{cC}	0.09^{hB}
		0.3	0.10^{dA}	0.10^{bA}	0.09^{bB}	0.17^{eC}
	0.7	0.1	0.09^{eA}	0.09^{cA}	0.07^{dB}	0.15^{fC}
		0.3	0.08^{fA}	0.07^{eB}	0.06^{eC}	0.20^{cD}
	0.8	0.1	0.08^{fA}	0.06^{fB}	0.05^{fC}	0.13^{gD}
		0.3	0.08^{fA}	0.06^{fB}	0.05^{fC}	0.24^{aD}
5.6	0.6	0.1	0.11^{cA}	0.09^{cB}	0.08^{cC}	0.09^{hB}
		0.3	0.10^{dA}	0.09^{cB}	0.08^{cC}	0.17^{eD}
	0.7	0.1	0.08^{fA}	0.08^{dA}	0.08^{cA}	0.18^{dB}
		0.3	0.08^{fA}	0.07^{eB}	0.07^{dB}	0.24^{aC}
	0.8	0.1	0.08^{fA}	0.07^{eB}	0.06^{eC}	0.09^{hD}
		0.3	0.06^{gA}	0.06^{fA}	0.05^{fB}	0.09^{hC}
SEM[3]			0.006	0.014	0.016	0.017

[1] C/F ratio = casein-to-fat ratio
[2] Mean value
[3] SE of the mean
[a-i] Means in each column with different letters are significantly different ($P < 0.05$) at same storage period
[A-D] Means in the same row with different capital letters are significantly different.

The initial low salt concentration in experimental cheeses enhanced the reduction of pH, leading to greater moisture discharge from the cheese matrix and thus less adhesiveness (Fox et al. 2000a; Fox et al. 2004b). The increased adhesiveness in cheeses at the end of storage (Day 180) may have resulted from activity of the bacterial proteolytic enzymes (cell wall proteinase and intracellular peptidases), which break down existing small amino acids in the cheese matrix and thus form a soft and sticky cheese (Fox et al. 2000a; Parente & Cogan 2004).

5.3.1.5 Springiness of Cheese

Springiness or elasticity is defined as the rate or percentage at which a deformed cheese returns to its original form after the deforming force is removed (O'Callaghan & Guinee

2004). Table 18 presents the springiness of all experimental cheeses made with a different pH, C/F ratio and rennet concentration during storage of 180 days. The springiness of all experimental cheeses decreased significantly ($P < 0.05$) during storage. From Day 60 onwards, cheeses made with 0.3 mL/L rennet concentration had lower springiness than did those made with 0.1 mL/L rennet. The breakdown of α_{sl}-casein to α_{s1}-I casein is the key reaction responsible for the initial softening of cheese texture, indicating that textural changes during ripening are related to proteolysis, particularly by residual rennet. The hardness and springiness of Cheddar cheese are correlated with proteolysis (Fox et al. 2004b). The springiness of cheeses containing a higher protein content (C/F ratio 0.7 and 0.8) showed greater values than did those with C/F ratio 0.6. A decrease in pH and subsequent decrease in moisture content affected springiness negatively.

Table 18: Springiness of Experimental Salt-reduced Cheddar Cheeses during 180 Days of Storage at 9 ± 0.5°C

pH	C/F ratio[1]	Rennet	Storage (day)			
			0	60	120	180
6.2	0.6	0.1	2.07^{fA2}	2.00^{aB}	1.54^{dC}	1.46^{eD}
		0.3	2.10^{eA}	1.54^{eB}	1.74^{bC}	1.42^{eD}
	0.7	0.1	2.28^{aA}	1.95^{abcB}	1.83^{aC}	1.78^{bD}
		0.3	2.26^{abA}	1.75^{cB}	1.62^{cC}	1.52^{dD}
	0.8	0.1	2.09^{efA}	2.02^{aB}	1.84^{aC}	1.75^{bD}
		0.3	2.19^{cA}	1.80^{bcdB}	1.65^{cC}	1.67^{cC}
5.9	0.6	0.1	2.07^{fA}	1.81^{bcdB}	1.67^{cC}	1.66^{cC}
		0.3	2.09^{efA}	1.76^{dB}	1.56^{dC}	1.37^{fD}
	0.7	0.1	2.26^{abA}	1.75^{dB}	1.65^{cC}	1.64^{cC}
		0.3	2.23^{bA}	1.82^{bcdB}	1.63^{cC}	1.44^{eD}
	0.8	0.1	2.08^{fA}	1.96^{abB}	1.83^{aC}	1.80^{aC}
		0.3	2.17^{dA}	1.77^{cdB}	1.54^{dC}	1.50^{dC}
5.6	0.6	0.1	2.01^{gA}	1.81^{bcdB}	1.57^{dC}	1.67^{cD}
		0.3	2.04^{cdeA}	1.80^{bcdB}	1.42^{eC}	1.32^{fD}
	0.7	0.1	2.22^{bA}	1.75^{dB}	1.73^{bB}	1.56^{dC}
		0.3	2.00^{gA}	1.45^{fB}	1.42^{eB}	1.43^{eB}
	0.8	0.1	2.08^{fA}	1.81^{bcdB}	1.77^{bC}	1.62^{cD}
		0.3	2.11^{eA}	1.54^{eB}	1.47^{eC}	1.72^{bD}
SEM[3]			0.02	0.03	0.02	0.03

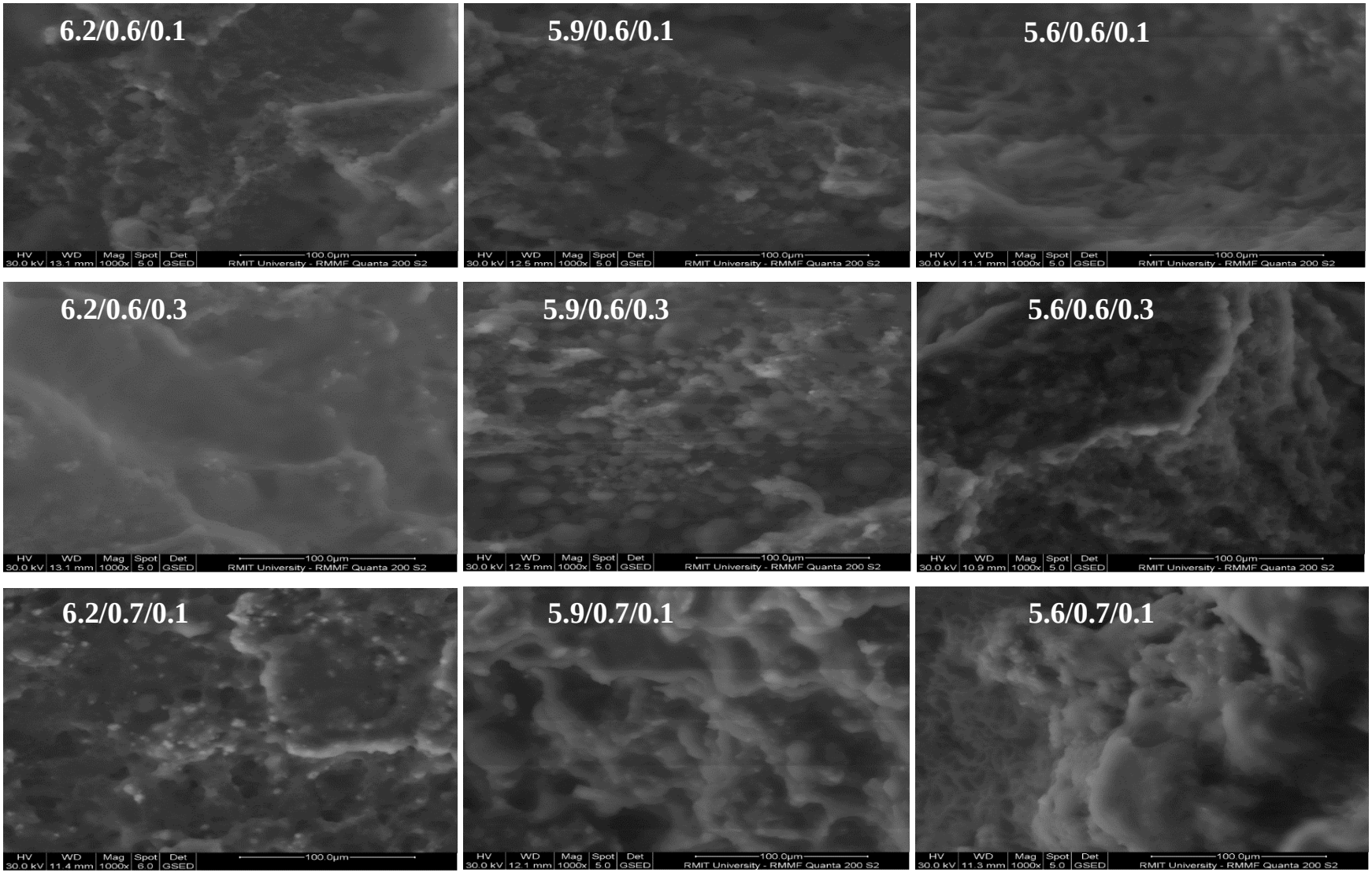
[1] C/F ratio = casein-to-fat ratio
[2] Mean value
[3] SE of the mean
[a-f] Means in each column with different letters are significantly different ($P < 0.05$) at same storage period
[A-D] Means in the same row with different capital letters are significantly different.

In general, the drop in pH (to 5.9 and 5.6) before drainage paralleled with higher whey expulsion (decrease in moisture content), which resulted in less springiness. The rate of proteolysis occurring during ripening had a major influence on all cheeses after Day 0 and onwards. The breaking down of the protein matrix due to the hydrolytic activities of rennet residues and bacterial enzymes (O'Callaghan & Guinee 2004) weakened the ability of the deformed cheeses to recover (low springiness).

5.3.2 Microstructure of Cheese

Figures 17 and 18 present the microstructure images of 18 experimental Cheddar cheeses at Days 0 and 180 of storage, respectively. Storage time affected the microstructure of all cheeses. A comparison between images at Days 0 and 180 per cheese showed that the microstructure became denser and more compact, with small gaps. As can be seen from Figure 17, the microstructures of all cheeses made with pH 5.6 were denser and more compact than those made with pH 5.9 and 6.2. This resulted from excessive expulsion of water out of the curd during prolonged cooking. Cheese made with a higher protein content (C/F ratio 0.7 and 0.8) showed more porous structure than cheese with a higher fat content (C/F ratio 0.6) at Day 0 of storage. This was due to increased aggregation of casein micelles upon addition of rennet in high-protein cheeses (Gunasekaran & Ak 2003). At Day 180, the images showed that the porosity and cavities vanished and microstructure became more homogenous than on Day 0.

Higher protein content in cheese enhances the activity of starter cultures, leading to the production of small size peptides and amino acids, which fill the pores and cavities in the cheese matrix (Fox & McSweeney 1996; Upadhyay et al. 2004). This was the reason for the denser and more compact structure of the cheeses made with a higher C/F ratio.


6.2/0.6/0.1
HV 30.0 kV WD 13.1 mm Mag 1000x Spot 5.0 Det GSED 100.0µm RMIT University - RMMF Quanta 200 S2
5.9/0.6/0.1
HV 30.0 kV WD 12.5 mm Mag 1000x Spot 5.0 Det GSED 100.0µm RMIT University - RMMF Quanta 200 S2
5.6/0.6/0.1
HV 30.0 kV WD 11.1 mm Mag 1000x Spot 5.0 Det GSED 100.0µm RMIT University - RMMF Quanta 200 S2
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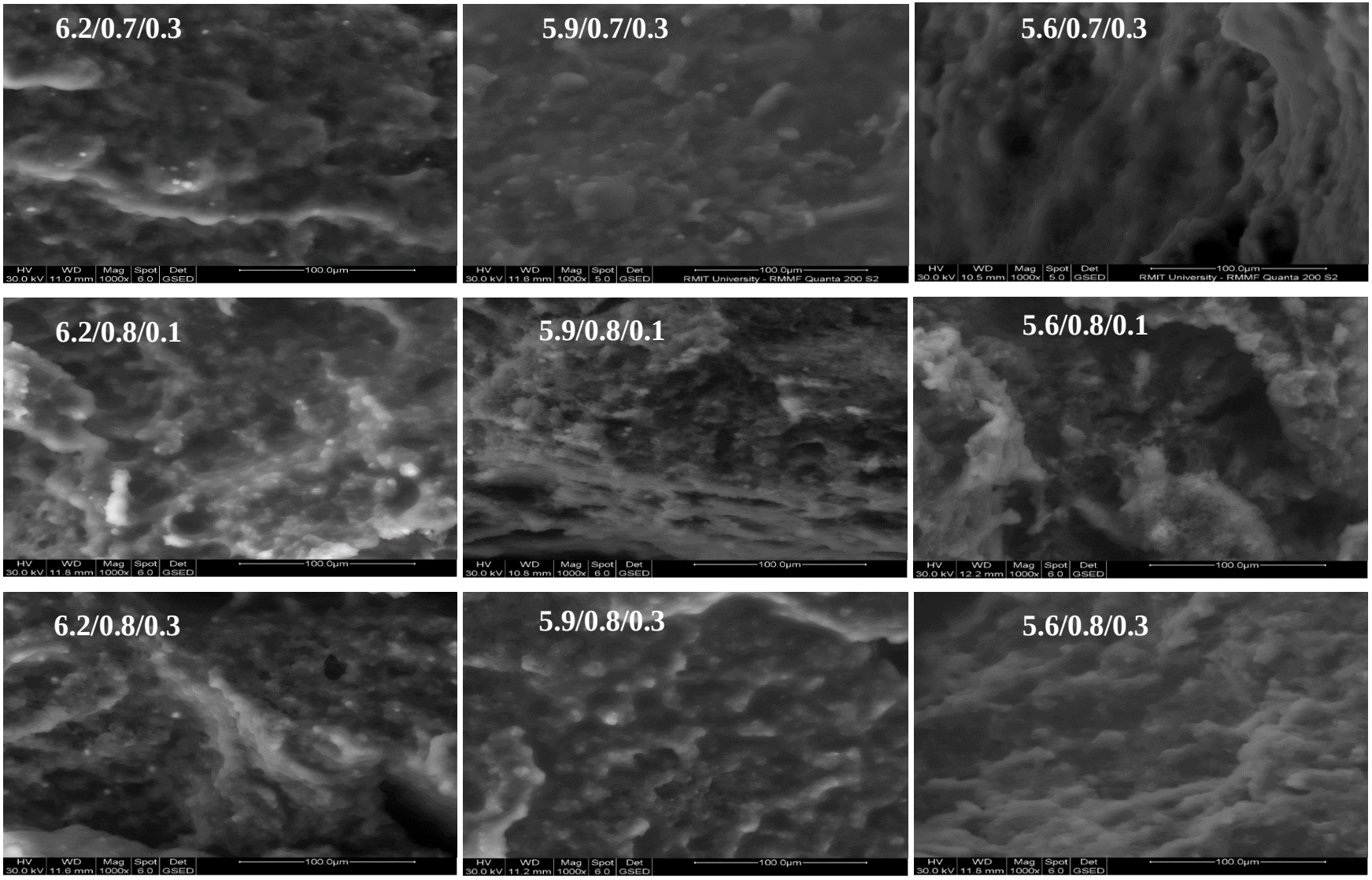
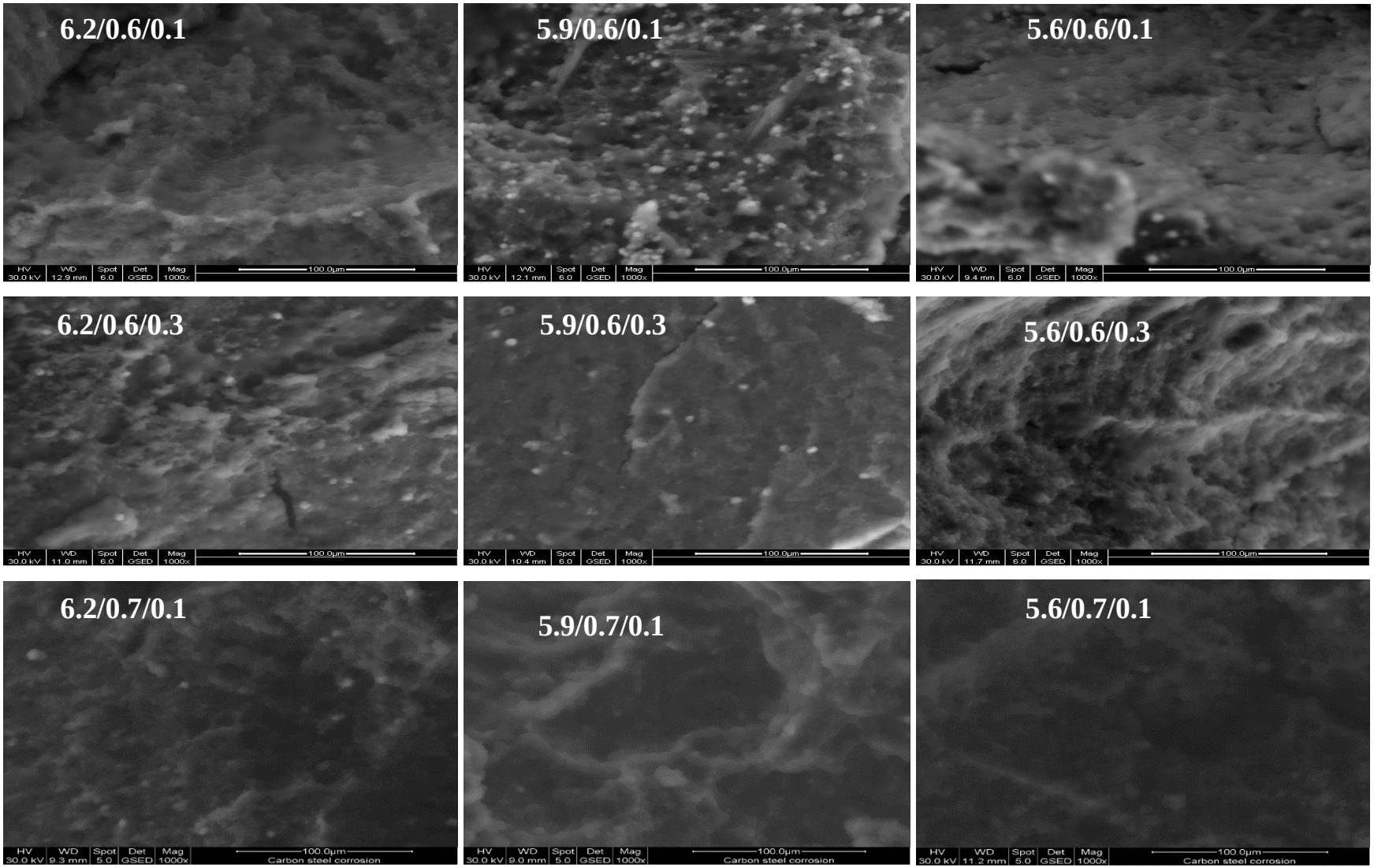


Figure 17: Microstructure of Experimental Cheddar Cheeses Made with Different Treatments at Day 0 of Storage


6.2/0.6/0.1
HV 30.0 kV | WD 12.9 mm | Spot 6.0 | Det GSED | Mag 1000x | 100.0µm
5.9/0.6/0.1
HV 30.0 kV | WD 12.1 mm | Spot 6.0 | Det GSED | Mag 1000x | 100.0µm
5.6/0.6/0.1
HV 30.0 kV | WD 9.4 mm | Spot 6.0 | Det GSED | Mag 1000x | 100.0µm
6.2/0.6/0.3
HV 30.0 kV | WD 11.0 mm | Spot 6.0 | Det GSED | Mag 1000x | 100.0µm
5.9/0.6/0.3
HV 30.0 kV | WD 10.4 mm | Spot 6.0 | Det GSED | Mag 1000x | 100.0µm
5.6/0.6/0.3
HV 30.0 kV | WD 11.7 mm | Spot 6.0 | Det GSED | Mag 1000x | 100.0µm
6.2/0.7/0.1
HV 30.0 kV | WD 9.3 mm | Spot 5.0 | Det GSED | Mag 1000x | 100.0µm | Carbon steel corrosion
5.9/0.7/0.1
HV 30.0 kV | WD 9.0 mm | Spot 5.0 | Det GSED | Mag 1000x | 100.0µm | Carbon steel corrosion
5.6/0.7/0.1
HV 30.0 kV | WD 11.2 mm | Spot 5.0 | Det GSED | Mag 1000x | 100.0µm | Carbon steel corrosion

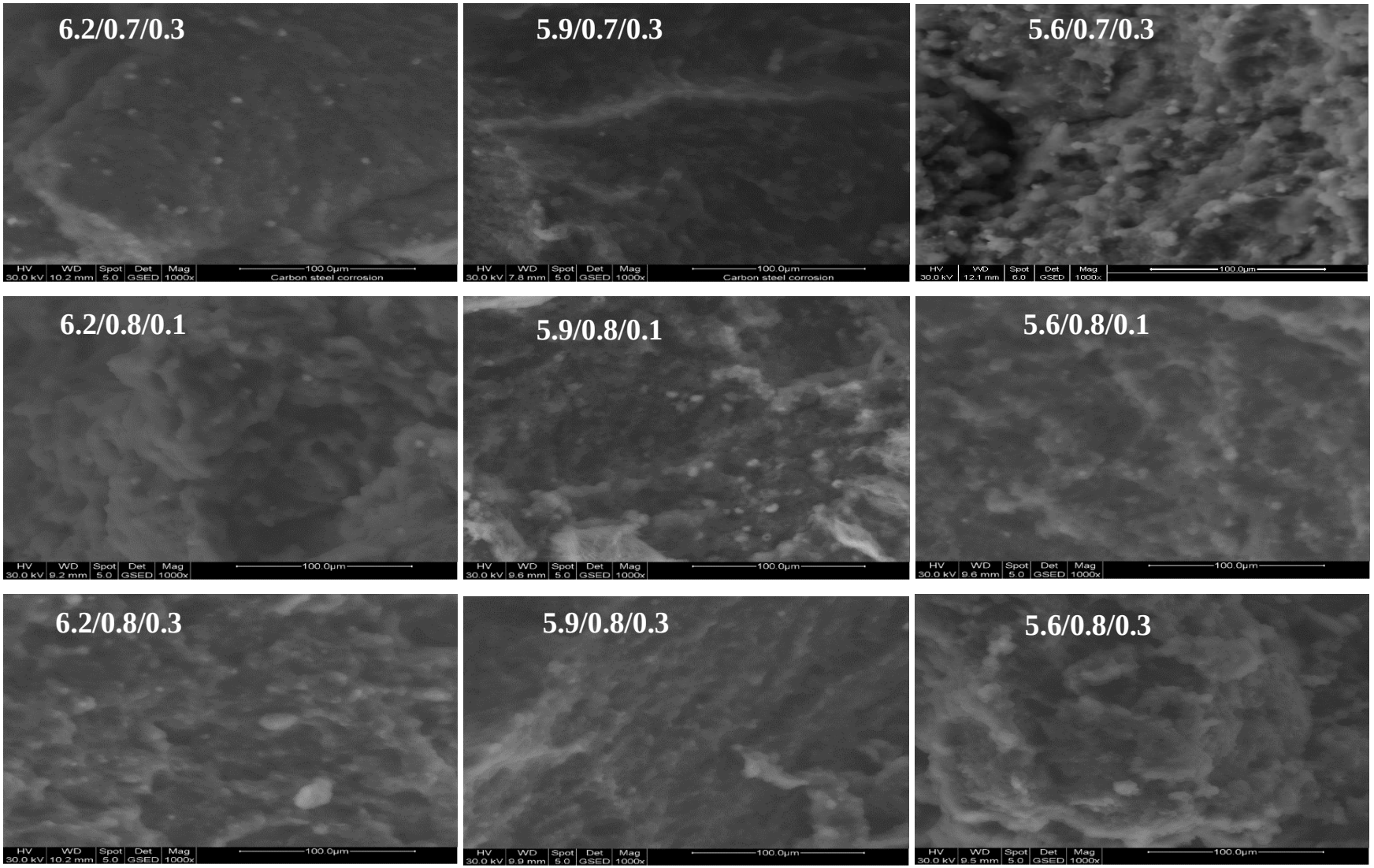


Figure 18: Microstructure of Experimental Cheddar Cheeses Made with Different Treatments at Day 180 of Storage at 9 ± 0.5ºC

5.4 Conclusion

The increase in C/F ratio and rennet concentration influenced the texture of the cheeses due to increased proteolysis, as indicated by an increase in the proteolytic parameters (Chapter 4). Hardness, adhesiveness and gumminess increased in all cheeses during storage, while springiness and cohesiveness decreased. Bryant et al. (1995) and Ong et al. (2013) reported similar findings (increase) on hardness of Cheddar cheese as a result of increase in protein content. Reducing the pH caused an increase in the rate of proteolysis, which directly affected the texture. Decrease in pH affects the CCP content in cheese resulting in harder texture (Lucey et al. 2003). The ash content of cheese may also reduce at a lower pH, which influences the proteolytic activity and texture. Changing the pH before drainage influenced the microstructure of the cheese. Reducing the pH particularly increased the demineralisation of CCP, which enhanced the association among the casein molecules. Therefore, the cheeses made at a lower pH had a more dense and compact structure and narrower voids than did those made at a higher pH (Lucey et al. 2003). Given that rennet concentration plays an influential role in proteolysis and consequently texture. manufacture of salt-reduced Cheddar cheese by changing the cheese milk composition is a possible technique to overcome the textural deficit due to salt reduction. However, more investigation needs to be done on wider ranges of pH and rennet concentrations in the future to confirm or change the pattern noted in this investigation.

Chapter 6: Diffusion and Release of Sodium from Reduced-salt Cheddar Cheese and Sensory Attributes as Affected by Change in the Casein-to-fat Ratio, Rennet Concentration and pH at Drainage

6.1 Introduction

Several studies have reported the influence of salt (NaCl) on the human body (Buemi et al. 2002; Heaney 2006; Kotchen 2005; Massey 2005; Turk et al. 2009). In Western countries, cheese is an important part of diets, and cheese containing salt contributes to daily intake of sodium (Anderson et al. 2010; Meneton et al. 2009). However, excessive salt intake can contribute to osteoporosis, kidney stones and hypertension (Buemi et al. 2002; Heaney 2006; Kotchen 2005; Massey 2005). Therefore, health authorities have recommended that food processors decrease salt content to prevent the incidence of these diseases and disorders (WHO 2007).

Salt has a multidimensional role in cheese, as it directly affects the texture, flavour and colour characteristics of cheese. Simple reduction of salt without replacement affects cheese composition, microstructure and extent of proteolysis, and consequently the quality and consumer acceptance of cheese (Guinee 2004a; Murtaza et al. 2014). Cheese manufacturers generally prefer to use the simple reduction technique; however, several issues need to be considered. Increased bitterness and uncontrolled microbial growth in cheese during storage are the main issues with low-salt cheeses.

One of the most noticeable changes resulting from salt reduction is undesirable taste and decreased perception of saltiness (Taylor & Roberts 2004). Although salt replacements may contribute to a salty taste, they may also provide undesirable aftertastes, such as bitter, metallic and sharp taste sensations (Lawless et al. 2003). For example, Grummer et al. (2012) reported that replacing salt with substitutes such as KCl and $MgCl_2$ may be a solution for the textural defects in cheese; however, they trigger a metallic and bitter taste. One solution proposed is to increase the perception of saltiness for a given amount

of salt, thereby enhancing the chances of acceptance of low-salt cheese by consumers, while reducing the consumption of salt. Hence, there is sufficient motivation for researchers to develop a low-salt cheese matrix that favours higher release of salt to the palate, thereby triggering a high perception of saltiness.

Changing the milk composition and manufacturing process affects the overall quality of cheese, including its texture and structure, which influences the release of salt from cheese into the mouth cavity during mastication. An increased amount of salt released from the cheese into the mouth during chewing may enhance the salt taste at low salt concentrations. There have been some attempts to manipulate cheese texture and composition to enhance salt release from cheese (Floury et al. 2009a; Guinee & Fox 1986; Guinee & O'Kennedy 2007; McMahon 2010; Murtaza et al. 2014; Simal et al. 2001). The cheese matrix is a structurally complex arrangement of water and dry matter (DM) through which salt has to migrate before release. Hence, the factors that influence the release of salt are the initial amount of salt in the cheese, moisture, and DM and its constituents (particularly protein, fat and minerals, including calcium). The manufacturing steps of pH at the time of drainage and ripening in Cheddar cheese also affect the release of salt due to their influence on structure and texture (see Chapters 3 and 5).

The transport of salt in cheese is characterised by diffusive mass transfer (Floury et al. 2009b; Floury et al. 2010; Guinee & Fox 1986; Morris et al. 1985; Turhan 1996; Turhan & Kaletunç 1992). The literature on this topic has been presented in reviews (Floury et al. 2010; Morris et al. 1985) and the methods of determination of diffusivity in foods, including cheese, were discussed by Gros and Ruegg (1987) based on review of experimental data sourced from several studies.

Diffusion of salt in cheese is slow and at least in real cheese systems the salt equilibrium in the matrix may not be possible to attain at all, as suggested by Guinee (2004a) unlike in tightly controlled small experimental situations. The effective diffusion coefficients (D_{eff}) in cheese cover a wide range of values (Floury et al. 2010) due to differences in the structure and composition of different cheese types. The salt D_{eff} may range from 1 to 5.5×10^{-10} m^2 s^{-1} in cheeses, and is about an order of magnitude lower than that in water (1.16×10^{-10} m^2 s^{-1}). This underlines the role of the cheese matrix constituents,

other than water, in affecting the diffusion of salt in cheese, in addition to the structure. However, during the salting of cheese, there is movement of water in the opposite direction to that of salt (Geurts et al. 1974; Geurts et al. 1980); thus, salt diffusivity will be affected by the change in viscosity of the moisture present in cheese and the degree of water binding by the matrix components. While it is known that salt, fat, protein and moisture contents contribute to the mass transfer in the matrix, it is difficult to quantify their individual effects due to the strong interactions between several factors. Based on a regression analysis of diffusivity data in several types of cheeses, Floury et al. (2010) suggested employing a best-fit equation (R^2 = 0.75) using DM, fat in dry matter (FDM)and temperature as dependent variables. This equation is written as:

Equation 4:

$$D_{\text{eff}} = 3.39 - 1.25 \times \text{DM} + 0.24 \times \text{fat/DM} - 0.14 \times \text{T}$$

Since DM was the only significant parameter, Floury et al. (2010) concluded that DM alone can be used to predict D_{eff} in cheese. While this appears to be too simplistic because it ignores the structure of the matrix, it does provide a practical basis for the relationship between the easily measured compositional variables of DM, FDM and temperature.

The matrix composition and pH influence the texture and microstructure of cheese by affecting the droplet size and distribution of the fat within (Floury et al. 2009a). The measurement of salt transport in the matrix is difficult due to structural inconsistencies hindering transport even in the model systems. A coarser structure in the matrix due to low protein content normally leads to higher salt movement, as a result of higher tortuosity and a more dominant role of fat content, at least in high-fat Cheddar cheese. A softer texture may also result from low protein and the use of higher pH during renneting (Floury et al. 2009a; Floury et al. 2009b; Lawrence et al. 2012). This would increase the release of salt and improve the perception of saltiness (Lawrence et al. 2012).

Ripening is the key stage in the development of the flavour and texture of Cheddar cheese, and depends on hydrolysis of protein and lipids. During the ripening period, there must be uniform distribution of salt in the cheese matrix for desired enzymatic and

microbiological activity to assure good quality Cheddar cheese. Simal et al. (2001) investigated the diffusion of water and salt during the ripening of Mahon cheese. They brined cheeses in a mixture of 28% NaCl and 1.5% $CaCl_2$ at 12°C, and then ripened them for 70 days. They concluded that the mass transfer phenomenon during ripening was affected by the complex protein matrix of the cheese offering internal and external resistance in the surrounding environment. The diffusivity values for salt (5.3×10^{-10} m^2 s^{-1}) were higher than that of water (7.8×10^{-10} m^2 s^{-1}), suggesting a higher movement of salt in the matrix during ripening, and relatively slower dehydration of cheese at different investigated levels of relative humidity.

There is little information available regarding the effect of changes in the composition and texture of Cheddar cheese, and their effect on salt release. Most of the previous studies on the release of salt from cheese were performed on model cheese matrices (Floury et al. 2009b; Lawrence et al. 2012; Phan et al. 2008; Simal et al. 2001; Turhan 1996). Floury et al. (2009b) studied diffusion in model cheeses, as a lipoprotein-containing matrix, simulating a hard cheese with different compositions (DM, FDM and salt) and rennet concentration, fat content and pH. The D_{eff} values determined from the experimental data ranged from 2.81 to 3.43×10^{-10} m^2 s^{-1} at 15°C. Similarly, Lawrence et al. (2012) found that the effect of cheese composition on salt release and the perception of saltiness in the mouth appeared to be different in a lipoprotein matrix. In both of these studies, the effect of ripening was not investigated; hence, there is a gap in the knowledge.

The objective of the current study was to investigate the effect of changes in drainage pH, rennet concentration and casein-to-fat (C/F) ratio on the diffusion and release of salt into artificial saliva from salt-reduced Cheddar cheeses (1.5% w/w salt), with a view to identify the treatment combination corresponding to the maximum release of salt. The transport of salt in the experimental cheese matrices was considered as a diffusive mass transfer. The experiments included the effect of storage (ripening) on salt transport.. Finally, sensory assessment of the samples of cheese with the highest rate of diffusion (suggesting higher release of sodium) was conducted and compared with the control cheese containing 2.5% salt.

6.2 Materials and Methods

6.2.1 Experimental Design

The experimental design is similar to the one described in chapter 4 (Table 12). For calculation of diffusivity salt profiling in the cheese cylinders was labelled alphabetically as given in the appendix.

6.2.2 Cheese Making

The process of cheese making was undertaken according to the method of Kosikowski (1977), with some modifications, as described in Chapter 4.

6.2.3 Salt Release and Diffusivity Measurement

An experimental unit (Figure 19) was designed to measure the amount of sodium ion released from the cheese samples into the saliva, as described by Floury et al. (2009b), with minor changes. Artificial saliva was prepared according to Van Ruth et al. (1994). Table 19 shows the ingredients (Merck Pty Ltd, Victoria, Australia) used to create the artificial saliva.

Cheese cylinders (7 cm long with a 2 cm diameter) from each experimental batch of cheese were prepared by cutting from cheese blocks at Day 0 and after Day 120, and coated with wax, except one side (the flat surface) of the cylinder that was exposed to the artificial saliva to allow unidirectional movement of the salt from the cheese matrix to the saliva. The un-waxed surface of the cylinder was dipped into the saliva and kept in place by using pronged clamps (Labtek, Brendale, Queensland, Australia). The volume of artificial saliva was sufficiently large (4 L) to allow the solute concentration differences in the saliva to be insignificant throughout the experiment (Pajonk et al. 2003). In addition, the saliva was agitated constantly throughout the entire duration of the experiment in order to overcome the external resistance to mass transfer in saliva.

The experimental unit was placed into a temperature- and humidity-controlled environmental chamber (Steridium Pty Ltd, Brisbane, Queensland, Australia) and the

temperature and humidity inside the chamber were kept constant at 15ºC and 75%, respectively. The entire experimental unit was covered with aluminium foil to prevent evaporation of the moisture from the saliva. Constant contact between the surface of the cheese cylinder and the saliva was maintained, and samples were taken after Days 1 and 6 of exposure.

In order to be able to duplicate the salt release measurement, two cylindrical samples of cheeses were removed simultaneously from the experimental unit, followed by cutting into 2 mm slices using a Vernier calliper. The salt released was calculated by subtracting the amount of salt retained in the matrix from the initial salt content of the cylinders. It was difficult to cut slices from the cheeses stored for 180 days due to high friability; thus, only Days 0 and 120 of the storage samples were used for diffusivity measurements.

6.2.4 Determination of Salt in Cheese Slices

The collected slices were fully digested in an acid mixture consisting of five parts of HNO_3 (69%) and one part of $HClO_4$ (70%) sourced from Merck Pty Ltd, Victoria, Australia, according to Cortez et al. (2008), using an oil bath (120ºC). Afterwards, 1 mL of each of the clear digest was diluted in 10 mL of deionised water, followed by filtering through a 0.45 µm filter (Millex). The sodium content of the samples was analysed using a multi-type inductively coupled plasma atomic emission spectrometer (ICPE-9000; Shimadzu Scientific Instruments [Oceania] Pty Ltd, Rydalmere, New South Wales, Australia). A standard curve was prepared at 1, 10, 20, 30 and 40 ppm sodium in order to calculate the concentration of Na^+ in the samples. All analytical measurements were performed in duplicate.

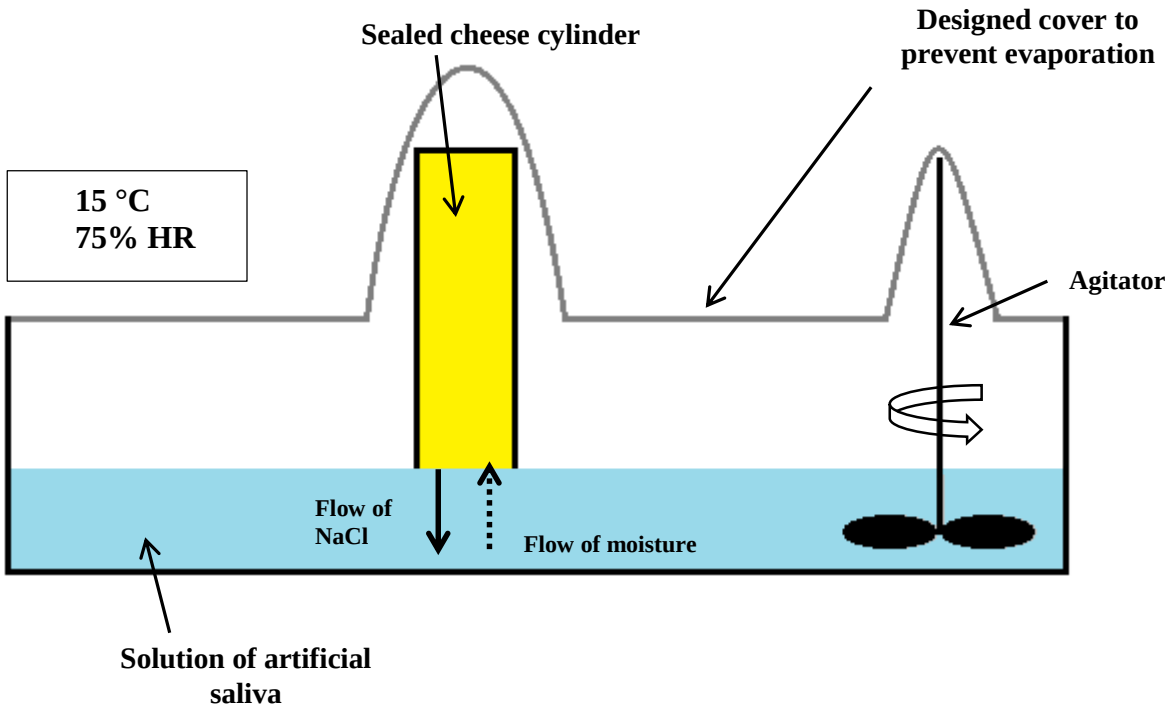


Figure 19: Illustration of the Experimental Unit Set Up for Measurement of Diffusion

Table 19: Ingredients of Artificial Saliva (Van Ruth et al. 1994)

Substance	Concentration[1]
NaH_2PO_4-H_20	0.1 mol L^{-1}
K_2HPO_4	0.1 mol L^{-1}
NaCl	15 mmol L^{-1}
KCl	6.4 mmol L^{-1}
NaN_3	0.005 g/100 g

[1] In deionised water with pH adjusted to 7.

6.2.5 Determination of Effective Diffusion Coefficient

Cheddar cheese has a complex heterogeneous structure, which poses difficulties for the exact measurement of the solute penetration and tracking the path of migration in the matrix. The movement of solutes is normally described as a diffusion process (Floury et al. 2009b; Gros & Ruegg 1987) with an apparent or effective diffusivity characterising the salt transfer. While the salt in cheese is moving downward into the saliva, there is a concomitant uptake of water from the saliva into the cheese, as is the case during the salting of curd. In the absence of convective mass transfer, salt movement in the cheese

cylinder can be described by Fick's second law of diffusion as an unsteady state mass transfer. The equation is written as follows:

Equation 5:

$$\frac{\partial C}{\partial t} = \nabla [D_{eff} \times \nabla(C)]$$

where *C* represents the Na concentration in the cheese (mol kg^{-1}), *t* is the time (seconds) and D_{eff} is the effective diffusion coefficient of NaCl in the cheese ($m^2.s^{-1}$).

Consider a special case of unidirectional transfer of salt with the following initial and boundary conditions:

$t = 0$, then $C(x, 0) = C_0$

$t > 0$, then $C(0, t) = C_s$, $C(\infty, t) = C_0$

For given dimensions of the cheese cylinders, the Fourier number ($D \times t/l^2$) is < 0.05 (Floury et al. 2009b; Floury et al. 2010; Pajonk et al. 2003; Turhan & Kaletunç 1992); hence, the cheese cylinders can be considered semi-infinite solids. The analytical solution of the Fick's diffusion equation is given in Equation 6 for these conditions (Crank 1975).

Equation 6:

$$\frac{C(x,t) - Cs}{C0 - Cs} = erf\left(\frac{x}{2\sqrt{D_{eff}}.t}\right)$$

where *Cs* is the Na or Cl concentration in the artificial saliva solution, *erf* is the error function and D_{eff} is the effective diffusion coefficient ($m^2 \cdot s^{-1}$).

This equation was used to determine the D_{eff} values for cheese (1.5% salt) prepared using different C/F ratios, rennet concentrations and pH at the stage of drainage at zero and 120 days of storage. The details of the experimental treatments are given in Section 4.2.1. Similar protocols were used by other researchers (Floury et al. 2009b; Gros &

Ruegg 1987). The amount of salt released into the saliva after one and six days from different cheese matrices ripened at zero, 60 and 120 days was calculated by subtracting the remaining salt in the cheese cylinders from their initial salt concentration.

6.2.6 Sensory Evaluation

Sensory evaluation was conducted after approval by Victoria University's Human Ethics Committee (approval number: HRE13-005). Fourteen panellists were recruited from staff and research students of Victoria University to assess the sensory attributes of the experimental cheese samples, using a 10-point hedonic scale. Panellists were trained in the ability to detect and distinguish Cheddary, creamy, sour-acid, vinegary and bitter tastes in Cheddar cheese.

The sensory evaluation was conducted for cheeses at zero and 180 days of storage. Cheese samples were tempered at room temperature (20°C) for one hour, cut into pieces and placed on white plates coded with random three-digit numbers. Each panellist evaluated four samples per session. Crackers and water were provided to the judges between samples to change the taste and rinse the palate and tastebuds, respectively. Panellists ranked the sample based on seven attributes using a hedonic scale, with extreme values of zero = lowest sensation and 10 = highest sensation. These ratings were given for the Cheddary, creamy, sour-acid, vinegary and bitter taste sensations. The overall acceptability of the samples was ranked from zero (not accepted) to 10 (highly accepted). The forms for information, consent and rating are presented in the appendices.

6.2.7 Data Analysis

Data were analysed using the one-way ANOVA procedure of Minitab 16, with the level of significance set at 5% to test the effects of treatments on the release of salt. Tukey's honest significant difference test was used to assess the significant difference between the means. Correlation and general regression features were used to establish the relationship between the compositional variables and diffusivity. All statistical analysis was conducted with Minitab 16.

6.3 Results and Discussion

6.3.1 Salt Diffusion Coefficient as Affected by Treatments

The appendices present the sodium profiles of all experimental cheese matrices after one and six days of contact with the artificial saliva, for all treatments, with the normalised concentration difference plotted again the diffusive path depicted as 'x'. The concentration profile pattern was similar to those of Turhan (1996) and Pajonk et al. (2003), who reported that Fick's law of diffusion represented the sodium diffusion in the matrix of soft cheese (white cheese) and hard cheese (Emmental), respectively. Similar profiles were reported by Floury et al. (2009b) for a lipoprotein matrix simulating cheese. Table 20 presents the Na^+ diffusion coefficients of the experimental cheese matrices after one and six days of the experiment.

The values of the effective diffusion coefficients calculated from the cheese matrix varied between 2.62 and 4.97 × 10^{-10} m^2 s^{-1} at 15°C, depending on the type of matrix, which differed in both composition and structure as affected by the treatments. These values are in general agreement with those of Turhan (1996), who reported a salt diffusion coefficient of 2.2 and 3.7 × 10^{-10} m^2 s^{-1} at 4ºC, and 3.9 and 4.2 × 10^{-10} m^2 s^{-1} at 20ºC in a semi-hard, ripened Turkish white cheese (~6 to 7% salt). However, Guinee and Fox (1983), Morris et al. (1985) and Floury et al. (2009b) reported diffusion coefficients ranging from around 1.4 to 3.63 × 10^{-10} m^2 s^{-1}. As stated by Floury et al. (2010) in their review, variations in D value are expected due to the complex nature of the cheese matrix, which prohibits direct comparison of the data, even for the same type of cheese produced using identical conditions. Thus, many studies have used a model lipoprotein matrix to simulate cheese to assess D_{eff} (Floury et al. 2009b; Grummer & Schoenfuss 2011). As reported by Turhan and Kaletunç (1992), diffusivity in this study also increased with the time allowed for diffusion, as D_{eff} was higher after six days than after one day, irrespective of the storage or ripening time. In contrast, salt transport in all cheeses decreased upon storage or ripening, as the D_{eff} values were lower than those before ripening. This was due to compaction of the microstructure of cheese upon ripening, as reported in Chapter 5. This is a known characteristic of Cheddar cheeses (Guinee et al. 2000). These effects are normally associated with composition mainly;

DM content (Floury et al. 2009b), temperature, proteolysis, calcium content and the homogenisation of milk (Guinee & Fox 1983).

The ANOVA showed that the treatments (pH, C/F ratio and rennet concentration) significantly affected the sodium content and D values in cheese ($P < 0.05$). The differences between the D values among different treatments were due to differences in cheese compositions and microstructural and textural properties, as influenced by changes in C/F ratio, pH at drainage and rennet concentration during the cheese making process. These effects are discussed individually as below.

Table 20: Diffusion Coefficients of Sodium Ion ($\times 10^{-10}$ m^2 s^{-1}) Determined for Cheese Matrices Prepared by Different Treatments

Cheese matrix composition Drainage pH/C:F ratio/rennet conc.	Storage (day)			
	0		120	
	Contact with saliva (day)		Contact with saliva (day)	
	1	6	1	6
6.2/0.6/0.1	3.51	4.97	3.39	4.25
6.2/0.6/0.3	3.49	4.88	3.36	4.22
6.2/0.7/0.1	3.48	4.86	3.33	3.97
6.2/0.7/0.3	3.45	4.83	3.29	3.92
6.2/0.8/0.1	3.38	4.79	3.24	3.91
6.2/0.8/0.3	3.33	4.74	3.16	3.88
5.9/0.6/0.1	3.48	4.72	3.07	3.85
5.9/0.6/0.3	3.44	4.69	3.03	3.85
5.9/0.7/0.1	3.38	4.60	3.01	3.79
5.9/0.7/0.3	3.34	4.56	2.94	3.68
5.9/0.8/0.1	3.28	4.51	2.90	3.67
5.9/0.8/0.3	3.23	4.49	2.89	3.63
5.6/0.6/0.1	3.42	4.45	2.88	3.61
5.6/0.6/0.3	3.39	4.44	2.86	3.59
5.6/0.7/0.1	3.28	4.40	2.82	3.51
5.6/0.7/0.3	3.22	4.37	2.77	3.49
5.6/0.8/0.1	3.17	4.24	2.68	3.45
5.6/0.8/0.3	3.10	4.19	2.62	3.41

6.3.2 Salt Release from Cheese

The results of the salt released in the artificial saliva are presented in Figures 20, 21 and 22 for storage period Days 0, 60 and 120, respectively. The pH, C/F ratio and rennet

concentration significantly ($P < 0.05$) affected the salt released from the cheese cylinders, and the pattern followed a similar trend as observed in the diffusivity values. The increase in C/F ratio from 0.6 to 0.8 decreased the amount of sodium released from all cheeses, irrespective of pH at drainage and rennet concentration for zero, 60 and 120 days of storage. This may be attributed to the higher moisture content of cheeses made with 0.6 C/F ratio (Lawrence et al. 2004) and higher protein in the matrix impeding diffusion. The higher moisture content may facilitate Na^+ ion movement inside the cheese matrix. Cheese made with 0.8 C/F ratio had a lower moisture content and higher protein content (data shown in Chapter 4); thus, the interaction between Na^+ ions and cheese proteins could be stronger at higher C/F ratios.

Figure 20 shows that the drop in pH at drainage from 6.2 to 5.6 decreased the release of sodium from the cheese cylinders. This may be due to the lower moisture content of cheeses drained at lower pH (such as 5.6) (Tunick et al. 2007), and the pH reduced as a result of lactic acid formation during cooking time. Thus, the increase in cooking time led to the expulsion of more moisture from the curd, and thereby reduced the final moisture content of the final product (Tunick et al. 2007). The lower moisture content reduced salt movements inside the cheese matrix, as shown by Geurts et al. (1980); thus, the Na^+ ions were retained in the cheese cylinder. It may be recalled that the moisture content of the samples with a high pH at drainage was higher than that of the cheese samples prepared at lower pH at drainage.

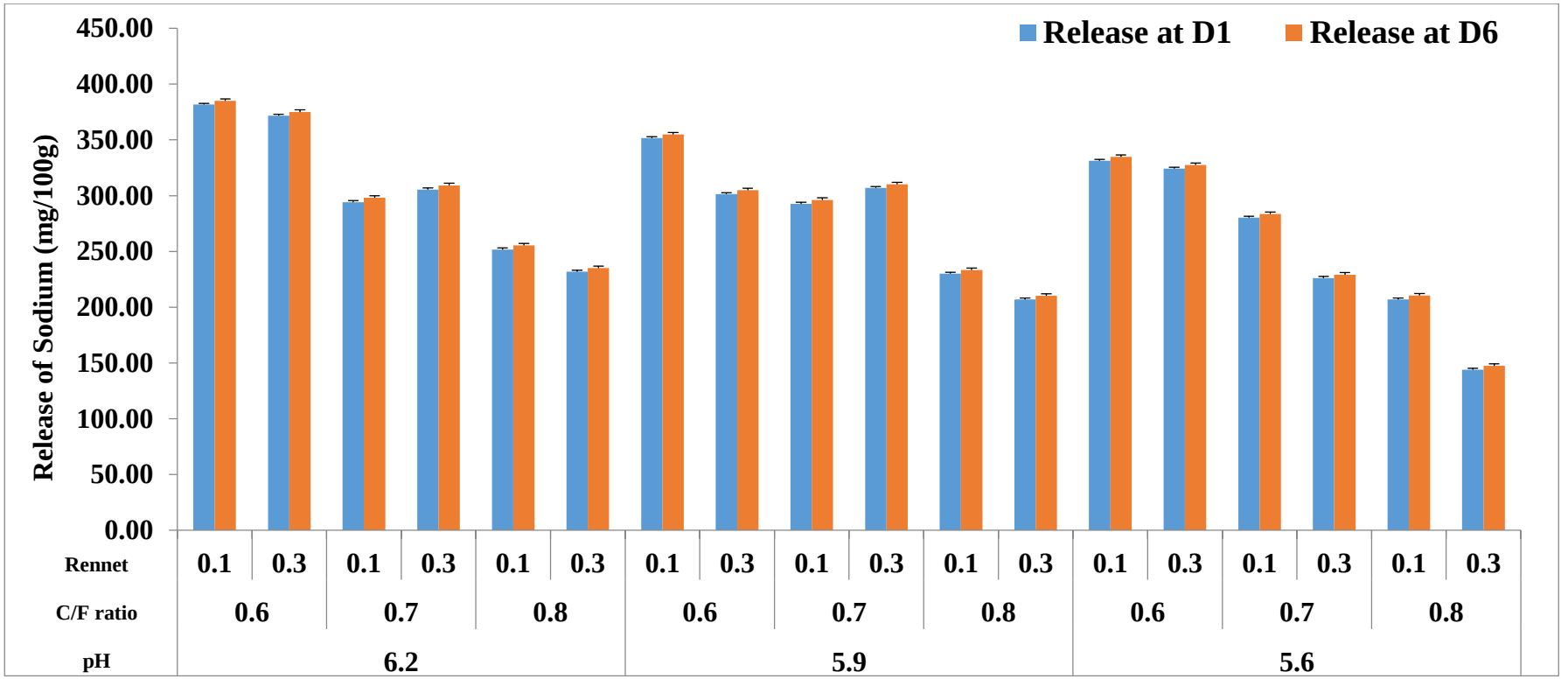


Figure 20: Release of Sodium from Cheese Cylinders Made with Different Drainage pH, C/F Ratio and Rennet Concentrations Stored for 0 Day

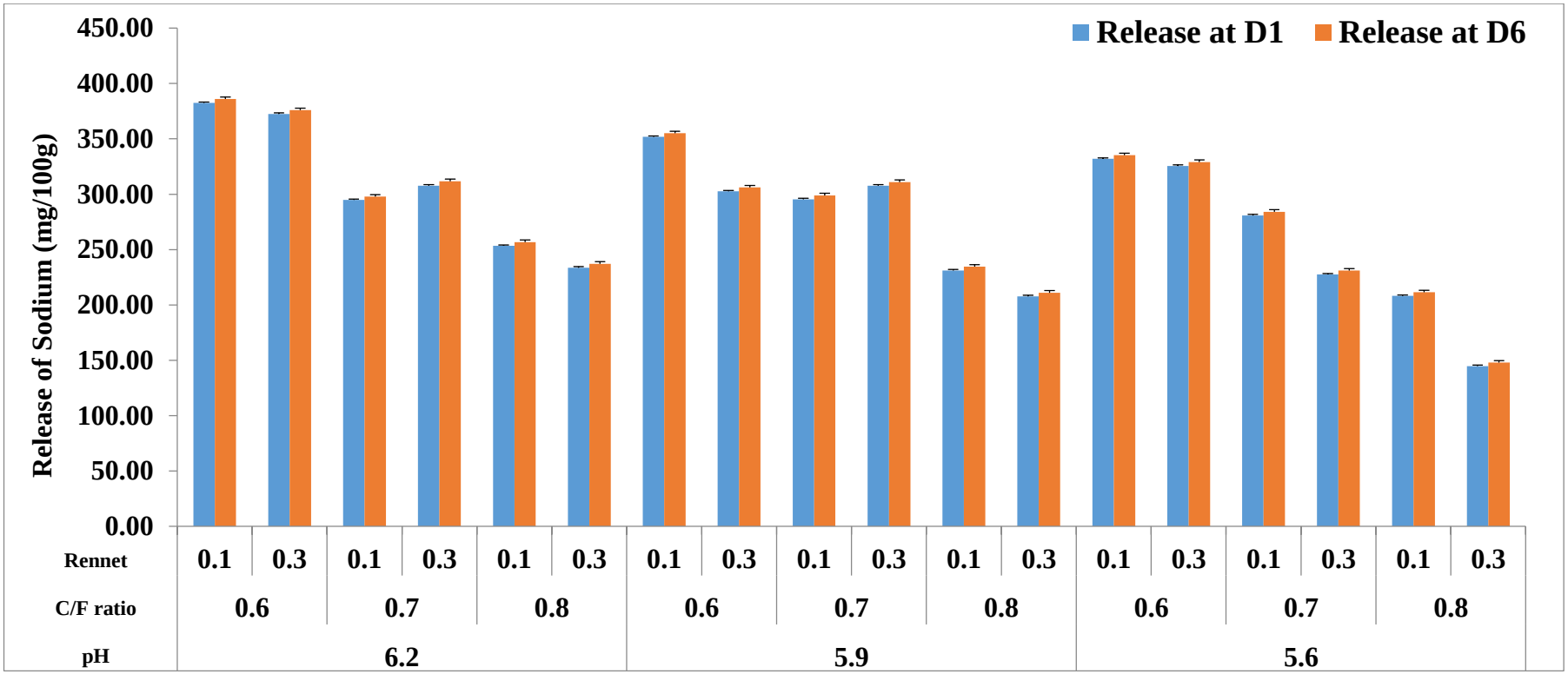


Figure 21: Release of Sodium from Cheese Cylinders Made with Different Drainage pH, C/F Ratio and Rennet Concentrations Stored for 60 Days at 9 ± 0.5°C

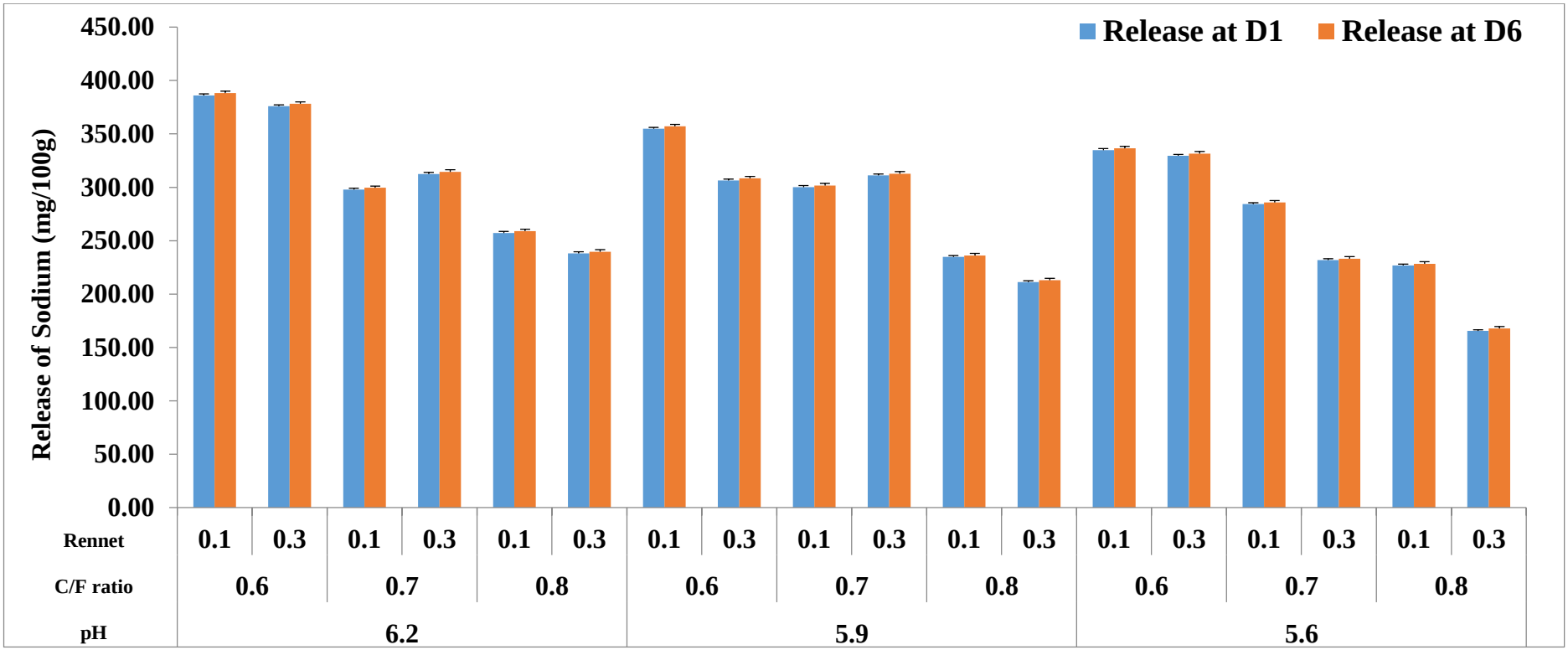


Figure 22: Release of Sodium from Cheese Cylinders Made with Different Drainage pH, C/F Ratio and Rennet Concentrations Stored for 120 Days at 9 ± 0.5°C

6.3.3 Effect of C/F Ratio

As can be seen from the above figures, the increase in C/F ratio from 0.6 to 0.8 decreased the release of sodium from the cheeses. This suggests that the highest transport of sodium was in the cheeses made with the lowest C/F ratio that is, high fat content and lower protein compared with C/F 0.7 and 0.8. The diffusivity of sodium increased in order of 0.6 > 0.7 > 0.8. This may be attributed to the higher moisture content (34.86 to 37.06 %) and coarser microstructure of cheeses made with a C/F ratio of 0.6 compared with the higher C/F ratio cheeses (Lawrence et al. 2004), given that higher moisture content facilitates better sodium movement in the matrix. This is reflected in Figure 23, which shows that the salt diffusivity was higher in the cheese with lower protein content. At the same time, the diffusivity was consistently lower in the cheese stored for 120 days. This means that high-protein cheese will have a lower salt perception, as a lower amount of salt will be released from such a matrix.

The presence of fat globules in cheese tends to affect the diffusivity of salt by its effects on the volume fraction of the rest of the non-fat component, including proteins. As presented in Figure 24, diffusivity consistently increased upon increase in the fat content, and decreased as the protein content increased in all experimental cheese samples. Similar effects of varying fat content were reported by Geurts et al. (1974) in cheeses with equal moisture content. However, Floury et al. (2009b) did not observe this trend in a more recent work using a lipoprotein model without any moisture content control. In the later study, the moisture content was not controlled. In the current study, the diffusivity values of salt were consistently lower for cheese samples stored for 120 days than zero days. This suggests that continued hydrolysis of protein and fat made the cheese matrix more compact, thereby reducing the salt movement in the ripened cheese. Release will be higher in ripened cheese than in fresh cheese with the same salt content. In terms of the perception of saltiness, high-fat and low-protein cheese will induce a higher sensation of saltiness in the mouth, as reported by Lawrence et al. (2012). It is also possible to deduce from Figures 23 and 24 that salt diffusivity values are more sensitive to changes in FDM content than to protein content, as the slope of the lines in Figure 24 is steeper, irrespective of fresh or ripened cheese. The negative correlation of protein (that is, lower salt diffusion at a high C/F ratio) may be due to the existence of stronger interactions between sodium ion and protein. Sodium (Na^+) is a positively

charged ion that interacts with amino acids that contain both negative (COOH) and positive (NH_2) groups in their structure. Higher protein content triggers greater production of amino acids during ripening, which entrap more sodium ions in the cheese matrix (Buxbaum 2007; Fox et al. 2000a). This supports the current study's results, in which diffusivity was lower in the cheese containing a higher C/F ratio (Table 20).

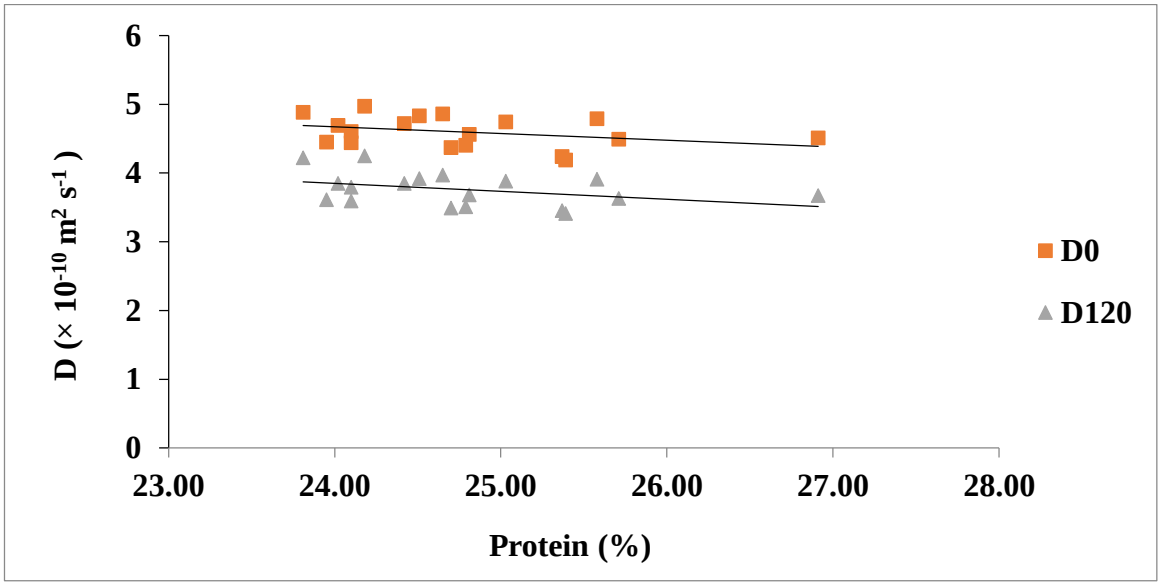


Figure 23: Effect of Protein in Cheese on Diffusivity of Salt Measured after Six Days of Exposure to Saliva before (D0) and after Storage (D120)

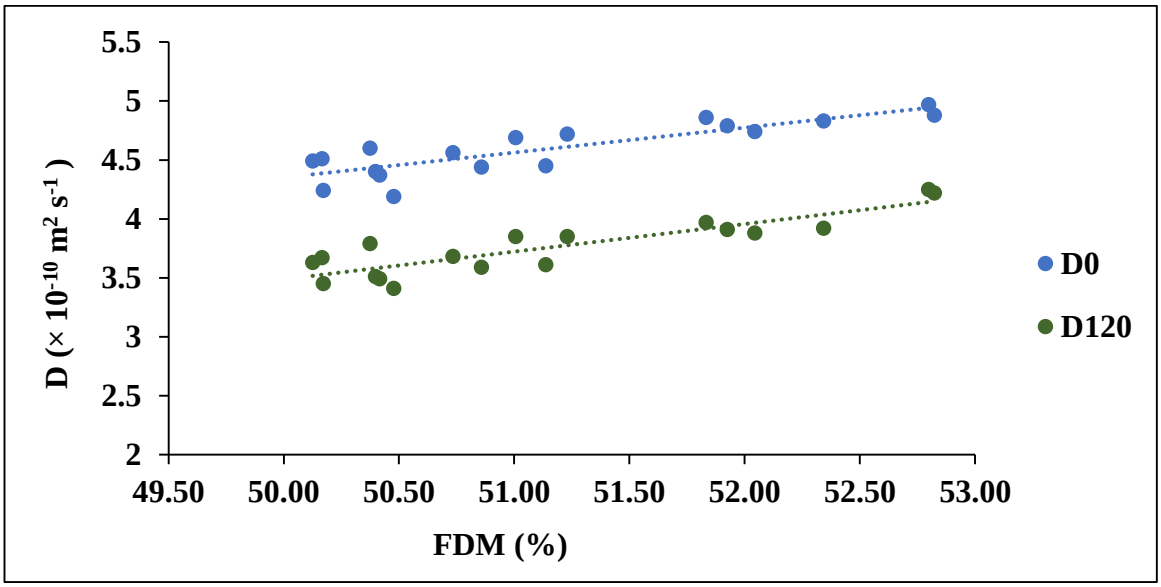


Figure 24: Effect of Fat Content in Cheese on Diffusivity of Salt Measured after Six Days of Exposure to Saliva before (D0) and after Storage (D120)

6.3.4 Effect of DM

DM and moisture are important compositional variables that are correlated with salt transport in cheese (Floury et al. 2010; Guinee 2004a; McMahon 2010). Cheeses made with higher DM content affect the movement of salt and thus reduce diffusivity values, as observed at a higher DM content (Figure 25). Water is required for salt movement and a high DM entails low moisture content (Fox et al. 2000a). This is also due the compacted microstructure of protein networks as a result of higher crosslinking of proteins (Floury et al. 2009b). These results are consistent with those of Floury et al. (2009b), who observed that the apparent salt diffusion coefficient decreased from 3.43 to 3.02 $\times 10^{-10}$ $m^2.s^{-1}$ (decrease of 12%) when the DM content increased from 370 to 440 g kg^{-1}.

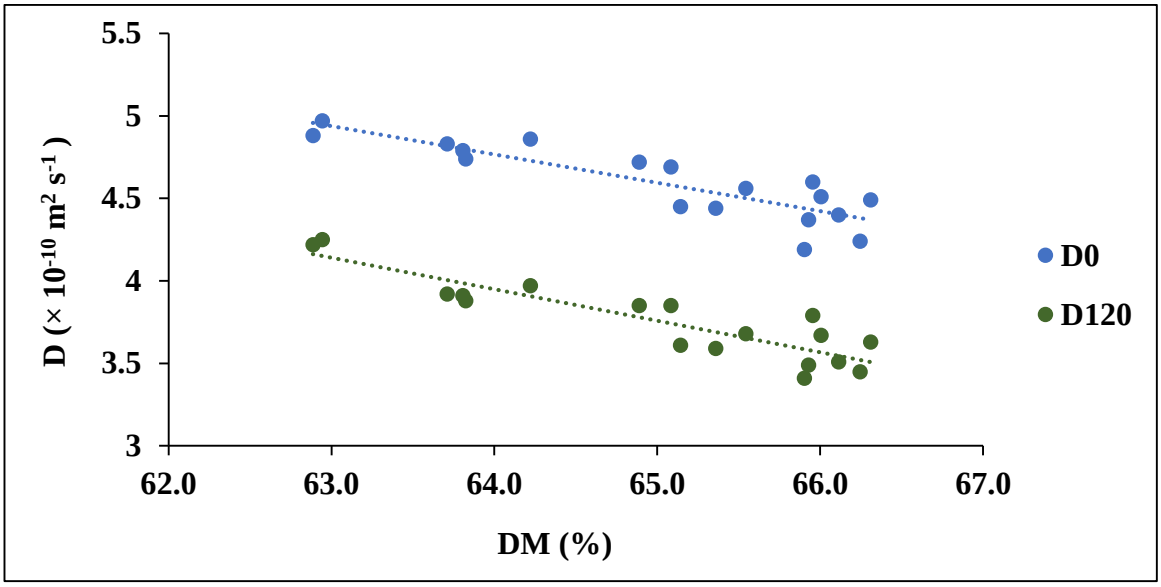


Figure 25: Effect of DM in Cheese on Diffusivity of Salt Measured after Six Days of Exposure to Saliva before (D0) and after Storage (D120)

Once again, the diffusivity of salt in both the unripened cheese (D0) and the cheese ripened for 120 days showed a decrease in D values as the DM content increased in the matrices. In addition, the unripened cheese D values were consistently lower than those in the ripened cheese by about 15%. Based on multiple regression analysis of diffusivity data and the composition of different cheeses as reported in the literature, Floury et al. (2010) claimed that diffusivity can be predicted in different types of cheese using DM, FDM and temperature via Equation 4 with R^2 of 0.75. However, of these three, DM was

the most significant factor influencing diffusivity; thus, it was used to map the diffusivity of salt in 18 different types of cheese (Floury et al. 2010).

6.3.5 Effect of pH at Drainage

The pH at drainage showed a significant influence on the diffusivity of sodium. Regardless of the C/F ratio and rennet concentration, the diffusion coefficient decreased from 4.97 to 2.62 × 10^{-10} m^2 s^{-1}, which meant a reduction to ~53%.

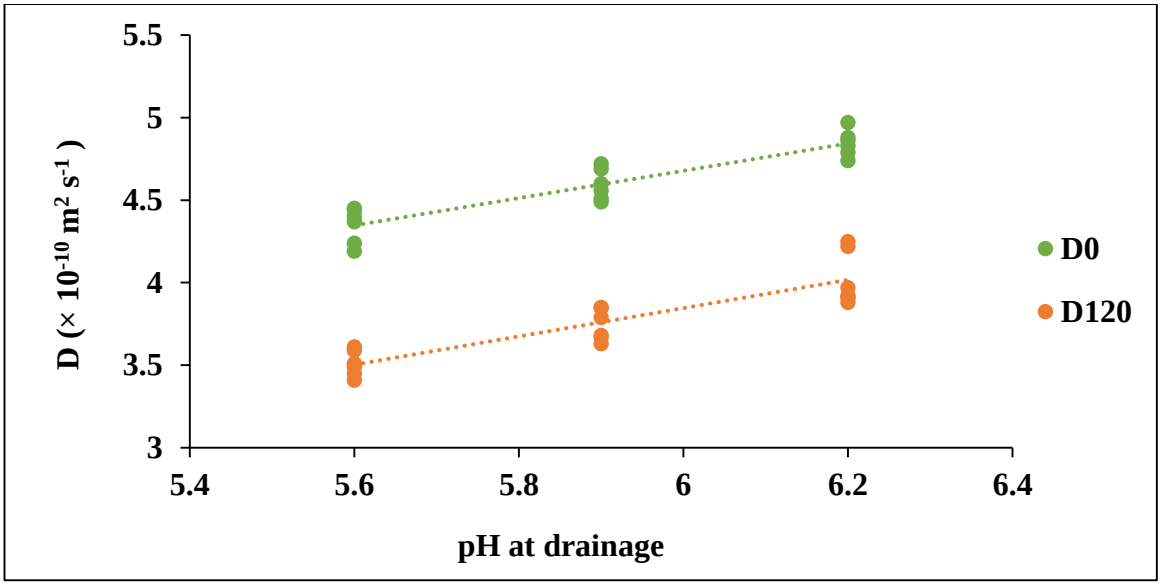


Figure 26: Effect of Drainage pH on Diffusivity of Salt after Six Days in Cheese before (D0) and after Storage (D120)

The differences in pH influence casein hydration and aggregation, which affect sodium diffusivity. An increase in pH at drainage at the renneting stage improves the hydration of the casein micelles due to the increased negative charges. This results in higher solubilisation of proteins, thereby facilitating diffusion (Famelart et al. 1996). Floury et al. (2009a) also reported a significant effect of pH at renneting on the structural and textural properties of the model cheese matrices. As pH decreased, the hardness and springiness of the model cheeses significantly increased, while their adhesiveness showed a reduction. Excessive loss of moisture due to increased cooking time led to increased interaction between casein micelles. This produced a compacted and integrated protein network that limited the movement of Na^+ (Geurts et al. 1980; Lawrence et al. 2004; Tunick et al. 2007).

The occurrence of a pH drop at the drainage stage influences the initial structure of curd, which affects the overall texture of cheese during ripening (Fox et al. 2000a; Gunasekaran & Ak 2003). The current study's results do not agree with Geurts et al. (1974) and Guinee and Fox (1983), who reported a constant diffusion coefficient despite differences in the matrix composition of the cheese. In the current study, the decrease in D value corresponded to the lower water content (~33 to 35%) of cheeses drained at a lower pH (such as 5.6), compared to drainage at higher pH (Tunick et al. 2007).

The prolonged cooking time required for the pH to drop to 5.8 enhanced the expulsion of moisture from the curd, thereby decreasing the moisture content in the final product (Tunick et al. 2007). The lower moisture content limited the diffusivity of the salt and hence its movements inside the cheese matrix (Geurts et al. 1980), thereby releasing less salt into the saliva.

6.3.6 Effect of the Rennet

Proteolysis is the major factor affecting the cheese matrix and its structure by hydrolysing the protein network (para-casein). The initial hydrolysis of caseins is caused by the coagulant and, to a lesser extent, by the plasmin and perhaps the somatic cell proteinases (such as cathepsin D). These steps result in the formation of large (water-insoluble) and intermediate (water-soluble) sized peptides that are subsequently hydrolysed by the coagulant and enzymes from the starter and non-starter flora of the cheese. The production of small peptides and amino acids is caused by the action of microbial proteinases and peptidases, respectively (Fox et al. 2004b). Rennet concentration affects salt diffusion indirectly through the proteolysis process. The initial structure of the protein network in the cheese matrix is formed by addition of an enzyme (rennet) and is influenced by residual rennet and bacterial enzymes during ripening (Fox et al. 2004b).

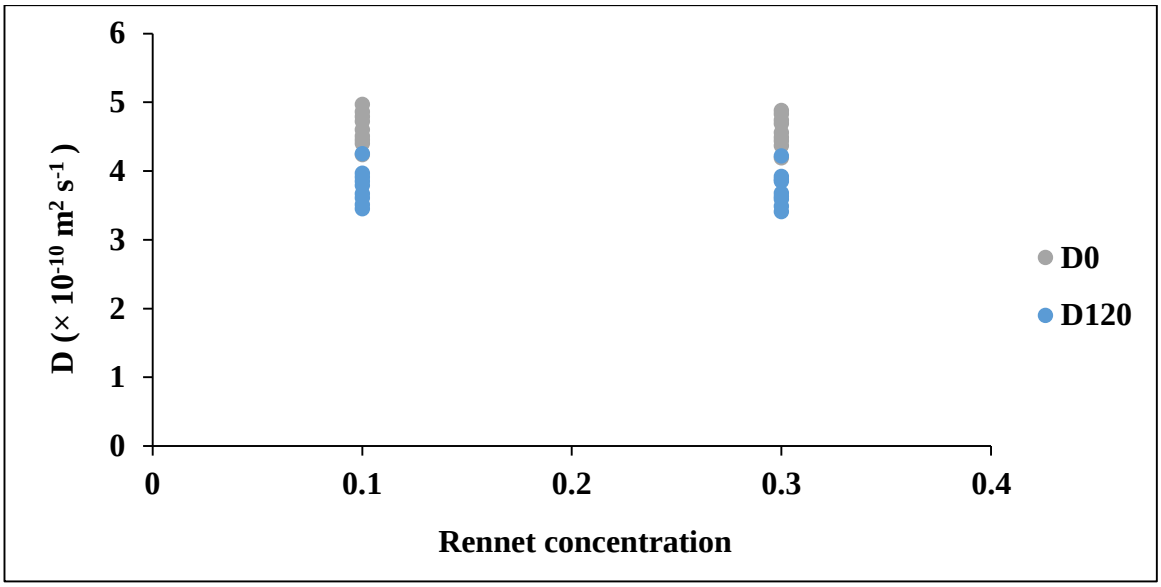


Figure 27: Effect of Rennet Concentration on Diffusivity of Salt after Six Days in Cheese before (D0) and after Storage (D120)

Up to 30% of the rennet added to the cheese milk is retained in the curd, depending on the enzyme type, cooking temperature, pH at drainage and moisture content of the curd. The initial enzyme concentration influences the amount of retained rennet in the cheese after whey drainage. In Cheddar cheese, about 6% of the chymosin added to the milk is retained in the curd, but the amount increases with decreasing pH upon whey drainage (Fox et al. 2004b).

An increase in rennet (chymosin) concentration (larger amounts of rennet added to cheese milk) reduces the total time required for rennet clotting. As a result, the secondary phase of rennet action also proceeds earlier, with the net result of an increase in the rate of curd firmness. This property of chymosin could be more effective in the presence of higher protein content. An increase in the amount of rennet added to the milk during cheese manufacture would increase the residual amount of rennet retained in the cheese. As a result, the primary step of proteolysis would be enhanced and result in more intermediate and small molecular weight peptides. These peptides have high water-binding capacity and the ability to bind free water in cheese. Thus, the mobility of Na+ is reduced due to less availability of free water (Fox et al. 2004b). In this study, rennet was used at 0.1 and 0.3% levels, and Figure 27 depicts the effects of their influence on the diffusion coefficient of salt. The lower D values for cheeses stored for 120 days than cheeses stored for zero days suggest that the effects of continued

proteolysis during ripening produce more polar molecules that reduce the availability of water. However, in this study, the level of rennet used produced a non-significant difference on D values for a given storage time.

6.3.7 Relationship between D Value, Composition Variable and pH at Drainage, as Affected by Different Treatments

The effect of C/F ratio, rennet concentration and pH at drainage affect salt transport and release in saliva through their collective influence on the composition and structure of cheese before and after storage, as discussed earlier. Significant changes occurred in the cheese matrix during storage (ripening), as reported in the previous chapters, which reduced the movement of salt and its release, as evidenced by lower D values (Table 20). The presence of interactions between the formulation (C/F ratio and rennet) and process (pH at drainage) variables predict the release of salt from the cheese matrix. However, the role of DM, moisture, fat (composition) and pH at drainage have been shown to influence the transport of salt, as shown in Figures 23 to 26. With the experimental conditions used in this study, it is possible to relate the effects of changes in these variables to diffusion coefficients and release. Table 21 shows the regression equations that describe the influence of D values (and therefore the release of salt from cheese) on DM, FDM and pH at drainage. These equations are valid only for the Cheddar cheese made in this study with 1.5% salt. High and significant ($P < 0.05$) R^2 values were found for all the regression equations, which can be used to predict the diffusivity of salt by knowing the DM and FDM contents.

As reported previously by Floury et al. (2010), D values are negatively correlated with DM consisting of protein, fat and minerals with a R^2 of 0.874 and 0.887 in unripened and ripened cheese samples, respectively. The D value was positively correlated with FDM, with R^2 values of 0.730 and 0.774 in unripened and ripened cheese samples, respectively. The current study found that both of these compositional parameters were significant in affecting D values, unlike Floury et al. (2010), who found FDM to be insignificant. Hence, the current study sought to incorporate both DM and FDM in the regression equation, and the results of the fit suggest superior relationship as significantly higher R^2 values (> 0.9) were achieved. Thus, this study proposes using these regression equations to predict D values.

The last two regression equations allowed prediction of D values from pH at drainage, a key parameter in Cheddar cheese manufacturing. This parameter had a positive correlation with D values, suggesting that high pH of drainage would facilitate more salt transport and salt release. The predictability of these equations, as presented in Table 21, is limited to the conditions used in this study.

Table 21: Effect of Composition Variables and Drainage pH on Diffusivity ($D \times 10^{-10}$ m^2 s^{-1}) Measured at 15°C after Six Days of Exposure of Cheddar Cheese Samples Made with 1.5% Salt in Saliva before (D_0) and after Ripening for 120 days (D_{120})

Equation	R^2	Remarks
D_0 = 15.309-0.0165 DM	0.874	DM = DM in cheese, g/kg
D_{120} = 15.374 - 0.179 DM	0.887	
D_0 = -6.244 + 0.212 FDM	0.730	FDM = FDM in cheese, g/100 g
D_{120}= -8.245 + 0.235 FDM	0.774	
D_0 = 30.84 - 0.16 FDM – 0.0278 DM	0.904	
D_{120} = 25.51 – 0.106 FDM – 0.0251 DM	0.898	
D_0 = -0.288 + 0828 pHD	0.846	pHD = pH at drainage
D_{120} = -1.304 + 0.858 pHD	0.786	

6.3.8 Sensory Evaluation

The diffusivity values determined for different treatments showed that the maximum D value was at a drainage pH of 6.2, C/F ratio of 0.6 and rennet concentration of 0.1; thus, the cheese produced using this treatment would have the highest release of sodium and subsequently the highest *in vivo* saltiness. The predictability of these equations is limited to the conditions used in this study. The cheeses (1.5% salt) made using this treatment at Days 0 and 180 were used for sensory analysis and compared with the control cheese (2.5% salt). Table 22 presents the scores for the Cheddary, creamy, sour-acid, bitter and vinegary flavours, as well as the overall acceptability attributes.

Except the vinegary and sour-acid tastes, no significant difference was observed between the control cheese and the cheese with the highest diffusion coefficient value (6.2/0.6/0.1) at the same storage period. This suggests that the highest amount of release of Na^+ ions occurs from this particular treatment (6.2/0.6/0.1). Higher pH at drainage (higher moisture) and a higher amount of fat (C/F ratio 0.6) led to formation of fat micelles surrounding water in the cheese matrix. This occurs as a result of the

hydrophobicity of fat globules, which improves the movement of sodium ions in the cheese matrix (Geurts et al. 1980; Lawrence et al. 2004; Tunick et al. 2007). Moreover, due to a lower concentration of rennet (0.1 mL/L) in this treatment, the amount of residual rennet decreased, leading to production of a smaller amount of peptides in cheese (Upadhyay et al. 2004). A more porous cheese matrix accordingly produces more Na^+ ion diffusional movement (Guinee 2004a; Guinee & Fox 2004), leading to an increase in the sensory perception of saltiness in salt-reduced cheeses (Taylor & Roberts 2004).

Table 22: Sensory Attributes of the Cheese with the Highest Amount of Salt Release at Days 0 and 180 of Storage at 9 ± 0.5°C

Treatment	Storage (day)	Cheddary	Creamy	Sour-acids	Vinegary	Bitterness	Saltiness	Acceptability
Control[1]	0	4.98±0.43[a2]	6.36±0.29[a]	4.02±0.27[a]	3.07±0.41[a]	1.93±0.34[a]	5.81±0.32[a]	5.71±0.32[a]
	180	8.07±0.29[b]	6.43±0.64[b]	4.61±0.46[ab]	3.21±0.59[a]	3.07±0.62[b]	6.12±0.46[b]	8.00±0.35[b]
6.2/0.6/0.1	0	5.29±0.46[ab]	6.50±0.40[a]	4.24±0.36[ab]	3.14±0.47[a]	2.00±0.41[ab]	5.76±0.47[a]	5.93±0.44[a]
	180	7.43±0.39[bc]	6.79±0.28[b]	5.46±0.34[c]	4.21±0.41[b]	3.43±0.48[b]	6.37±0.26[b]	7.36±0.31[ab]

[1] Control cheese containing 2.5% salt w/w
[2] Mean value ± SE
[a-c] Means in each column with different letters are significantly different ($P < 0.05$) at same storage period.

6.4 Conclusion

Salt diffusion in Cheddar cheeses can be assessed by using Fick's law of diffusion. The effective diffusion coefficient values in the cheese samples prepared by varying the C/F ratio, rennet concentration and pH at drainage ranged between 2.6 to 4.97 × 10^{-10} m^2 s^{-1}, depending on the contact time between the sample and saliva, time of ripening (zero days and 120 days) and treatments. Irrespective of treatments, the D values increased as a result of ripening at constant contact time and vice versa. These experimental D values obtained in this study are in general agreement with the values previously published in the literature.

A low C/F ratio and rennet concentration increased the diffusion of sodium due to higher retention of moisture and lower production of peptides and amino acids, as reported in Chapter 4. Higher moisture content at a low C/F ratio was due to reduced expulsion of water.

Since the transport of salt from cheese to saliva was controlled by the internal diffusion of salt in the cheese matrix, a higher D value corresponding to the treatment drainage pH/CF ratio/ rennet concentration of 6.2/0.6/0.1 showed the highest release of salt in saliva.

Salt mobility and release are dependent on the composition and microstructure of the cheese matrix. This study sought to determine the effects of the treatments on diffusivity to the DM and FDM of cheese before and after ripening. The sensory attributes of the cheeses with the highest rate of sodium diffusion from reduced salt (1.5%) were compared to the attributes of the control cheese made with 2.5% salt at both zero and 180 days of ripening. The assessment showed that the reduced-salt cheese had similar overall acceptability scores. Except for the vinegary and sour-acid flavours, the rest of the attributes were similar to the control cheeses.

Chapter 7: Conclusions and Recommendations for Further Research

7.1 Conclusions

Although salt plays an essential role in preserving and processing cheese, as well as contributing to flavour (Silva et al. 2003), it is known as one of the risk factors for the development of osteoporosis, kidney stones and hypertension in humans (Buemi et al. 2002; Heaney 2006; Kotchen 2005; Massey 2005). Salt content in some cheese can be as high as 3 to 5%; thus, cheeses are targeted to reduce salt to comply with the WHO's (2007) recommendations. Due to the multidimensional role of salt in cheese, reducing salt seriously affects the overall quality of cheese. Cheeses made without salt or with low amounts of salt are often low-grade cheese with poor texture and flavour (Guinee & O'Kennedy 2007). These effects result from the influence of salt on the growth of starter and non-starter bacteria and the enzymes responsible for proteolysis and lipolysis.

This research began with a hypothesis that the formulation of milk used for hard-type cheeses (such as Cheddar) and manufacturing practices influence the quality of salt-reduced cheese. Thus, this study examined the influence of pH at drainage, change in the C/F ratio and concentration of coagulant (rennet). This study also examined the effect of these factors on the release of salt, with a view to identify which factors would enhance the salt release in the mouth from low-salt cheese in order to improve salt perception in the mouth. For Cheddar-type cheeses, ripening plays a significant role in dictating the end quality; hence, this study also investigated the effect of ripening time (storage time).

This study showed that salt reduction significantly influences the chemical composition (moisture, protein, fat, ash, S/M content and pH), proteolysis, texture profile (hardness, adhesiveness, cohesiveness, springiness and gumminess), microstructure and sensory properties of cheese made with a different C/F ratio, rennet concentration and pH at drainage. This study also demonstrated that the moisture content of cheese is affected by several factors during the production and ripening of cheese. Salt (NaCl) content is a

major factor affecting the moisture and ash content. Reducing the salt from 2.5% to 1% increased the cheese moisture content by ~5.7%. The protein and fat content of cheese did not differ significantly from changes in the salt concentration.

This study found that the microbial growth and proteolysis (WSN, TCA-SN, PTA-SN and TFAA) of cheeses with a lower salt content were higher ($P < 0.05$) than the control (2.5% salt). The sensory quality of cheese was also predominately influenced by salt reduction due to its effect on proteolysis. The influence of salt reduction (particularly below 2% salt) was mainly attributed to the higher proteolytic activity of the starter culture, as indicated by the PTA-SN values for the same storage time. Production of lower molecular weight peptides enhanced ACE inhibition, with maximum inhibition (19%) corresponding to 1% salt cheese stored for eight weeks. Hardness and gumminess were reduced significantly when the salt concentration was ≤ 1.5% at the same storage time for the entire eight weeks of storage. Differences in the cheese matrices due to proteolysis were also noted on ESEM due to lower salt content. More hydrophobic peptides were produced as a result of an increase in rennet concentration during the storage (ripening) in reduced salt cheeses.

An increase in C/F ratio and rennet concentration, especially at low pH, showed similar proteolysis to cheese containing normal salt concentration of 2.5 to 3%, as previously reported by other studies (Kuchroo & Fox 1982; McSweeney & Fox 1997; Murtaza et al. 2014). Even the hydrophilic, hydrophobic and extra-hydrophobic peptide profiles were similar. Increased production of peptides in low-salt (1.5%) Cheddar cheese should be further investigated to examine the health benefits, in addition to those related to ACE-inhibitory effects. The increase in C/F ratio and rennet concentration influenced the texture of cheeses due to higher proteolysis. Hardness and adhesiveness increased in all cheeses during storage, while springiness and cohesiveness decreased. Reducing the pH caused an increase in the rate of proteolysis, which directly affected texture. The ash content of cheese may also reduce at lower pH, which influences the proteolytic activity and texture. The change in pH at drainage influenced the microstructure of cheese. Reducing the pH particularly increased the demineralisation of CCP, which enhanced association among casein molecules. Therefore, the cheeses made with lower pH had a denser and more compact structure and narrower voids than did the cheeses made at higher pH. As rennet concentration plays an important role in proteolysis and

consequently texture, a wider range of rennet concentrations should be investigated in the future.

At the lower C/F ratio and rennet concentration used in the study, a higher diffusion coefficient was observed for sodium. Similarly, release of sodium ions from cheese was also higher, indicating that higher salt perception is possible at lower C/F ratio and rennet concentration. These observations are due to the higher retention of moisture and less protein degradation, as reported in Chapter 4, facilitating higher transport of salt in the cheese matrix. Higher moisture content at a low C/F ratio is due to reduced expulsion of water from the curd. The effective diffusion coefficient in cheese matrices ranged between 2.6 to 4.97 $\times$ 10^{-10} m^2 s^{-1}, depending on the contact time between the sample and saliva, time of ripening (zero and 120 days) and treatments. The highest D value was found in the treatment drainage pH/CF ratio/rennet concentration of 6.2/0.6/0.1, which corresponded to the highest release of salt in saliva.

The ripening period had a positive effect on D values, which increased as ripening progressed over 160 days due to continued proteolysis causing changes in the cheese matrix over time. Changes in the matrix microstructure supported these observations.

Salt mobility and release were dependent on the composition and manufacturing process (storage time or ripening and pH at drainage) of the cheese matrix (containing 1.5% salt), resulting from changes in the C/F ratio and rennet concertation (composition). Salt diffusivity was related to DM and FDM and, for the given range of treatments, the proposed regression equations can be used to predict diffusivity. The reduced-salt (1.5%) Cheddar cheese matrix that provided the highest D value and maximum release of sodium was the one resulting from the treatment with a drainage pH of 6.2, C/F ratio of 0.6 and rennet concentration of 0.1%. Sensory analysis of this cheese sample showed similar acceptance for overall quality to the cheese samples prepared using 2.5% salt. The reduced-salt cheese showed similar overall acceptability scores to the control cheese, except for the vinegary and sour-acid flavours, which resulted from increased activity of starter and non-starter bacteria (Beeren 2013; Guinee & Fox 2004). These defects may be overcome by use of flavour marking agents.

No direct comparison can be made between the cheeses made in this study and those made by replacing salt by other salts, such as, KCl, $MgCl_2$, and $CaCl_2$. Since cheese made using this approach are known to cause defects such as softness, bitterness, metallic and soapy taste (Grummer et al. 2012), there appears to be no need for the use of replacers,

No economic analysis was carried out in this study; however, comparison between salt reduction along with modification of cheese milk composition and replacement of salt proves that the cost of ingredients used in this study (milk and cream) is expected to be lower than when salt replacers are used. For instance, 1 kg of $MgCl_2$ costs approximately AUD$ 350.00 to 700.00, 1 kg of KCl costs around AUD $160.00 to 250.00. Hence, for a given quality of cheese, cost associated with salt reduction would be lesser than with salt replacement.

7.2 Further Research and Recommendations

The effects of reducing salt in Cheddar cheese are complex and depend on the interplay of many variables. Thus, there remain many questions that need answering, and these questions form the basis of further research. In cheeses that are ripened, such as Cheddar, proteolysis is of central importance in dictating the overall cheese quality. Sufficient evidence was provided in this study that reducing salt affects proteolysis. Thus, the effect of salt reduction on proteolytic agents in cheese (such as rennet, indigenous milk enzymes and bacterial enzymes) requires further investigation. A wider range of rennet concentrations and C/F ratios should be examined.

Model cheeses are useful to examine the effect of salt reduction on the activities of the remaining chymosin and indigenous milk enzymes in cheese. In addition, the activity of starter culture proteinases at different salt levels should be investigated to segregate the effects from coagulants. Instead of using milk, pure milk caseins should be used as a substrate to separately examine the effect of salt reduction on enzymes from the starter cultures and coagulants. Artificial media offers the possibility of precisely controlling the factors that would affect the structure due to salt reduction. Extraction and purification of these proteinases must be done to monitor the amount of remaining

enzymes present at a given time, and their proteolytic activity in lower salt concentrations. Each extracted enzyme and its proteolytic effect can be assessed separately on a salt-reduced model cheese. Moreover, the effect of salt reduction on caseins hydration needs further investigation in order to provide better understanding of how salt reduction influences proteolysis in cheese. Also, there is still much to understand when using different starters, reduced salt levels and process conditions to establish commercially adequate cheese quality parameters when formulating a reduced salt cheese product. Therefore, Model cheeses can be used to explore the effect of salt reduction, milk composition and processing factors (such as drainage pH and ripening) on casein hydration.

One of the major hurdles associated with salt reduction is the adverse sensory quality of cheese. The effect of salt reduction without substitution on the sensory properties of Cheddar cheeses during storage requires in-depth investigation to separately determine the effect of less salt concentration on each sensory attribute. For example, greater focus should be given to discover the bitter-taste-producing peptides and the techniques to disguise this defect in order to increase the reduction of NaCl. Parallel application of salt reduction techniques, use of natural flavour enhancers, and use of different strains of starter cultures could be solutions to compensate for the sensory defects in salt-reduced cheeses.

In addition to proteolytic enzymes, lipolytic enzymes have been known to be associated with the flavour of cheese. Thus, further research on the effect of salt reduction on lipolytic enzymes and their activity is required to understand the role these play in cheese quality. It is proposed to monitor their effects on sensory attributes in the reduced salt cheeses.

Finally, examining salt reduction applications with other types of cheeses, especially those with high salt content, will lead to the further development of valuable knowledge based on the individuality of each cheese's characteristics and manufacturing processes. This will support the overall aim of reducing dietary consumption of salt in cheese in order to make cheese a healthier food product.

References

Agboola, S, Chen, S & Zhao, J 2004, 'Formation of bitter peptides during ripening of ovine milk cheese made with different coagulants', *Lait*, vol. 84, pp. 567–578.

Albarracín, W, Sánchez, IC, Grau, R & Barat, JM 2011, 'Salt in food processing; usage and reduction: A review', *International Journal of Food Science and Technology*, vol. 46**,** pp. 1329–1336.

Alderman, MH 2006, 'Evidence relating dietary sodium to cardiovascular disease', *Journal of the American College of Nutrition*, vol. 25, pp. 256S–261S.

Anderson, CAM, Appel, LJ, Okuda, N, Brown, IJ, Chan, Q, Zhao, L, Ueshima, H, Kesteloot, H, Miura, K, Curb, JD, Yoshita, K, Elliott, P, Yamamoto, ME & Stamler, J 2010, Dietary sources of sodium in China, Japan, the United Kingdom, and the United States, women and men aged 40 to 59 years: the INTERMAP study', *Journal of the American Dietetic Association*, vol. 110, pp. 736–745.

AOAC International 1995, *Official methods of analysis of AOAC International*, Author, Arlington, VA, US.

Appel, LJ & Anderson, CAM 2010, 'Compelling evidence for public health action to reduce salt intake', *New England Journal of Medicine*, vol. 362, pp. 650–652.

Ash, A & Wilbey, A 2010, 'The nutritional significance of cheese in the UK diet', *International Journal of Dairy Technology*, vol. 63, pp. 305–319.

Aston, JW, Durward, IG & Dulley, JR 1983, 'Proteolysis and flavour development in Cheddar cheese', *Australian Journal of Dairy Technology*, vol. 38, pp. 59–65.

Ayyash, MM & Shah, NP 2010, 'Effect of partial substitution of NaCl with KCl on halloumi cheese during storage: chemical composition, lactic bacterial count, and organic acids production', *Journal of Food Science*, vol. 75, pp. C525–C529.

Ayyash, MM & Shah, NP 2011a, 'Effect of partial substitution of NaCl with KCl on proteolysis of halloumi cheese', *Journal of Food Science*, vol. 76, pp. C31–C37.

Ayyash, MM & Shah, NP 2011b, 'The effect of substitution of NaCl with KCl on chemical composition and functional properties of low-moisture mozzarella cheese', *Journal of Dairy Science*, vol. 94, pp. 3761–3768.

Ayyash, MM & Shah, NP 2011c, 'Proteolysis of low-moisture mozzarella cheese as affected by substitution of NaCl with KCl', *Journal of Dairy Science*, vol. 94, pp. 3769–3777.

Ayyash, MM, Sherkat, F, Francis, P, Williams, RP & Shah, NP 2011, 'The effect of sodium chloride substitution with potassium chloride on texture profile and microstructure of halloumi cheese', *Journal of Dairy Science*, vol. 94, pp. 37–42.

Beeren, C 2013, Salt reduction. UK: Food science and technology information.

Belitz, HD & Kaiser, KP 1993, 'Monitoring Cheddar cheese ripening by chemical indices of proteolysis-3. Identification of several high-molecular mass peptides', *Zeitschrift fÃ¼r Lebensmittel-Untersuchung und -Forschung*, vol. 197, pp. 118–122.

Beresford, T & Williams, A 2004, 'The microbiology of cheese ripening', in PF Fox, PLH McSweeney, TM Cogan & TP Guinee (eds), *Cheese: chemistry, physics and microbiology*, Academic Press, London, UK.

Blaustein, MP, Iwamoto, T & Lingrel, JB 2004, *Scientists uncover how excess salt leads to hypertension*, viewed 25 March 2015, viewed 20-03-2015, http://umm.edu/news-and-events/news-releases/2004/scientists-uncover-how-excess-salt-leads-to-hypertension

Børsting, MW, Qvist, KB, Rasmussen, M, Vindeløv, J, Vogensen, FK & Ardö, Y 2012, 'Impact of selected coagulants and starters on primary proteolysis and amino acid release related to bitterness and structure of reduced-fat Cheddar cheese', *Dairy Science and Technology*, vol. 92, pp. 593–612.

Bryant, A, Ustunol, Z & Steffe, J 1995, 'Texture of Cheddar cheese as influenced by fat reduction', *Journal of Food Science*, vol. 60, pp. 1216–1219.

Buemi, M, Senatore, M, Corica, F, Aloisi, C, Romeo, A, Tramontana, D & Frisina, N 2002, 'Diet and arterial hypertension: is the sodium ion alone important?', *Medicinal Research Reviews*, vol. 22, pp. 419–428.

Buxbaum, E 2007, *Fundamentals of protein structure and function*, Sprinter Science, New York, US.

Califano, AN & Bevilacqua, AE 2000, 'Multivariate analysis of the organic acids content of gouda type cheese during ripening', *Journal of Food Composition and Analysis*, vol. 13, pp. 949–960.

Campbell, IL, Abraham, CR, Masliah, E, Kemper, P, Inglis, JD, Oldstone, MBA & Mucke, L 1993, 'Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin 6', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, pp. 10061–10065.

Castelli, H & Du Vale, L 2013, *Handbook on cheese: production, chemistry and sensory properties*, Nova Science Publishers Inc., New York, US.

Chamba, JF & Irlinger, F 2004, 'Secondary and adjunct cultures', in PF Fox, PLH McSweeney, TM Cogan & TP Guinee (eds), *Cheese: chemistry, physics and microbiology*, Academic Press, London, UK.

Chobanian, AV, Bakris, GL, Black, HR, Cushman, WC, Green, LA, Izzo Jr, JL, Jones, DW, Materson, BJ, Oparil, S, Wright Jr, JT & Roccella, EJ 2003, 'Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure', *Hypertension*, vol. 42, pp. 1206–1252.

Cliffe, AJ, Marks, JD & Mulholland, F 1993, 'Isolation and characterization of non-volatile flavours from cheese: peptide profile of flavour fractions from Cheddar cheese, determined by reverse-phase high-performance liquid chromatography', *International Dairy Journal*, vol. 3, pp. 379–387.

Cortez, MAS, Furtado, MM, Gigante, ML & Kindstedt, PS 2008, 'Effect of pH on characteristics of low-moisture mozzarella cheese during refrigerated storage', *Journal of Food Science*, vol. 73, pp. S443–S448.

Crabbe, MJC 2004, Rennets: general and molecular aspects. *Cheese: chemistry, physics and microbiology.*

Crank, J 1975, *The mathematics of diffusion*, Clarendon Press, Bristol, England.

Cushman, DW & Cheung, HS 1971, 'Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung', *Biochemical Pharmacology*, vol. 20, pp. 1637–1648.

Dairy Australia 2015, *Cheese*, viewed 20-08-2015, http://www.dairyaustralia.com.au/Markets-and-statistics/Production-and-sales/Cheese.aspx

Dalgleish, DG & Law, AJR 1989, 'pH-induced dissociation of bovine casein micelles. II. Mineral solubilization and its relation to casein release', *Journal of Dairy Research*, vol. 56, pp. 727–735.

Davidson, PM 2001, ‘Chemical preservatives and natural antimicrobial compounds’, in MP Doyle, LR Beauchat & TJ Montville (eds), *Food microbiology: fundamentals and frontiers*, ASM Press, Washington DC, US.

De Loubens, C, Panouillé, M, Saint-Eve, A, Déléris, I, Tréléa, IC & Souchon, I 2011, ‘Mechanistic model of in vitro salt release from model dairy gels based on standardized breakdown test simulating mastication’, *Journal of Food Engineering*, vol. 105, pp. 161–168.

Dimitreli, G & Thomareis, AS 2007, ‘Texture evaluation of block-type processed cheese as a function of chemical composition and in relation to its apparent viscosity’, *Journal of Food Engineering*, vol. 79, pp. 1364–1373.

Durack, E, Alonso-Gomez, M & Wilkinson, MG 2008, ‘Salt: a review of its role in food science and public health’, *Current Nutrition and Food Science*, vol. 4, pp. 290–297.

Duran-Meras, I, De La Pena, AM, Espinosa-Mansilla, A & Salinas, F 1993, ‘Multicomponent determination of flavour enhancers in food preparations by partial least squares and principal component regression modelling of spectrophotometric data’, *Analyst*, vol. 118, pp. 807–813.

Elliott, P & Brown, I 2006, ‘Sodium intakes around the world’, *WHO forum and technical meeting on reducing salt intake in populations*, viewed 10-06-2015, http://www.who.int/dietphysicalactivity/Elliot-brown-2007.pdf

Emmons, DB & Modler, HW 2010, ‘Invited review: a commentary on predictive cheese yield formulas’, *Journal of Dairy Science*, vol. 93, pp. 5517–5537.

Famelart, MH, Lepesant, F, Gaucheron, F, Le Graet, Y & Schuck, P 1996, ‘pH-induced physicochemical modifications of native phosphocaseinate suspensions: influence of aqueous phase’, *Lait*, vol. 76, pp. 445–460.

Farkye, NY, 2004, ‘Cheese technology’, *International Journal of Dairy Technology*, vol. 57, pp. 91–98.

Farkye, NY, Kiely, LJ, Allshouse, RD & Kindstedt, PS 1991, ‘Proteolysis in mozzarella cheese during refrigerated storage’, *Journal of Dairy Science*, vol. 74, pp. 1433–1438.

FDA 2014. *Nutrition labeling of food*, viewed 25-02-2015, http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?fr=101.9

Fenelon, MA, O'Connor, P & Guinee, TP 2000, 'The effect of fat content on the microbiology and proteolysis in Cheddar cheese during ripening', *Journal of Dairy Science*, vol. 83, pp. 2173–2183.

Fennema, OR 1996, *Food chemistry*, Marcel Dekker, New York, US.

Fernandes, R 2008, *Microbiology handbook, dairy products*, Leatherhead Food International Ltd, Cambridge, UK.

Flock, MR & Kris-Etherton, PM 2011, 'Dietary guidelines for Americans 2010: implications for cardiovascular disease', *Current Atherosclerosis Reports*, vol. 13, pp. 499–507.

Floury, J, Camier, B, Rousseau, F, Lopez, C, Tissier, JP & Famelart, MH 2009a, 'Reducing salt level in food: part 1. Factors affecting the manufacture of model cheese systems and their structure–texture relationships', *LWT—Food Science and Technology*, vol. 42, pp. 1611–1620.

Floury, J, Jeanson, S, Aly, S & Lortal, S 2010, 'Determination of the diffusion coefficients of small solutes in cheese: a review,' *Dairy Science and Technology*, vol. 90, pp. 477–508.

Floury, J, Rouaud, O, Le Poullennec, M & Famelart, MH 2009b, 'Reducing salt level in food: part 2. Modelling salt diffusion in model cheese systems with regards to their composition', *LWT—Food Science and Technology*, vol. 42, pp. 1621–1628.

Folkertsma, B & Fox, PF 1992, 'Use of the Cd-ninhydrin reagent to assess proteolysis in cheese during ripening', *Journal of Dairy Research*, vol. 59, pp. 217–224.

Forbes, RJ 1965, *Studies in ancient technology*, Brill, Leiden, The Netherlands.

Forde, A & Fitzgerald, GF 2000, 'Biotechnological approaches to the understanding and improvement of mature cheese flavour', *Current Opinion in Biotechnology*, vol. 11, pp. 484–489.

Fox, PF 1987, 'Significance of salt in cheese ripening', *Dairy Industries International*, vol. 52, pp. 19–22.

Fox, PF 1989, 'Proteolysis during cheese manufacture and ripening', *Journal of Dairy Science*, vol. 72, pp. 1379–1400.

Fox, PF & Brodkorb, A 2008, 'The casein micelle: historical aspects, current concepts and significance', *International Dairy Journal*, vol. 18, pp. 677–684.

Fox, PF & Guinee, TP 2013, 'Cheese science and technology', in *Milk and dairy products in human nutrition: production, composition and health*, John Wiley and Sons.

Fox, PF, Guinee, TP, Cogan, TM & McSweeney, PLH 2000a, *Fundamentals of cheese science*, Aspen Publishers Inc., Maryland, US.

Fox, PF, Guinee, TP, Cogan, TM & McSweeny, PLH 2000b, 'Starter culture', in P Fox & PLH McSweeney (eds), *Fundamentals of cheese science*, Aspen Publishers Inc., Gaithersburg, MD.

Fox, PF, Lucey, JA & Cogan, TM 1990, 'Glycolysis and related reactions during cheese manufacture and ripening', *Critical Reviews in Food Science and Nutrition*, vol. 29, pp. 237–253.

Fox, PF & McSweeney, PLH 1996, 'Proteolysis in cheese during ripening', *Food Reviews International*, vol. 12, pp. 457–509.

Fox, PF & McSweeney, PLH 2004, 'Cheese: an overview', in PF Fox, PLH McSweeney, TM Cogan & TP Guinee (eds), *Cheese: chemistry, physics and microbiology—general aspects*, 3rd edn, Elsevier Academic Press, London, UK.

Fox, PF, McSweeney, PLH, Cogan, TM & Guinee, TP 2004a, *Cheese: chemistry, physics, and microbiology*, Elsevier, London, New York.

Fox, PF, McSweeney, PLH, Cogan, TM & Guinee, TP 2004b, *Cheese chemistry, physics, and microbiology—volume 1: general aspects*, Elsevier, London, New York.

Fox, PF & Mulvihill, DM 1983, 'Functional properties of caseins, caseinates and casein and co-precipitates [production figures 1980, producing countries, physico-chemical, association, micelle, heat stability, solubility, gelation, coagulation, hydration, water sorption, milk protein, viscosity, interfaces]', in *Physico-chemical aspects of dehydrated protein-rich milk products*, Statens Forsoegsmejeri, Helsingoer, Denmark.

Ganesan, B, Brown, K, Irish, DA, Brothersen, C & McMahon, DJ 2014, 'Manufacture and sensory analysis of reduced- and low-sodium Cheddar and mozzarella cheeses', *Journal of Dairy Science*, vol. 97, pp. 1970–1982.

Geleijnse, JM, Witteman, JCM, Stijnen, T, Kloos, MW, Hofman, A & Grobbee, DE 2007, 'Sodium and potassium intake and risk of cardiovascular events and all-cause mortality: the Rotterdam study', *European Journal of Epidemiology*, vol. 22, pp. 763–770.

Geurts, TJ 1978, ‘Some factors which affect the moisture content of cheese before salting’, *Netherlands Milk and Dairy Journal*, vol. 32 (1978), pp. 112–124.

Geurts, TJ, Walstra, P & Mulder, H 1974, ‘Transport of salt and water during salting of cheese 1—analysis of the processes involved’, *Netherlands Milk and Dairy Journal*, vol. 28, pp. 102–129.

Geurts, TJ, Walstra, P & Mulder, H 1980, ‘Transport of salt and water during salting of cheese 2—quantities of salt taken up and of moisture lost’, *Netherlands Milk and Dairy Journal*, vol. 34, pp. 229–254.

Go, AS, Chertow, GM, Fan, D, McCulloch, CE & Hsu, CY 2004, ‘Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization’, *New England Journal of Medicine*, vol. 351, pp. 1296–1305+1370.

Gomes, AP, Cruz, AG, Cadena, RS, Celeghini, RMS, Faria, JAF, Bolini, HMA, Pollonio, MAR & Granato, D 2011, ‘Manufacture of low-sodium minas fresh cheese: effect of the partial replacement of sodium chloride with potassium chloride’, *Journal of Dairy Science*, vol. 94, pp. 2701–2706.

Gomez, MJ, Garde, S, Gaya, P, Medina, M & Nuñez, M 1997, ‘Relationship between level of hydrophobic peptides and bitterness in cheese made from pasteurized and raw milk’, *Journal of Dairy Research*, vol. 64, pp. 289–297.

Grappin, R, Rank, TC & Olson, NF 1985, ‘Primary proteolysis of cheese proteins during ripening: a review’, *Journal of Dairy Science*, vol. 68, pp. 531–540.

Gros, JB & Ruegg, M 1987, ‘Determination of the apparent diffusion coefficient of NaCl in model foods and cheese’, in R Jowitt, F Escher, M Kent, B Mckenna, & M Roques (eds.), *Physical Properties of Foods*, Elsevier, London.

Grummer, J, Bobowski, N, Karalus, M, Vickers, Z & Schoenfuss, T 2013, ‘Use of potassium chloride and flavor enhancers in low sodium Cheddar cheese’, *Journal of Dairy Science*, vol. 96, pp. 1401–1418.

Grummer, J, Karalus, M, Zhang, K, Vickers, Z & Schoenfuss, TC 2012, ‘Manufacture of reduced-sodium Cheddar-style cheese with mineral salt replacers’, *Journal of Dairy Science*, vol. 95, pp. 2830–2839.

Grummer, J & Schoenfuss, TC 2011, ‘Determining salt concentrations for equivalent water activity in reduced-sodium cheese by use of a model system’, *Journal of Dairy Science*, vol. 94, pp. 4360–4365.

Guerreiro, JS, Fernandes, P & Bardsley, RG 2013, 'Cheese ripening and proteolysis', in H. Castelli & L. du Vale (eds.), *Handbook on cheese: production, chemistry and sensory properties*, Nova Science Publishers Inc., New York.

Guinee, TP 2004a, 'Salting and the role of salt in cheese', *International Journal of Dairy Technology*, vol. 57, pp. 99–109.

Guinee, TP 2004b, 'Scrap the salt', *Dairy Industries International*, vol. 69, pp. 36–38.

Guinee, TP, Auty, MAE, Mullins, C, Corcoran, MO & Mulholland, EO 2000, 'Preliminary observations on effects of fat content and degree of fat emulsification on the structure-functional relationship of Cheddar-type cheese', *Journal of Texture Studies*, vol. 31, pp. 645–663.

Guinee, TP & Fox, PF 1983, 'Sodium chloride and moisture changes in Romano-type cheese during salting', *Journal of Dairy Research*, vol. 50, pp. 511–518.

Guinee, TP & Fox, PF 1986, 'Transport of sodium chloride and water in Romano cheese slices during brining', *Food Chemistry*, vol. 19, pp. 49–64.

Guinee, TP & Fox, PF 2004, 'Salt in cheese: physical, chemical and biological aspects', in *Cheese: chemistry, physics and microbiology—general aspects*, 3rd edn, Academic Press, London, UK.

Guinee, TP & O'Kennedy, BT 2007, 'Reducing salt in cheese and dairy spreads', in D Kilcast & F Angus (eds), *Reducing salt in foods: practical strategies*, CRC Press LLC, Washington, DC, US.

Guinne, TP & Fox, PF 1984, 'Salt in cheese: physical, chemical and biological aspects', *Cheese: Chemistry, Physics and Microbiology*, vol. 1, pp. 31–35.

Gunasekaran, S & Ak, MM 2003, *Cheese rheology and texture*, CRC Press, Boca Raton, FL.

Guo, MR, Gilmore, JA & Kindstedt, PS 1997, 'Effect of sodium chloride on the serum phase of mozzarella cheese', *Journal of Dairy Science*, vol. 80, pp. 3092–3098.

Guo, MR & Kindstedt, PS 1995, 'Age-related changes in the water phase of mozzarella cheese', *Journal Dairy Science*, vol. 78, pp. 2099–2107.

Guven, M & Karaca, OB 2001, 'Proteolysis levels of white cheeses salted and ripened in brines prepared from various salts', *International Journal of Dairy Technology*, vol. 54, pp. 29–33.

Hall, JE, Brands, MW & Henegar, JR 1999, 'Mechanisms of hypertension and kidney disease in obesity', *Annals of the New York Academy of Sciences*, vol. 18, pp. 91–107.

Halmos, AL, Pollard, A, Sherkat, F & Seuret, MG 2003, 'Natural Cheddar cheese texture variation as a result of milk seasonality', *Journal of Texture Studies*, vol. 34, pp. 21–40.

Hassan, AV & Lucey, JA 2001, 'Characterization of the melt properties of Cheddar cheese during aging using dynamic low amplitude oscillatory rheology and melt profile analysis', *Journal of Dairy Science*, vol. 84, p. 145.

He, FJ & Macgregor, GA 2007, 'Dietary salt, high blood pressure and other harmful effects on health', in D Kilcast & F Angus (eds), *Reducing salt in foods: practical strategies*, CRC Press LLC, Washington, DC, US.

He, FJ & Macgregor, GA 2010, 'Reducing population salt intake worldwide: from evidence to implementation', *Progress in Cardiovascular Diseases*, vol. 52, pp. 363–382.

Heaney, RP 2006, 'Role of dietary sodium in osteoporosis', *Journal of the American College of Nutrition*, vol. 25, pp. 271–276.

Hennelly, PJ, Dunne, PG, O'Sullivan, M & O'Riordan, D 2005, 'Increasing the moisture content of imitation cheese: effects on texture, rheology and microstructure', *European Food Research and Technology*, vol. 220, pp. 415–420.

Henney, JE, Taylor, CL & Boon, CS 2010, *Strategies to reduce sodium intake in the United States*, National Academies Press, Washington DC, US.

Hoare, J, Ruston, D & Henderson, L 2003, 'The national diet and nutrition survey: adults aged 19 to 64 years', in *The National Diet and Nutrition Survey*, vol. 3, London, UK, viewed, 10-02-2015, http://www.food.gov.uk/multimedia/pdfs/ndnsv3.pdf

Irudayaraj, J, Chen, M & McMahon, DJ 1999, 'Texture development in Cheddar cheese during ripening', *Canadian biosystems engineering/Le Genie des biosystems au Canada*, vol. 41, pp. 253–258.

Irvine, DM & Price, WV 1961, 'Influence of salt on the development of acid by lactic starters in skimmilk and in curd submerged in brine', *Journal of Dairy Science*, vol. 44, pp. 243–248.

Janitha, PK, Wanasundara, PD, Ross, ARS, Amarowlcz, R, Ambrose, SJ, Pegg, RB & Shand, PJ 2002, 'Peptides with angiotensin I-converting enzyme (ACE) inhibitory activity from defibrinated, hydrolyzed bovine plasma', *Journal of Agricultural and Food Chemistry*, vol. 50, pp. 6981–6988.

Joossens, JV, Hill, MJ, Elliott, P, Stamler, R, Stamler, J, Lesaffre, E, Dyer, A, Nichols, R & Kesteloot, H 1996, 'Dietary salt, nitrate and stomach cancer mortality in 24 countries', *International Journal of Epidemiology*, vol. 25, pp. 494–504.

Jordan, KN & Cogan, TM 1993, 'Identification and growth of non-starter lactic acid bacteria in Irish Cheddar cheese', *Irish Journal of Agricultural and Food Research*, vol. 32, pp. 47–55.

Kaiser, KP, Belitz, HD & Fritsch, RJ 1992, 'Monitoring Cheddar cheese ripening by chemical indices of proteolysis—2: peptide mapping of casein fragments by reverse-phase high-performance liquid chromatography', *Zeitschrift für Lebensmittel-Untersuchung und -Forschung*, vol. 195, pp. 8–14.

Kaminarides, S, Stamou, P & Massouras, T 2007, 'Changes of organic acids, volatile aroma compounds and sensory characteristics of halloumi cheese kept in brine', *Food Chemistry*, vol. 100, pp. 219–225.

Katsiari, MC, Alichanidis, E, Voutsinas, LP & Roussis, IG 2000a, 'Proteolysis in reduced sodium feta cheese made by partial substitution of NaCl by KCl', *International Dairy Journal*, vol. 10, pp. 635–646.

Katsiari, MC, Voutsinas, LP, Alichanidis, E & Roussis, IG 1997, 'Reduction of sodium content in feta cheese by partial substitution of NaCl by KCl', *International Dairy Journal*, vol. 7, pp. 465–472.

Katsiari, MC, Voutsinas, LP, Alichanidis, E & Roussis, IG 1998, 'Manufacture of kefalograviera cheese with less sodium by partial replacement of NaCl with KCl', *Food Chemistry*, vol. 61, pp. 63–70.

Katsiari, MC, Voutsinas, LP, Alichanidis, E & Roussis, IG 2000b, 'Lipolysis in reduced sodium feta cheese made by partial substitution of NaCl by KCl', *International Dairy Journal*, vol. 10, pp. 369–373.

Katsiari, MC, Voutsinas, LP, Alichanidis, E & Roussis, IG 2000c, 'Proteolysis in reduced-sodium feta cheese made by partial substitution of NaCl by KCl', *International Dairy Journal*, vol. 10, pp. 635–646.

Katsiari, MC, Voutsinas, LP, Alichanidis, E & Roussis, IG 2001a, 'Lipolysis in reduced sodium kefalograviera cheese made by partial replacement of NaCl with KCl', *Food Chemistry*, vol. 72, pp. 193–197.

Katsiari, MC, Voutsinas, LP, Alichanidis, E & Roussis, IG 2001b, 'Proteolysis in reduced sodium kefalograviera cheese made by partial replacement of NaCl with KCl', *Food Chemistry*, vol. 73, pp. 31–43.

Kaya, S 2002, ‘Effect of salt on hardness and whiteness of Gaziantep cheese during short-term brining’, *Journal of Food Engineering*, vol. 52, pp. 155–159.

Kelly, M, Fox, PF & McSweeney, PLH 1996, ‘Effect of salt-in-moisture on proteolysis in Cheddar-type cheese’, *Milchwissenschaft*, vol. 51, pp. 498–501.

Kilcast, D & Angus, F 2007, *Reducing salt in foods: practical strategies*, Woodhead Publishing Limited, Cambridge, England.

Kilic, M & Isin, TG 2004, ‘Effects of salt level and storage on texture of Dil cheese’, *Journal of Texture Studies*, vol. 35, pp. 251–262.

Kongo, JM 2013, ‘Lactic acid bacteria as starter-cultures for cheese processing: past, present and future developments’, *Lactic Acid Bacteria—R & D for Food, Health and Livestock Purposes.* InTech.

Kosikowski, FV 1977, ‘Cheddar cheese and related types’, in *Cheese and fermented milk foods*, Edward Brothers Inc., Ann Arbor, MI, US.

Kotchen, TA 2005, ‘Contributions of sodium and chloride to NaCl-induced hypertension’, *Hypertension*, vol. 45, pp. 849–850.

Kubiš, I, Sousa, MJ, Walsh-O’Grady, D, Kelly, AL & McSweeney, PLH 2001, ‘Proteolysis in Cheddar-type cheese made from goat’s milk’, *Milchwissenschaft*, vol. 56, pp. 557–560.

Kuchroo, CN & Fox, PF 1982, ‘Soluble nitrogen in Cheddar cheese: comparison of extraction procedures’, *Milchwissenschaft*, vol. 53, pp. 262–269.

Kumar, S, Kanawjia, SK & Kumar, S 2011, ‘The effect of varying casein/fat ratio on physicochemical and sensory qualities of Feta-type cheese made using buffalo milk’, International Journal of Dairy Technology, vol. 64, pp. 380-385.

LK, M 1995, ‘Dietary salt, urinary calcium, and kidney stone risk’, *Nutrition Reviews*, vol. 53, pp. 131–134.

Laborda, MA & Rubiolo, AC 1999, ‘Proteolysis of Fynbo cheese salted with NaCl/KCl and ripened at two temperatures’, *Journal of Food Science*, vol. 64, pp. 33–36.

Lambert, R & Hainaut, P 2007, ‘Epidemiology of oesophagogastric cancer’, *Best Practice and Research: Clinical Gastroenterology*, vol. 21, pp. 921–945.

Lau, KY, Barbano, DM & Rasmussen, RR 1991, ‘Influence of pasteurization of milk on protein breakdown in Cheddar cheese during aging’, *Journal of Dairy Science*, vol. 74, pp. 727–740.

Law, BA 1997, *Microbiology and biochemistry of cheese and fermented milk*, Springer, US.

Lawless, HT, Rapacki, F, Horne, J & Hayes, A 2003, 'The taste of calcium and magnesium salts and anionic modifications', *Food Quality and Preference*, vol. 14, pp. 319–325.

Lawrence, G, Buchin, S, Achilleos, C, Bérodier, F, Septier, C, Courcoux, P & Salles, C 2012, 'In vivo sodium release and saltiness perception in solid lipoprotein matrices 1: effect of composition and texture', *Journal of Agricultural and Food Chemistry*, vol. 60, pp. 5287–5298.

Lawrence, RC, Creamer, LK & Gilles, J 1987, 'Texture development during cheese ripening', *Journal of Food Science*, vol. 49, pp. 1098–1101.

Lawrence, RC, Gilles, J, Creamer, LK, Crow, VL, Heap, HA, Honoré, CG, Johnston, KA & Samal, PK 2004, 'Cheddar cheese and related dry-salted cheese varieties', in PF Fox, PLH McSweeney, TM Cogan & TP Guinee (eds), *Cheese: chemistry, physics and microbiology*, Academic Press, London, UK.

Lefier, D, Grappin, R, Grosclaude, G & Curtat, G 1987, 'Sensory properties and nutritional quality of low-sodium Gruyere cheese', *Lait*, vol. 67, pp. 451–463.

Liem, DG, Miremadi, F & Keast, RSJ 2011, 'Reducing sodium in foods: the effect on flavor', *Nutrients*, vol. 3, pp. 694–711.

Lin, PH, Ginty, F, Appel, LJ, Aickin, M, Bohannon, A, Garnero, P, Barclay, D & Svetkey, LP 2003, 'The DASH diet and sodium reduction improve markers of bone turnover and calcium metabolism in adults', *Journal of Nutrition*, vol. 133, pp. 3130–3136.

Lucey, JA, Johnson, ME & Horne, DS 2003, 'Invited review: perspectives on the basis of the rheology and texture properties of cheese', *Journal of Dairy Science*, vol. 86, pp. 2725–2743.

Lucey, JA & Singh, H 1997, 'Formation and physical properties of acid milk gels: a review', *Food Research International*, vol. 30, pp. 529–542.

Maldonado, R, Melendez, B, Arispe, I, Boeneke, C, Torrico, D & Prinyawiwatkul, W 2013, 'Effect of pH on technological parameters and physicochemical and texture characteristics of the pasta filata cheese Telita', *Journal of Dairy Science*, vol. 96, pp. 7414–7426.

Martens, R, Vanderpoorten, R & Naudts, M 1976, 'Production, composition and properties of low-sodium gouda cheese', *Revue de l'Agriculture*, vol. 29, pp. 681–698.

Massey, LK 2005, ‘Effect of dietary salt intake on circadian calcium metabolism, bone turnover, and calcium oxalate kidney stone risk in postmenopausal women’, *Nutrition Research*, vol. 25, pp. 891–903.

Massey, LK & Whiting, SJ 1995, ‘Dietary salt, urinary calcium, and kidney stone risk’, *Nutrition Reviews*, vol. 53, pp. 131–139.

Massey, LK & Whiting, SJ 1996, ‘Dietary salt, urinary calcium, and bone loss’, *Journal of Bone and Mineral Research*, vol. 11, pp. 731–736.

McCarron, DA, Geerling, JC, Kazaks, AG & Stern, JS 2009, ‘Can dietary sodium intake be modified by public policy?’, *Clinical Journal of the American Society of Nephrology*, vol. 4, pp. 1878–1882.

McIntosh, GH, Royle, PJ & Playne, MJ 1999, ‘A probiotic strain of L. Acidophilus reduces DMH-induced large intestinal tumors in male Sprague-Dawley rats’, *Nutrition and Cancer*, vol. 35, pp. 153–159.

McMahon, DJ 2010, ‘Issues with lower fat and lower salt cheeses’, *Australian Journal of Dairy Technology*, vol. 65, pp. 200–205.

McMahon, DJ, Motawee, MM & McManus, WR 2009, ‘Influence of brine concentration and temperature on composition, microstructure, and yield of feta cheese’, *Journal of Dairy Science*, vol. 92, pp. 4169–4179.

McSweeney, PLH 2007, *Cheese problems solved*, Woodhead Publishing Limited, Cambridge, England.

McSweeney, PLH & Fox, PF 1997, ‘Chemical methods for the characterization of proteolysis in cheese during ripening’, *Dairy Science and Technology*, vol. 77, pp. 41–76.

McSweeney, PLH & Sousa, MJ 2000, ‘Biochemical pathways for the production of flavour compounds in cheeses during ripening: a review’, *Lait*, vol. 80, pp. 293–324.

Meneton, P, Lafay, L, Tard, A, Dufour, A, Ireland, J, Ménard, J & Volatier, JL 2009, ‘Dietary sources and correlates of sodium and potassium intakes in the French general population’, *European Journal of Clinical Nutrition*, vol. 63, pp. 1169–1175.

Merćep, A, Kirin, S, Zdolec, N, Fleck, ZC, Filipović, I, Njari, B, Mitak, M & Kozačinski, L 2010, ‘Quality of Trappist cheese from Croatian dairy plant’, *Mljekarstvo*, vol. 60, pp. 288–298.

Mistry, VV & Kasperson, KM 1998, 'Influence of salt on the quality of reduced fat Cheddar cheese', *Journal of Dairy Science*, vol. 81, pp. 1214–1221.

Moller, KK, Rattray, FP, Bredie, WLP, Hoier, E & Ardö, Y 2013, 'Physicochemical and sensory characterization of Cheddar cheese with variable NaCl levels and equal moisture content', *Journal of Dairy Science*, vol. 96, pp. 1953–1971.

Morris, HA, Guinee, TP & Fox, PF 1985, 'Salt diffusion in Cheddar cheese', *Journal of Dairy Science*, vol. 68, pp. 1851–1858.

Murtaza, MA, Huma, N, Sameen, A, Murtaza, MS, Mahmood, S, Mueen-Ud-Din, G & Meraj, A 2014, 'Texture, flavor, and sensory quality of buffalo milk Cheddar cheese as influenced by reducing sodium salt content', *Journal of Dairy Science*, vol. 97, pp. 6700–6707.

Murtaza, MA, Rehman, SU, Anjum, FM, Huma, N, Tarar, OM & Mueen-Ud-Din, G 2012, 'Organic acid contents of buffalo milk Cheddar cheese as influenced by accelerated ripening and sodium salt', *Journal of Food Biochemistry*, vol. 36, pp. 99–106.

Nateghi, L 2012, 'Effects of different adjunct starter cultures on proteolysis of reduced fat Cheddar cheese during ripening', *African Journal of Biotechnology*, vol. 11, pp. 12491–12499.

NHMRC 2003, *Dietary guidelines for children and adolescents in Australia*, viewed 10-04-2003, http://www.nhmrc.gov.au/_files_nhmrc/file/publications/synopses/n34.pdf

NHMRC 2005, *Nutrient reference values for Australia and New Zealand*, viewed 09-09-2015, http://www.nhmrc.gov.au/_files_nhmrc/publications/attachments/n35.pdf

NHMRC 2013, *Australian dietary guidelines*, Author, Canberra.

Nolan, D 2009, 'International Dairy Federation/Dairy Industry Association of Australia functional dairy foods conference: 24–25 February 2009, Melbourne, Australia', *Nutrition and Dietetics*, vol. 66, pp. 121–122.

Noort, MWJ, Bult, JHF, Stieger, M & Hamer, RJ 2010, 'Saltiness enhancement in bread by inhomogeneous spatial distribution of sodium chloride', *Journal of Cereal Science*, vol. 52, pp. 378–386.

O'Callaghan, DJ & Guinee, TP 2004, 'Rheology and texture of cheese', in PF Fox, PLH McSweeney, TM Cogan & TP Guinee (eds), *Cheese: chemistry, physics and microbiology—general aspects*, vol. 1, Elsevier Academic Press, London, UK.

O'Shaughnessy, KM & Karet, FE 2006, 'Salt handling and hypertension', *Annual Review of Nutrition*, vol. 26, pp. 343–365.

Ong, L, Dagastine, RR, Kentish, SE & Gras, SL 2013, 'Microstructure and composition of full fat Cheddar cheese made with ultrafiltered milk retentate', *Foods*, vol. 2, pp. 310–331.

Ong, L & Shah, NP 2008a, 'Influence of probiotic Lactobacillus acidophilus and L. helveticus on proteolysis, organic acid profiles, and ACE-inhibitory activity of Cheddar cheeses ripened at 4, 8, and 12°C', *Journal of Food Science*, vol. 73, pp. M111–M120.

Ong, L & Shah, NP 2008b, 'Release and identification of angiotensin-converting enzyme-inhibitory peptides as influenced by ripening temperatures and probiotic adjuncts in Cheddar cheeses', *LWT—Food Science and Technology*, vol. 41, pp. 1555–1566.

Otten, J, Hellwig, J & Meyers, L 2006, *Dietary reference intakes: the essential guide to nutrient requirements*, National Academies Press, Washington DC, US, viewed 15-04-2014, http://www.nap.edu/catalog/11537.html

Pajonk, AS, Saurel, R & Andrieu, J 2003, 'Experimental study and modeling of effective NaCl diffusion coefficients values during Emmental cheese brining', *Journal of Food Engineering*, vol. 60, pp. 307–313.

Parente, E & Cogan, TM 2004a, 'Starter cultures: general aspects', in PF Fox, PLH McSweeney, TM Cogan & TP Guinee (eds), *Cheese: chemistry, physics and microbiology, —general aspects*, vol. 1, Academic Press, London, UK.

Paulsan, BM, McMahon, DJ & Oberg, CJ 1998, 'Influence of sodium chloride on appearance, functionality and protein arrangements in nonfat mozzarella cheese', *Journal of Dairy Science*, vol. 81, pp. 2053–2064.

Penner, SB, Campbell, NRC, Chockalingam, A, Zarnke, K & Van Vliet, B 2007, 'Dietary sodium and cardiovascular outcomes: a rational approach', *Canadian Journal of Cardiology*, vol. 23, pp. 567–572.

Phan, VA, Yven, C, Lawrence, G, Chabanet, C, Reparet, JM & Salles, C 2008, 'In vivo sodium release related to salty perception during eating model cheeses of different textures', *International Dairy Journal*, vol. 18, pp. 956–963.

Phelan, JA, Guiney, J & Fox, PF 1973, 'Proteolysis of β-casein in Cheddar cheese', *Journal of Dairy Research*, vol. 40, pp. 105–112.

Pollard, A, Sherkat, F, Seuret, MG & Halmos, AL 2003, 'Textural changes of natural Cheddar cheese during the maturation process', *Journal of Food Science*, vol. 68, pp. 2011–2016.

Pons, M & Fiszman, SM 1996, 'Instrumental texture profile analysis with particular reference to gelled systems', *Journal of Texture Studies*, vol. 27, pp. 597–624.

Prasad, N & Alvarez, VB 1999, 'Effect of salt and chymosin on the physico-chemical properties of feta cheese during ripening', *Journal of Dairy Science*, vol. 82, pp. 1061–1067.

Pyne, GT & McGann, TCA 1960, 'The colloidal phosphate of milk II: influence of citrate', *Journal of Dairy Research*, vol. 27, pp. 9–17.

Rawson, NE & Li, X 2007, 'The cellular basis of flavour perception: taste and aroma', in *Flavor Perception*, Blackwell Publishing Ltd.

Reddy, KA & Marth, EH 1991, 'Reducing the sodium content of foods: a review', *Journal of Food Protection*, vol. 54, pp. 138–150.

Reddy, KA & Marth, EH 1993a, 'Composition of Cheddar cheese made with sodium chloride and potassium chloride either singly or as mixtures', *Journal of Food Composition and Analysis*, vol. 6, pp. 354–363.

Reddy, KA & Marth, EH 1993b, 'Proteolysis in Cheddar cheese made with sodium chloride, potassium chloride or mixtures of sodium and potassium chloride', *LWT—Food Science and Technology*, vol. 26, pp. 434–442.

Roefs, SPFM, Walstra, P, Dalgleish, DG & Horne, DS 1985, 'Preliminary note on the change in casein micelles caused by acidification', *Netherlands Milk and Dairy Journal*, vol. 39, pp. 119–122.

Rowney, MK, Roupas, P, Hickey, MW & Everett, DW 2004, 'Salt-induced structural changes in 1-day old mozzarella cheese and the impact upon free oil formation', *International Dairy Journal*, vol. 14, pp. 809–816.

Rulikowska, A, Kilcawley, KN, Doolan, IA, Alonso-Gomez, M, Nongonierma, AB, Hannon, JA & Wilkinson, MG 2013, 'The impact of reduced sodium chloride content on Cheddar cheese quality', *International Dairy Journal*, vol. 28, pp. 45–55.

Russell, NJ & Gould, GW 2003, *Food preservatives*, Springer-Verlag Inc., New York.

Sadat-Mekmene, L, Richoux, R, Aubert-Frogerais, L, Madec, MN, Corre, C, Piot, M, Jardin, J, Le Feunteun, S, Lortal, S & Gagnaire, V 2013, 'Lactobacillus

helveticus as a tool to change proteolysis and functionality in Swiss-type cheeses', *Journal of Dairy Science*, vol. 96, pp. 1455–1470.

SAS Institute Inc. 2008, *SAS/STAT ® 9.2 user's guide*. Author, Cary, NC, US.

Sato, RT, Vieira, ATB, Camisa, J, Vianna, PCB & De Rensis, CMVB 2012, 'Assessment of proteolysis and sensory characteristics of Prato cheese with adjunct culture', *Semina: Ciencias Agrarias*, vol. 33, pp. 3143–3152.

Schiffrin, EL, Lipman, ML & Mann, JFE 2007, 'Chronic kidney disease: effects on the cardiovascular system', *Circulation Research*, vol. 116, pp. 85–97.

Schroeder, CL, Bodyfelt, FW, Wyatt, CJ & McDaniel, MR 1988, 'Reduction of sodium chloride in Cheddar cheese: effect on sensory, microbiological, and chemical properties', *Journal of Dairy Science*, vol. 71, pp. 2010–2020.

Sheehan, JJ & Guinee, TP 2004, 'Effect of pH and calcium level on the biochemical, textural and functional properties of reduced-fat mozzarella cheese', *International Dairy Journal*, vol. 14, pp. 161–172.

Sheibani, A, Mishra, V, Stojanovska, L & Ayyash, MM 2013b, 'Salt in cheese: health issues, reduction, replacement and release', in *Handbook on cheese: production, chemistry and sensory properties*, Nova Science Publishers Inc.

Shikata, K, Kiyohara, Y, Kubo, M, Yonemoto, K, Ninomiya, T, Shirota, T, Tanizaki, Y, Doi, Y, Tanaka, K, Oishi, Y, Matsumoto, T & Iida, M 2006, 'A prospective study of dietary salt intake and gastric cancer incidence in a defined Japanese population: the Hisayama study', *International Journal of Cancer*, vol. 119, pp. 196–201.

Sieber, R, Bütikofer, U, Egger, C, Portmann, R, Walther, B & Wechsler, D 2009, 'ACE-inhibitory activity and ACE-inhibiting peptides in different cheese varieties', *Dairy Science and Technology*, vol. 90, pp. 47–73.

Silva, JG, Morais, HA & Silvestre, MPC 2003, 'Comparative study of the functional properties of bovine globin isolates and sodium caseinate', *Food Research International*, vol. 36, pp. 73–80.

Simal, S, Sánchez, ES, Bon, J, Femenia, A & Rosselló, C 2001, 'Water and salt diffusion during cheese ripening: effect of the external and internal resistances to mass transfer', *Journal of Food Engineering*, vol. 48, pp. 269–275.

Singh, TK & Cadwallader, KR 2008, 'Cheese' in RC Chandan, A Kilara & NP Shah (eds), *Dairy processing and quality assurance*, Willey-Blackwell, Iowa, US.

Singh, TK, Drake, MA & Cadwallader, KR 2003, 'Flavor of Cheddar Cheese: A Chemical and Sensory Perspective', *Comprehensive Reviews in Food Science and Food Safety*, vol. 2, pp. 166–189.

Soodam, K, Ong, L, Powell, IB, Kentish, SE & Gras, SL 2015, 'Effect of rennet on the composition, proteolysis and microstructure of reduced-fat Cheddar cheese during ripening', *Dairy Science and Technology*, vol. 95, pp. 665–686.

Sousa, MJ, Ardö, Y & McSweeney, PLH 2001, 'Advances in the study of proteolysis during cheese ripening', *International Dairy Journal*, vol. 11, pp. 327–345.

Strazzullo, P, D'elia, L, Kandala, NB & Cappuccio, FP 2009, 'Salt intake, stroke, and cardiovascular disease: meta-analysis of prospective studies', *BMJ (Online)*, vol. 339, p. 1296.

Taylor, AJ & Roberts, DD 2004, *Flavor perception*, Blackwell Publishing, Oxford, UK.

Tharmaraj, N & Shah, NP 2003, 'Selective enumeration of Lactobacillus delbrueckii ssp. bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus, bifidobacteria, Lactobacillus casei, Lactobacillus rhamnosus, and propionibacteria', *Journal of Dairy Science*, vol. 86, pp. 2288–2296.

Tunick, MH, Guinee, TP, Van Hekken, DL, Beresford, TP & Malin, EL 2007, 'Effect of whey drainage pH on composition, rheology, and melting properties of reduced-fat Cheddar cheese', *Milchwissenschaft*, vol. 62, pp. 443–446.

Turhan, M 1996, 'Modelling of salt transfer in white cheese during short initial brining', *Netherlands Milk and Dairy Journal*, vol. 50, pp. 541–550.

Turhan, M & Gunasekaran, S 1999, 'Analysis of moisture transfer in white cheese during brining', *Milchwissenschaft*, vol. 54, pp. 446–450.

Turhan, M & Kaletunç, G 1992, 'Modeling of salt diffusion in white cheese during long-term brining', *Journal of Food Science*, vol. 57, pp. 1082–1085.

Turk, MW, Tuite, PK & Burke, LE 2009, 'Cardiac health: primary prevention of heart disease in women', *Nursing Clinics of North America*, vol. 44, pp. 315–325.

Upadhyay, VK, McSweeney, PLH, Magboul, AAA & Fox, PF 2004, 'Proteolysis in cheese during ripening', in PF Fox, PLH McSweeney, TM Cogan & PG Timothy (eds), *Cheese: chemistry, physics and microbiology*, 3rd edn, Elsevier Academic Press, London, UK.

Upreti, P, McKay, LL & Metzger, LE 2006, 'Influence of calcium and phosphorus, lactose, and salt-to-moisture ratio on Cheddar cheese quality: changes in residual

sugars and water-soluble organic acids during ripening', *Journal of Dairy Science*, vol. 89, pp. 429–443.

Van Der Klaauw, NJ & Smith, DV 1995, 'Taste quality profiles for fifteen organic and inorganic salts', *Physiology and Behavior*, vol. 58, pp. 295–306.

Van Ruth, SM, Roozen, JP & Cozijnsen, JL 1994, 'Comparison of dynamic headspace mouth model systems for flavour release from rehydrated bell pepper cuttings', in *Trends in flavour research*, Elsevier Science, Amsterdam, The Netherlands.

Vélez-Ruiz, JF 2009, 'Rheology and texture of cheese', in *Food Processing and Engineering Topics*, Nova Science Publishers Inc.

Walker, GP, Williams, R, Doyle, PT & Dunshea, FR 2007, 'Seasonal variation in milk production and cheese yield from commercial dairy farms located in northern Victoria is associated with pasture and grazing management and supplementary feeding practices', *Australian Journal of Experimental Agriculture*, vol. 47, pp. 509–524.

Wang, L, Zollinger, T & Zhang, J 2013, 'Association between Helicobacter pylori infection and liver cancer mortality in 67 rural Chinese counties', *Cancer Causes and Control*, vol. 24, pp. 1331–1337.

WHO 2005, *Preventing chronic diseases: a vital investment*, viewed 22-03-2015, http://whqlibdoc.who.int/publications/2005/9241563001_eng.pdf

WHO 2007, *Reducing salt intake in populations*, viewed 10-08-2014, http://www.who.int/dietphysicalactivity/reducingsaltintake_EN.pdf

WHO 2007, *Reducing salt intake in populations*, viewed 10-08-2014, http://www.who.int/dietphysicalactivity/Salt_Report_VC_april07.pdf

Yun, JJ, Barbano, DM, Kindstedt, PS & Larose, KL 1995, 'Mozzarella cheese: impact of whey pH at draining on chemical composition, proteolysis, and functional properties, *Journal of Dairy Science*, vol. 78, pp. 1–7.

Zorrilla, SE & Rubiolo, AC 1999, 'Note: sensory analysis during ripening of Fynbo cheese salted with NaCl/KCl brine', *Food Science and Technology International*, vol. 5, pp. 251–254.

Appendices

Appendix 1: Sensory Evaluation Information Sheet

INFORMATION TO PARTICIPANTS INVOLVED IN RESEARCH

You are invited to participate

You are invited to participate in a research project entitled **'The Effects of Salt Reduction on the Physicochemical and Microbiological Properties and Salt Release from Cheddar Cheese'.**

This project is being conducted by a student researcher, **ALI SHEIBANI**, as part of a PhD study at Victoria University under the supervision of **Dr Vijay Mishra** from Biomedical and Health Sciences, Faculty of Health, Engineering and Sciences.

Project explanation

The project is going to reduce the salt concentration in Cheddar cheese by 1.5% and then analyse all related aspects that might be affected. The chemical composition, texture, microbiology and release of salt from cheese are going to be analysed. A sensory evaluation is going to be held containing 10 to 12 people to achieve deeper knowledge of the texture, flavour and aroma of salt-reduced cheeses. The project is aiming to find a way to reduce the daily salt intake by people, and consequently reduce salt-associated diseases, such as hypertension, kidney stone and osteoporosis.

What will I be asked to do?

---

1. Complete sensory evaluation for four different cheese samples salted at two different levels.
2. Fill out the sensory evaluation (questionnaire) form and mark the score according to your taste.

What will I gain from participating?

Data will be collected from participants that will provide critical and important information about our treatment. These descriptive data will provide valuable information to the researcher about whether salt reduction negatively or positively affects the sensory attributes of cheese.

How will the information I give be used?

The data will be entered in statistical analysis software, which allows the researcher to develop results and then compare the panellists' results.

What are the potential risks of participating in this project?

Participants with any of the following **ARE NOT** able to contribute to this study:

- lactose intolerance
- allergy to milk or cheese proteins
- hypertension disorder
- osteoporosis
- kidney stones
- cardiovascular diseases
- any health problem potentially affected by eating cheeses or dairy products.

It is the participant's responsibility to know whether he or she has one of the above disorders. The participant will be interviewed to confirm that he or she has no diseases that may be affected by our experiment. The participant will be asked to sign the consent form, which will include the previous awareness. For any risk that may arise during the cheese sensory evaluation, a first aid procedure will be implemented immediately by well-trained staff. Emergency help (calling 000) will be immediately accessed.

How will this project be conducted?

1. The panellists familiar with basic sensory evaluation techniques for Cheddar cheese and will be further trained for their ability to detect flavours and sour-acid, bitter and vinegary tastes.
2. Prior to sensory evaluation, you will participate in briefing sessions. All panellists will sign a Victoria University human subject's consent form.

3. Sensory evaluation will be conducted for the cheeses after ripening for one month. The cheese samples will be removed from the refrigerator and cut into pieces (about 1.5 × 1.5 × 1.5 cm in size) and placed on white plates coded with random three-digit numbers one hour prior to evaluation at room temperature (25ºC).
4. Several variations of cheeses will be presented to the panellists in random order over one day. The sensory evaluation will be repeated the following week, which will be as replicates of the cheeses will be presented.
5. Panellists will have access to deionised water and unsalted crackers to help cleanse their palates.
6. Prior to tasting, panellists will complete a questionnaire on the frequency of cheese consumption (< 1 or once per week, 2 to 3 times per week, 4 to 5 times per week, or > 5 times per week) and cheese preference (mild, medium or sharp matured cheese).
7. Panellists will evaluate specific flavour attributes, which will include the flavour intensity, bitterness, sour-acid and vinegary flavours using a 10-point intensity scale (1 = low intensity and 10 = high intensity).
8. Panellists will also evaluate texture attributes (hardness and crumbliness) using a 10-point scale (hardness: 0 = extremely soft, 10 = extremely hard; crumbliness: 0 = extremely cohesive, 10 = extremely crumbly).

Who is conducting the study?

The School of Biomedical and Health Sciences, Victoria University

Dr Vijay Mishra Principal Researcher (Werribee Campus, building 2, Room 22.14, email: Vijay.Mishra@vu.edu.au; tel: 9919 8130)

Mr Ali Sheibani (PhD student in food science, Werribee Campus, building 2, room 21.20, email: ali.sheibani@live.vu.edu.au; tel: 9919 8109)

Any queries about your participation in this project may be directed to the Chief Investigator listed above.

If you have any queries or complaints about the way you have been treated, you may contact the Research Ethics and Biosafety Manager, Victoria University Human Research Ethics Committee, Victoria University, PO Box 14428, Melbourne, VIC, 8001 or phone (03) 9919 4148.

Appendix 2: Sensory Evaluation Consent Form

CONSENT FORM FOR PARTICIPANTS INVOLVED IN RESEARCH

INFORMATION TO PARTICIPANTS:

We would like to invite you to be a part of a study into …

'[State briefly the aims, procedures involved and the nature of the project, including a clear indication of any potential risks associated with this project]'

CERTIFICATION BY SUBJECT

I, (participant name) _____
of (participant address) _____

certify that I am at least 18 years old* and that I am voluntarily giving my consent to participate in the study: 'The Effects of Salt Reduction on the Physicochemical and Microbiological Properties and Salt Release from Cheddar Cheese', being conducted at Victoria University by: **Dr Vijay Mishra** (Vijay.Mishra@vu.edu.au).

I certify that the objectives of the study, together with any risks and safeguards associated with the procedures listed hereunder to be carried out in the research, have been fully explained to me by: **Ali Sheibani,** PhD student in food science (ali.sheibani@live.vu.edu.au)

and that I freely consent to participation involving the below mentioned procedures:

- triangle tests
- cheese grading
- saltiness scoring.

I certify that I have had the opportunity to have any questions answered and that I understand that I can withdraw from this study at any time and that this withdrawal will not jeopardise me in any way.

I have been informed that the information I provide will be kept confidential.

Signed:

Date:

Any queries about your participation in this project may be directed to the researcher
Dr Vijay Mishra Tel: (03) 9919 8130

If you have any queries or complaints about the way you have been treated, you may contact the Research Ethics and Biosafety Manager, Victoria University Human Research Ethics Committee, Victoria University, PO Box 14428, Melbourne, VIC, 8001 or phone (03) 9919 4148.

[*Please note: Where the participant/s are aged under 18, separate parental consent is required; where the participant/s are unable to answer for themselves due to mental illness or disability, parental or guardian consent may be required.]

Appendix 3: Sensory Evaluation Panellist Questionnaire

Panellist Questionnaire

VICTORIA UNIVERSITY | A NEW SCHOOL OF THOUGHT

Name:

Please put a tick next to your answer for the following questions:

Sex: Male_____ Female _____

Age: < 18 years _____
18–25 years _____
25–35 years _____
35–55 years _____

Frequency of cheese consumption:
≤ 1 per week _____
1–2 per week _____
2–3 per week _____
3–4 per week _____
> 5 per week _____

Preferred type of cheese:
Mild_____ Tasty (medium)_____ Matured _____

Appendix 4: Sensory Evaluation Specific Attributes Scoring Form

Scoring for Specific Attributes

VICTORIA UNIVERSITY | A NEW SCHOOL OF THOUGHT

Product: Cheddar cheese

Name:

Date:

Instruction:

You have been given four three-coded samples. Please score the Cheddar cheese samples on a scale of 1 to 10 for all parameters listed:

1.Cheddary	1 = none (very mild)	10 = high intensity (mature)
2.Creamy/milky	1 = not creamy	10 = very creamy
3.Sour- acid	1 = not acidic	10 = very acidic
4.Vinegary	1 = not detected	10 = high intensity
5. Bitterness	1 = not bitter	10 = very bitter
6. Hardness	1 = soft	10 = hard
7. Crumbliness	1 = crumbly (do not hold together)	10 = firm, stick together
8. Acceptability	1 = not accepted	10 = highly accepted

Please score one sample at a time. Use the water crackers and water to wash your palate after tasting each sample.

SAMPLES

Attributes				
1. Cheddary				
2. Creamy/milky				
3. Sour-acid				
4. Vinegary				
5. Bitterness				
6. Hardness				

7. Crumbliness				
8. Acceptability				

Cheddary: general flavours of Cheddar cheese

Creamy/milky: flavour associated with fresh milk, creamy product, condensed milk

Sour-acid: sour, taste sensation of lactic or citric acid

Vinegary: flavour associated with vinegar

Bitterness: chemical-like, aspirin, taste sensation of caffeine.

Thank you very much for your participation

Appendix 5: Sensory Evaluation Triangle Test

Triangle Test

VICTORIA UNIVERSITY | A NEW SCHOOL OF THOUGHT

Product: Cheddar cheese

Name:

Date:

Instructions:

You have been given four sets of three-coded samples. In each set, two samples are taken from the same batch and the other from another batch. Within each set, mark the **ODD** sample with 'X'. Please taste samples in order from left to right. Use the water crackers and water to wash your palate after tasting each sample.

Set	Codes		
1.	733	409	651
2.	767	377	420
3.	283	612	229
4.	865	134	962

Appendix 6: Sensory Evaluation Overall Acceptability Form

Acceptability of Cheddar Cheeses

Product: Cheddar cheese

Name:

Date:

Instructions:

You have been given four three-coded samples. Please score the Cheddar cheese samples on a scale of 1 to 10 (1 = dislike extremely, 10 = like extremely). Use the crackers and water to wash your palate after tasting each sample.

Sample code	123	861	754	678
Acceptability				

Thank you very much for your participation

Appendix 7: Experimental Design for the Study of Salt Diffusion

Factors			Treatment codes
pH	**C/F ratio[1]**	**Rennet concentration (mL/L)**	
6.2	0.6	0.1	A
		0.3	B
	0.7	0.1	C
		0.3	D
	0.8	0.1	E
		0.3	F
5.9	0.6	0.1	G
		0.3	H
	0.7	0.1	I
		0.3	J
	0.8	0.1	K
		0.3	L
5.6	0.6	0.1	M
		0.3	N
	0.7	0.1	O
		0.3	P
	0.8	0.1	Q
		0.3	R

[1] C/F ratio = casein-to-fat ratio.

Appendix 8: Sodium Diffusion Profile of Experimental Cheese Matrices on Saliva at Day 1

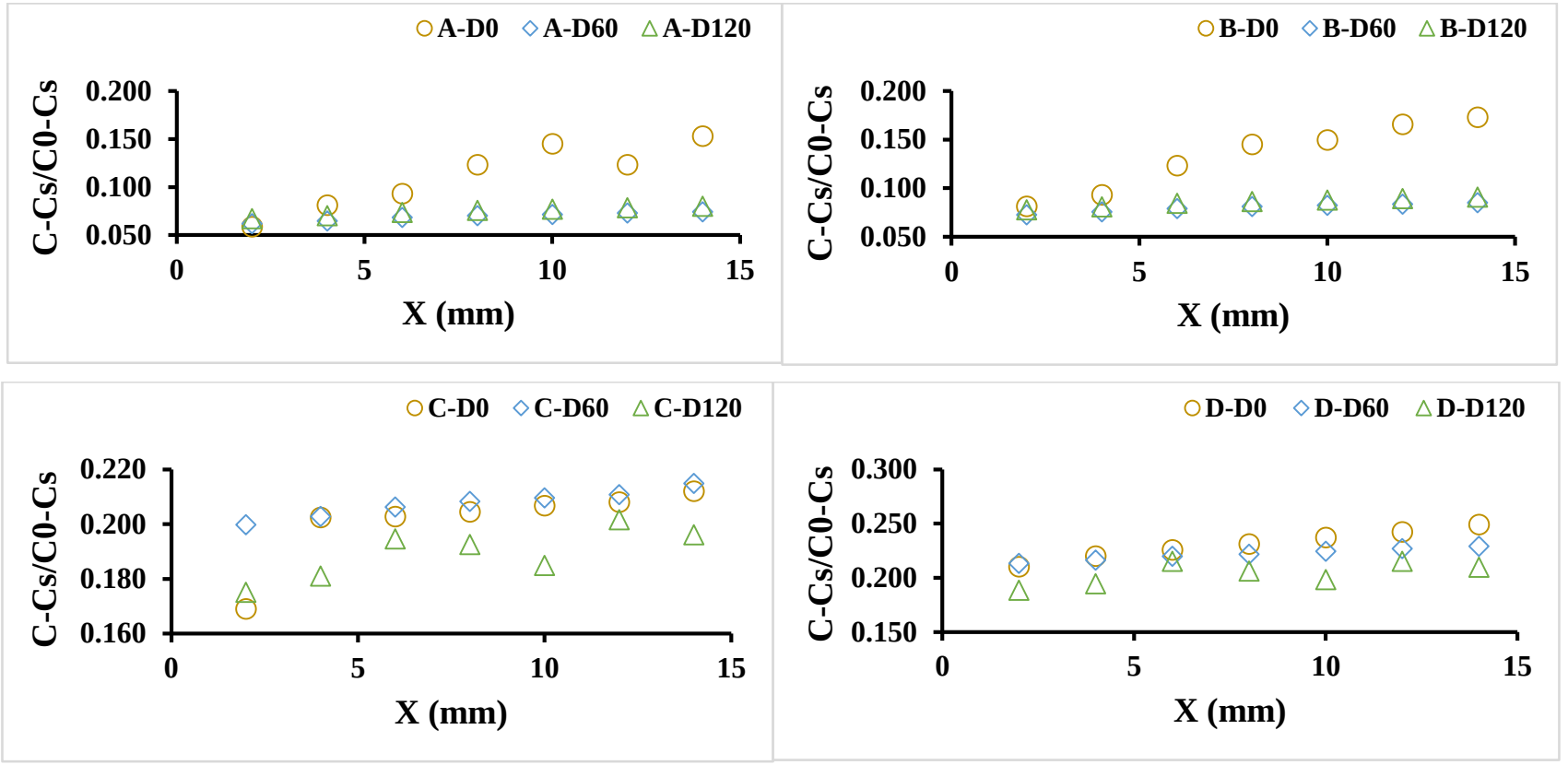


Figure 28 (cont'd):

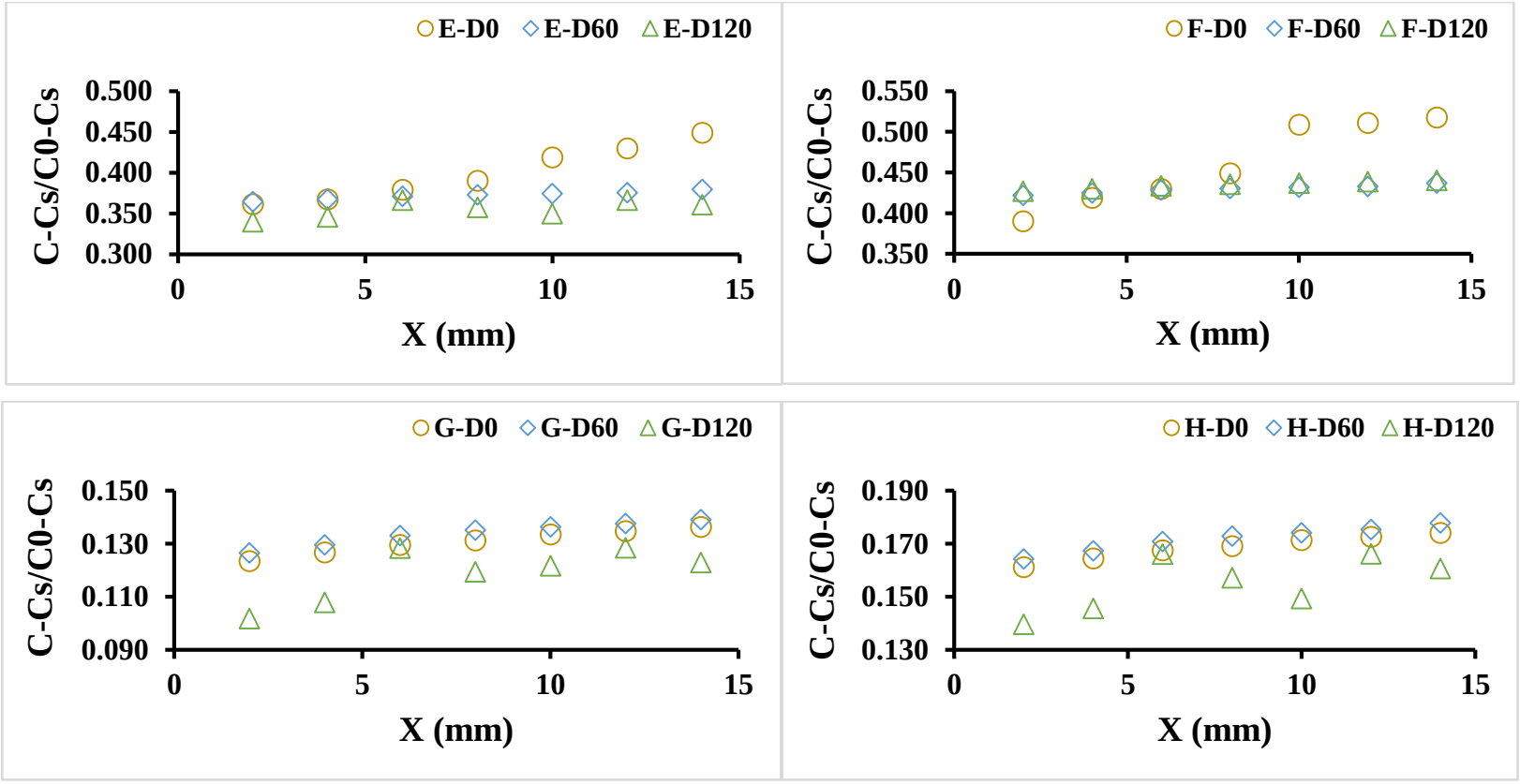


Figure 28 (cont'd):

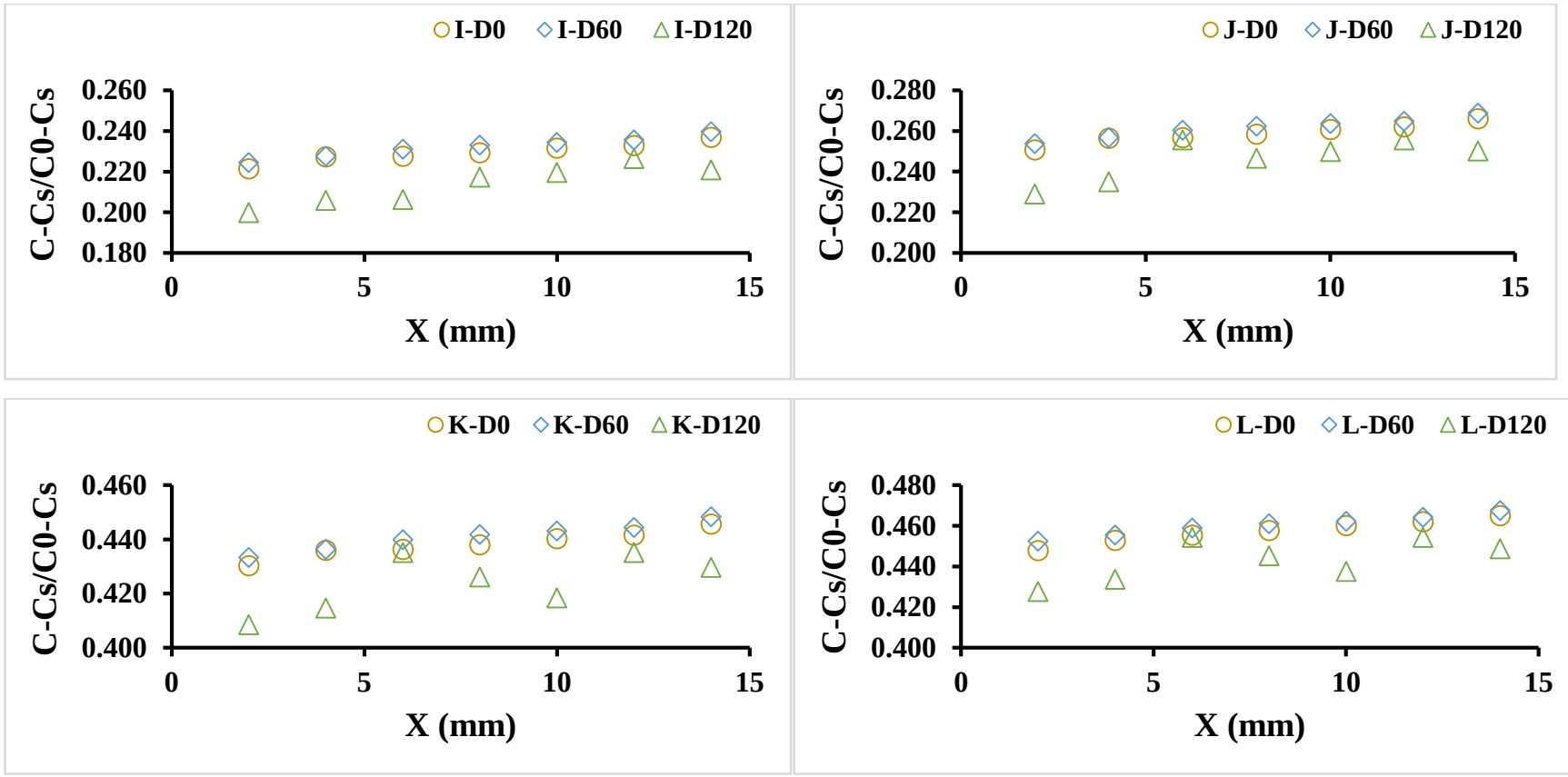


Figure 28 (cont'd):

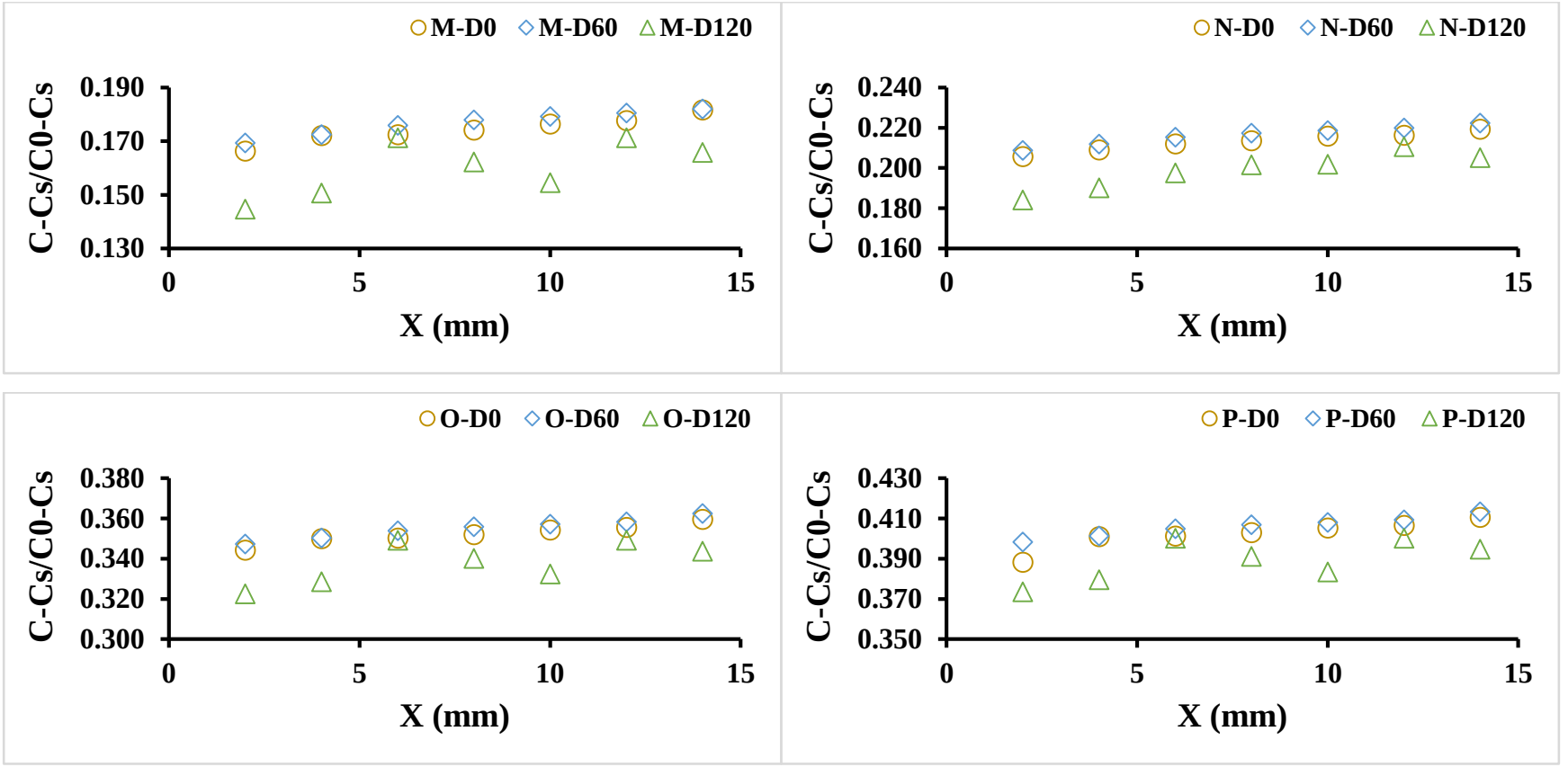


Figure 28 (cont'd):

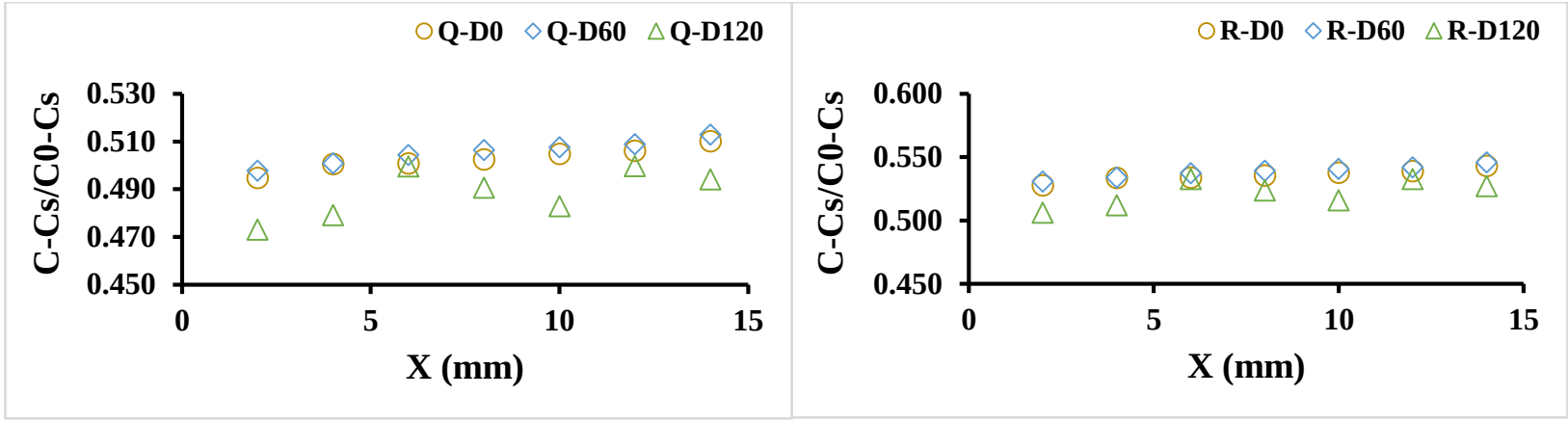


Figure 28: Sodium Diffusion Profiles of Experimental Cheese Matrices after One Day of Contact with the Solution of Artificial Saliva

Appendix 9: Sodium Diffusion Profile of Experimental Cheese Matrices at Day Six on Saliva

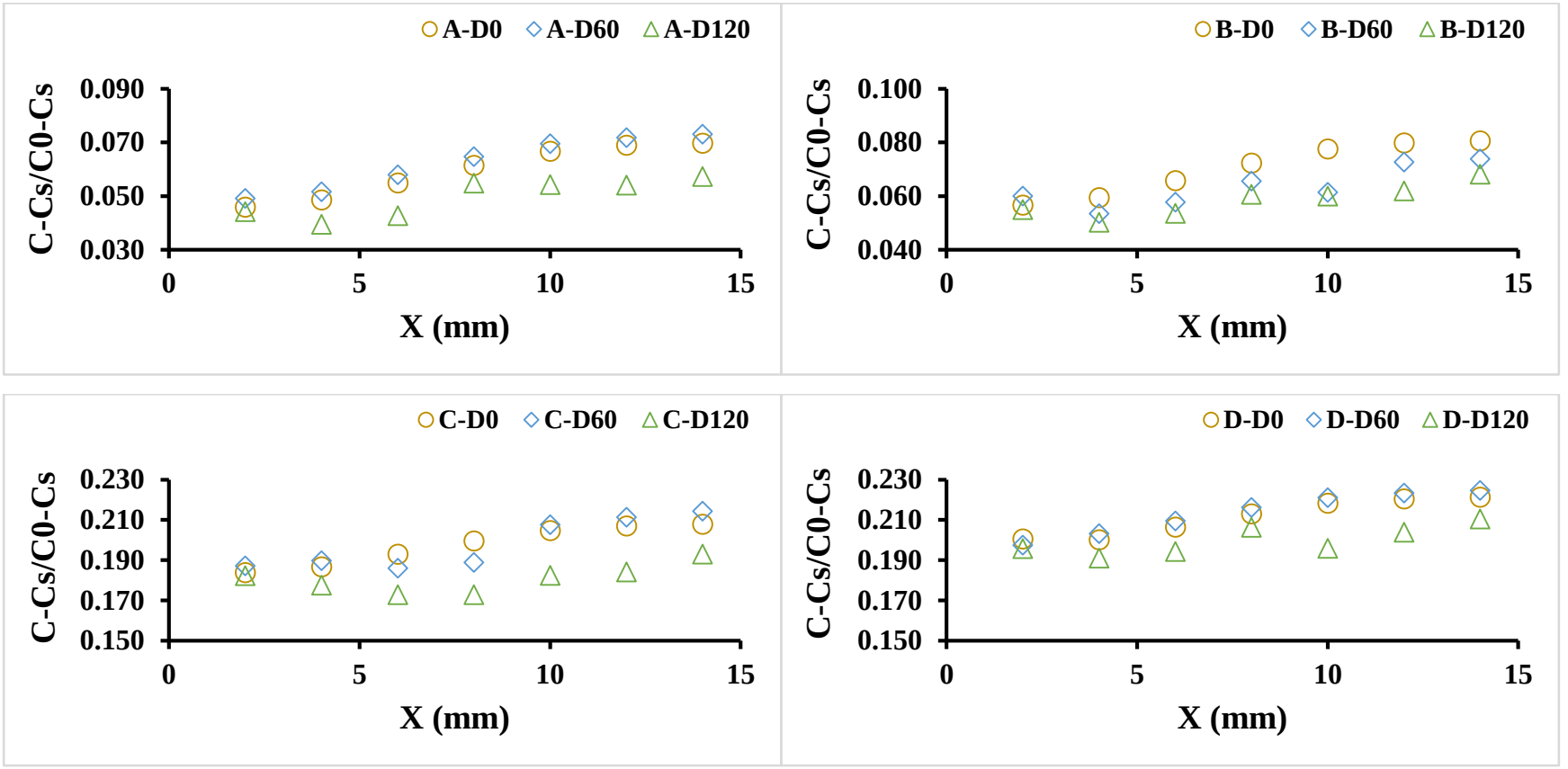


Figure 29 (cont'd):

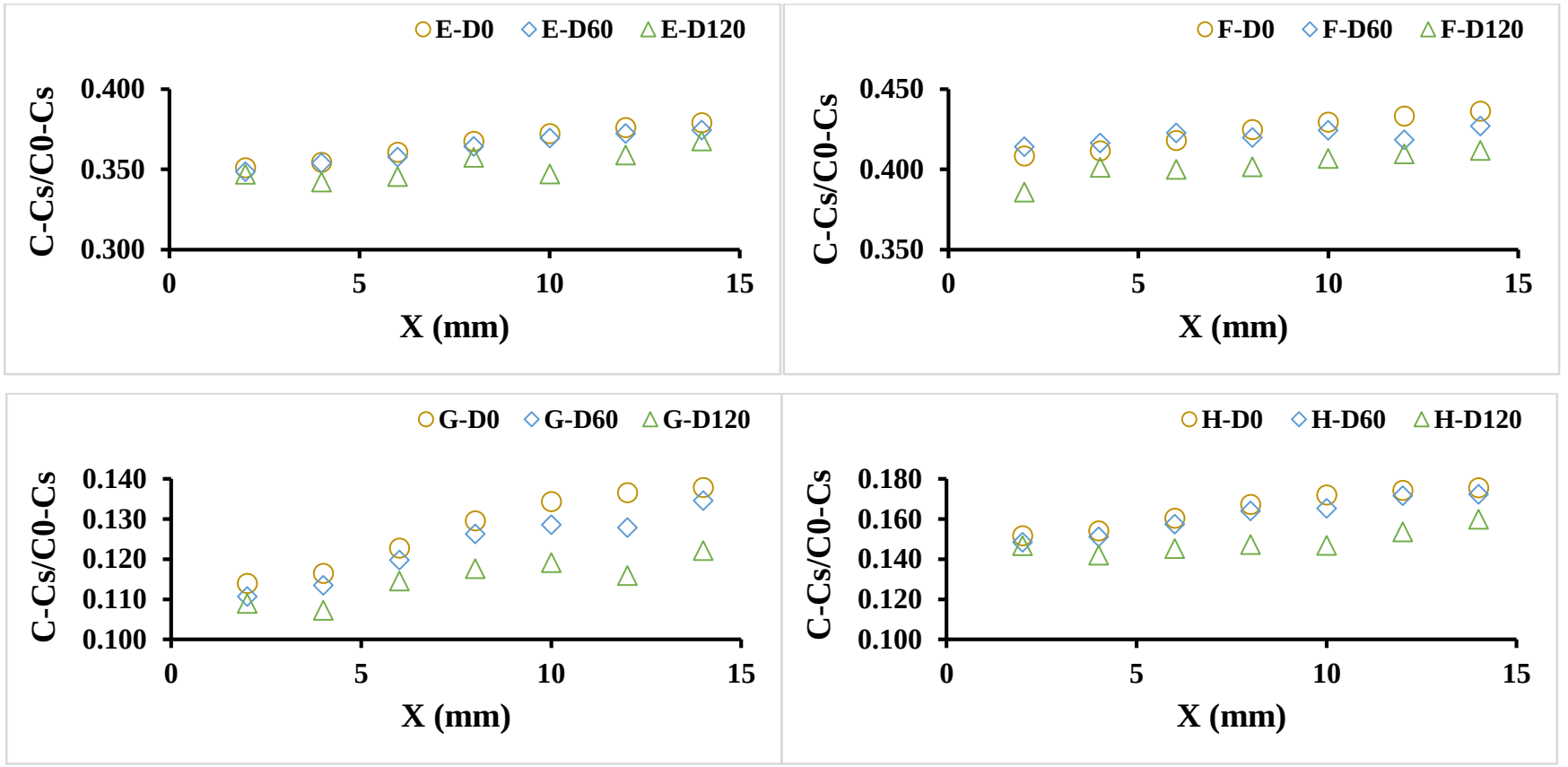


Figure 29 (cont'd):

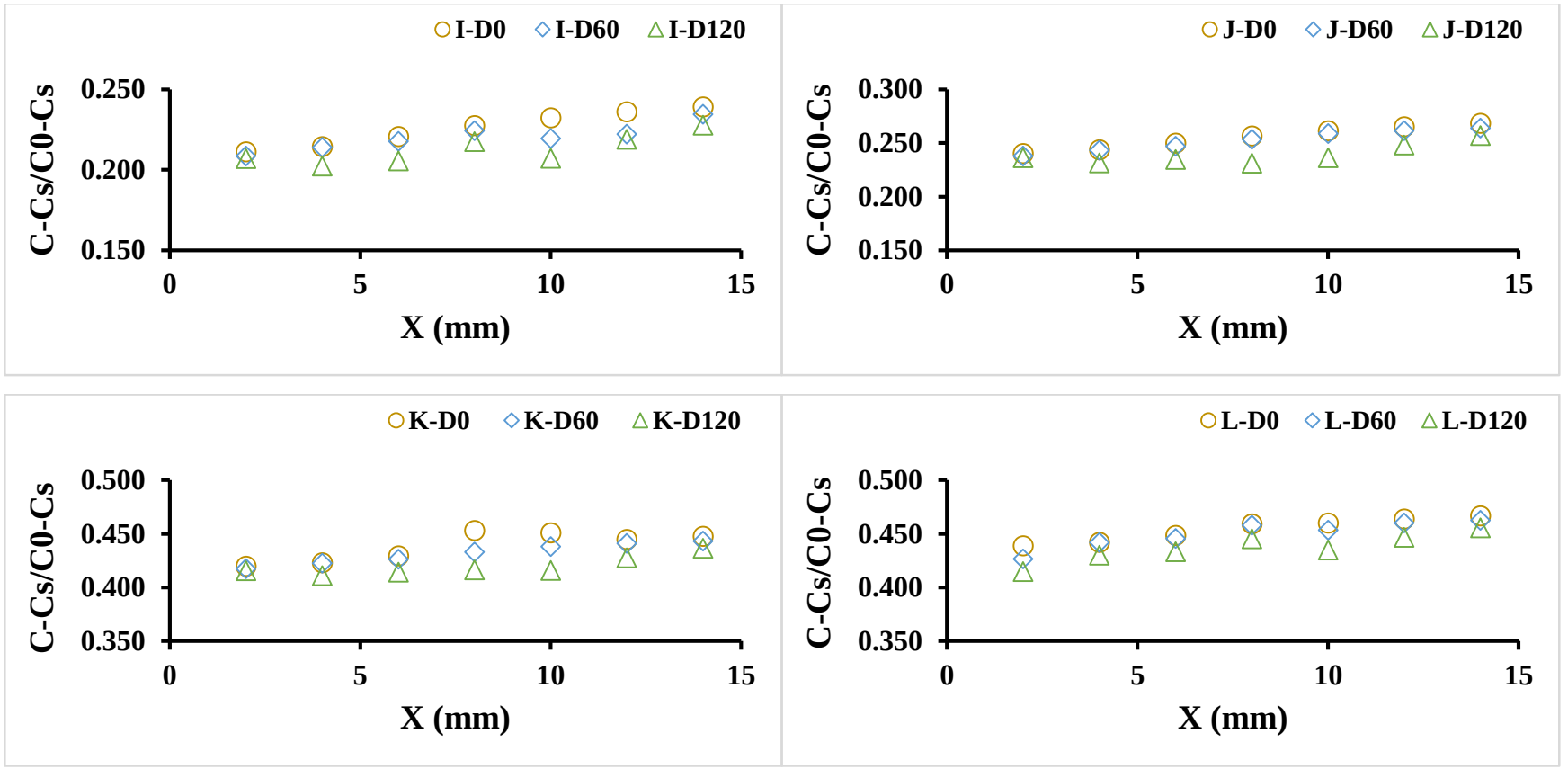


Figure 29 (cont'd):

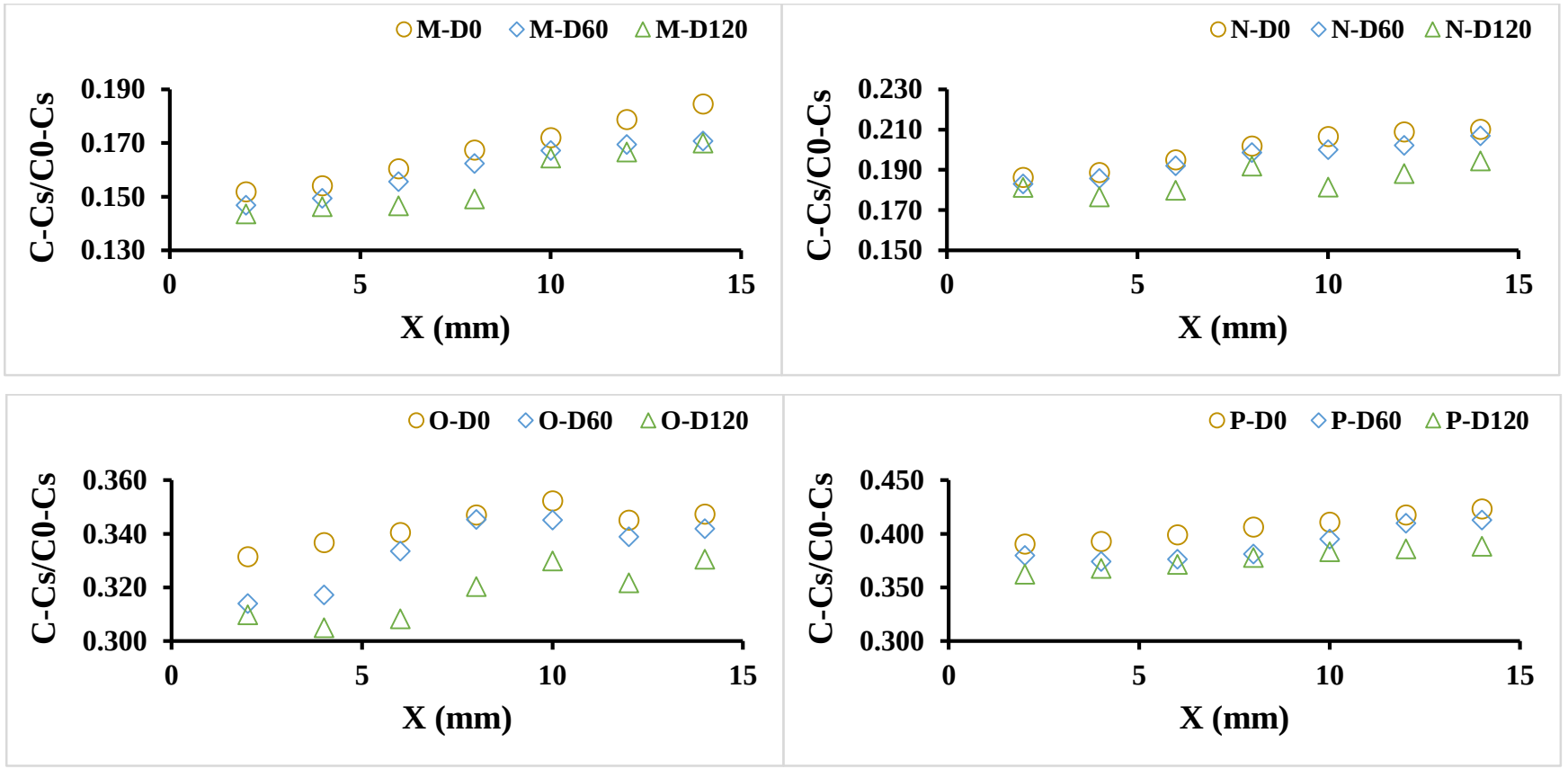


Figure 29 (cont'd):

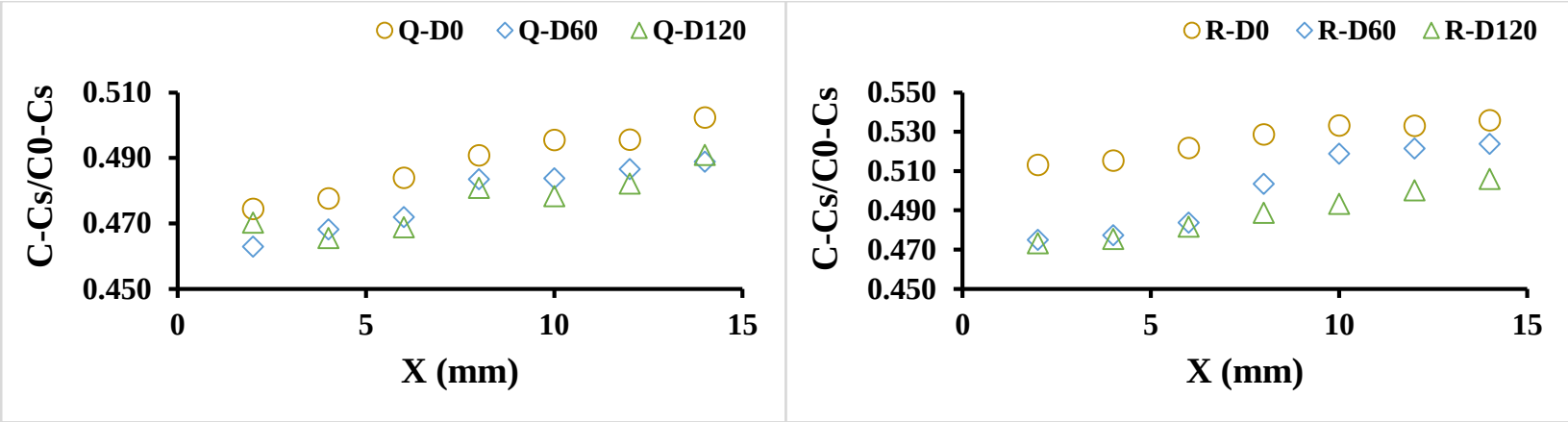


Figure 29: Sodium Diffusion Profiles of Experimental Cheese Matrices after Six Days of Contact with the Solution of Artificial Saliva