THE EFFECT OF SODIUM CHLORIDE SUBSTITUTION WITH POSTASSIUM CHLORIDE ON CHEMICAL, PHYSICAL AND MICROBIOLOGICAL CHARACTERISTICS OF MEDITERRANEAN CHEESES

A thesis submitted for the degree of Doctor of Philosophy

by

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June, 2013

It is my honour to dedicate this thesis to my beloved injured Palestine who is still bleeding under occupation

I. Abstract

The overall objective of this study was to investigate the influence of salt substitution with KCl on chemical, physical, microbiological and sensory properties of selected Mediterranean cheeses. Salt was substituted with KCl at four different levels (only NaCl, 3NaCl:1KCl, 1NaCl:1KCl, and 1NaCl:3KCl). Halloumi, Akawi and Nabulsi cheeses were prepared and brined in the four different salt treatments for 56 days at 4°C, 30 days at 4°C, and 5 months at room temperature, respectively. Low-moisture Mozzarella cheese was also produced and dry-salted and stretched in the four salt treatments and stored for 27 days at 4°C. Chemical, physical, microbiological and sensory characteristics were examined for all cheeses during storage. Cheeses salted with NaCl/KCl mixtures had similar chemical composition, texture profile, organic acids profile, and microstructure compared with control (made only with NaCl) at the same storage period. Organic acids significantly increased and hardness decreased significantly during storage time at the same salting treatment. The primary proteolysis (measured as water soluble nitrogen: WSN) was insignificantly affected by salt substitution, while the advanced proteolysis (measured as trichloroacetic acid-soluble nitrogen (TCA-SN), and phosphotungistic acid-soluble nitrogen (PTA-SN), and total free amino acids (TFAA) were significantly affected. All proteolysis parameters (WSN, TCA-SN, PTA-SN, and TFAA) increased significantly during storage time. Cheeses salted with NaCl/KCl mixtures at 3:1 and 1:1 ratios were the most similar to control cheese in term of sensory properties.

This study also examined the effect of salt substitution with KCl on the proteolytic activities of starter and probiotic bacterial enzymes in artificial media. MRS broths were separately mixed with four salt treatments (NaCl only, 1NaCl:1KCl, 1NaCl:3KCl, and KCl only) at 2 different concentrations (5% and 10%) followed by

inoculation with four bacteria (*L. bulgaricus, S. thermophiles, L. acidophilus* and *L. casei*) individually and then incubated at 37°C for 22 h. The cell-free extract (CFE) and the cell-free supernatants (CFS) were prepared and used as source of intercellular and extracellular proteinases, respectively. The CFE and CFS were incubated with 3 milk caseins (α-, β-, κ-casein) and subjected to angiotensin-converting-enzyme inhibitory (ACE-inhibitory) activity and proteolytic activity. Significant differences were observed in ACE-inhibitory and proteolytic activities between salt treatments of cell-free extract and cell-free supernatant of all bacteria at the same salt concentration and same pH level. The proteolytic activity of CFS (measured as azocasein) was significantly higher than that of CFE. Similarly, the proteolytic activities measured as OPA after incubation of CFS with milk caseins were significantly higher compared with CFE. The ACE-inhibitory activities of milk caseins incubated with CFS were significantly lower than CFE.

This study showed that KCl can be used successfully to partially substitute NaCl during cheese production and ripening. Chemical composition (moisture, protein, fat, ash, and pH), texture profile (hardness, adhesiveness, cohesiveness, and gumminess), microstructure and sensory properties of partially substituted cheeses were similar compared with the control. This study demonstrated that the diffusion of KCl molecules may occur in cheese in the same way as the NaCl molecules. Sensory attributes of experimental cheeses salted with different NaCl/KCl mixtures were similar compared with control. Among these, batches salted with 3NaCl:1KCl and 1NaCl:1KCl showed similar scores compared to that of the control. Proteolysis of cheeses salted with NaCl/KCl mixtures differed significantly compared with control. This study demonstrated that the primary and the intermediate stages of proteolysis in cheeses were

affected by salt replacement. KCl did not maintain the cheese proteolysis as it was with NaCl.

This project showed that the effect of NaCl substitution with KCl is dependent on cheese variety. Proteinases of the starter culture and probiotic had been significantly affected by NaCl substitution. This study showed that salt substitution with KCl successfully applied on Mediterranean cheeses with no adverse effect on its quality.

II. Certificate

Professor Nagendra P. Shah (M.Sc., PhD)
Food and Nutritional Science
School of Biological Sciences
The University of Hong Kong
Hong Kong

This is to certify that the thesis entitled "THE EFFECT OF SODIUM CHLORIDE SUBSTITUTION WITH POSTASSIUM CHLORIDE ON CHEMICAL, PHYSICAL AND MICROBIOLOGICAL CHARACTERISTICS OF MEDITERRANEAN CHEESES" submitted by **Mutamed Ayyash** in partial fulfilment of the requirement for the award of the Doctor of Philosophy in Food Technology at Victoria University is a record of bonafide research work carried out by 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.

Date:

Professor Nagendra P. Shah

III. Declaration

"I, Mutamed Ayyash, declare that this thesis entitled "THE EFFECT OF SODIUM CHLORIDE SUBSTITUTION WITH POSTASSIUM CHLORIDE ON CHEMICAL, PHYSICAL AND MICROBIOLOGICAL CHARACTERISTICS OF MEDITERRANEAN CHEESES" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Mutamed Ayyash

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IV. Acknowledgment

My sincere thanks and gratitude go to my Principal Supervisor, Professor Nagendra P. Shah, for his guidance, valuable discussions and suggestions throughout the course of study and in preparation of manuscripts. Prof. Shah was highly dedicating to this work and the manner in which he efficiently dealt with problems to the progress of this project was greatly positive..

I would like to express gratitude to my Co-supervisor Dr. Frank Sherkat (School of applied science, RMIT University) for his extensive support and guidance especially at the end of my candidature when Prof. Shah moved to University of Hong Kong.

From dept of my heart, I would like to express my sincere gratitude, endless love and infinite respect to my Mother Wijdan for her great prayers and support during my PhD study together with my father, brothers and sisters.

I am highly grateful to my colleagues at Victoria University for their kind friendship, and Technical Staff for their help and support during the PhD study. I would like to acknowledge the scholarship awarded to me by the Victoria University, which has enabled me to perform my research without financial difficulty. The School of Biomedical and Health Sciences also provided me with the financial support to broaden my scope by attending several international and domestic conferences

I would like to thank Mr. Phil Frances from RMIT University, department of applied physics, for his valuable help to get images of cheese microstructure. Also, a great thanks to Dr. Roderick Williams from The Commonwealth Scientific and Industrial Research Organisation (CSIRO) for his great help to analyse texture profile of all cheeses.

V. List of Publications

Peer-reviewed Journals

1. **Ayyash**, M. M., F. Sherkat, and N. P. Shah. 2013. The effect of NaCl substitution with KCl on proteinase activities cell-free extract and cell-free supernatant at different pH levels and salt concentrations: *Lactobacillus acidophilus* and *Lactobacillus casei*. Journal of Dairy Science, 96:40-49

2. **Ayyash**, M. M., F. Sherkat, and N. P. Shah. 2013. Effect of Partial NaCl Substitution with KCl on the Texture Profile, Microstructure, and Sensory Properties of Low-Moisture Mozzarella Cheese. Journal of Dairy Research, 80:7-13

3. **Ayyash** M. M., F. Sherkat, and N. P. Shah. 2012. The Effect of NaCl Substitution with KCl on Akawi Cheese: Chemical Composition, Proteolysis, ACE-inhibitory activity, Probiotic survival, Texture Profile and Sensory Properties. Journal of Dairy Science 95:4747-4759

4. **Ayyash**, M. M., F. Sherkat, and N. P. Shah. 2012. The impact of NaCl substitution with KCl on proteinase activities cell-free extract and cell-free supernatant at different pH levels and salt concentrations: *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* Journal of Food Science. 77:M490 – M498

5. **Ayyash** MM & Shah NP. 2011. Proteolysis of low-moisture Mozzarella cheese as affected by substitution of NaCl with KCl. Journal of Dairy Science 94(8):3769-3777.

6. **Ayyash**, M. M. and N. P. Shah. 2011. The effect of substitution of NaCl with KCl on chemical composition and functional properties of low-moisture Mozzarella cheese. Journal of Dairy Science 94(8):3761-3768.

7. **Ayyash**, M. M. and N. P. Shah. 2011. The effect of substituting NaCl with KCl on Nabulsi cheese: Chemical composition, total viable count, and texture profile. Journal of Dairy Science 94(6):2741-2751.

8. **Ayyash**, M. M., F. Sherkat, P. Francis, R. P. Williams, and N. P. Shah. 2011. The effect of sodium chloride substitution with potassium chloride on texture profile and microstructure of Halloumi cheese. Journal of Dairy Science 94(1):37-42.

9. **Ayyash**, M. M. and N. P. Shah. 2011. Effect of Partial Substitution of NaCl with KCl on Proteolysis of Halloumi Cheese. Journal of Food Science 76(1):C31-C37.

10. **Ayyash**, M. M. and N. P. Shah. 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 75(6):C525-C529.

Book Chapter

Ayyash M. M., F. Sherkat and NP Shah, Sodium chloride substitution of cheese. In Handbook of Cheese in Health: Production, Nutrition and Medical Sciences. Victor R. Preedy, Ronald Ross Watson and Vinood B. Patel (edrs). ISBN**:** 978-90-8686-766-0

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3	Effect of partial substitution of NaCl with KCl on Halloumi cheese during storage: Chemical composition, lactic bacterial count, and organic acids production	Published	**Journal of Food Science 75(6):C525-C529. Class A**
4	Effect of Partial Substitution of NaCl with KCl on Proteolysis of Halloumi Cheese	Published	**Journal of Food Science 76(1):C31-C37 Class A**
5	The effect of sodium chloride substitution with potassium chloride on texture profile and microstructure of Halloumi cheese	Published	**Journal of Dairy Science 94(1):37-42 Class A**
6	The effect of substituting NaCl with KCl on Nabulsi cheese: Chemical composition, total viable count, and texture profile	Published	**Journal of Dairy Science 94(6):2741-2751 Class A**
7	The effect of substitution of NaCl with KCl on chemical composition and functional properties of low-moisture Mozzarella cheese	Published	Journal of Dairy Science 94(8):3761-3768 Class A
8	Proteolysis of low-moisture Mozzarella cheese as affected by substitution of NaCl with KCl	Published	Journal of Dairy Science 94(8):3769-3777 Class A

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9	Effect of partial NaCl substitution with KCl on the texture profile, microstructure, and sensory properties of low-moisture mozzarella cheese	Published	**Journal of Dairy Research, 80:7-13 Class A**
10	The Effect of NaCl Substitution with KCl on Akawi Cheese: Chemical Composition, Proteolysis, ACE-inhibitory activity, Probiotic survival, Texture Profile and Sensory Properties.	Published	**Journal of Dairy Science 95:4747 - 4759 Class A**
11	The impact of NaCl substitution with KCl on proteinase activities of cell-free extract and cell-free supernatant at different pH levels and salt concentrations: Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus	Published	**Journal of Food Science 77(8):M490-M498 Class A**
12	The effect of NaCl substitution with KCl on proteinase activities of cell-free extract and cell-free supernatant at different pH levels and salt concentrations: Lactobacillus acidophilus and Latobacillus casei	Published	**Journal of Dairy Science 96:40-49 Class A**

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Mutamed M Ayyash 13-06-2013

VI. Oral and Poster Presentations

Oral presentation

Ayyash, M. M. and N. P. Shah (**2012**). The impact of NaCl substitution with KCl on proteinase activities cell-free extract and cell-free supernatant at different pH levels and salt concentrations: Lactic acid bacteria (*L. bulgaricus* and *S. thermophilus*) Probiotics (*L. acidophilus* and *L. casei.* JAM 2012 Joint Annual Meeting of ADSA – AMPA – ASAS – CSAS – WSASAS (July 15-19, 2012) in Phoenix, Arizona, USA.

Ayyash, M. M. and N. P. Shah **(2011).** The effect of substituting NaCl with KCl on Nabulsi cheese: Chemical composition, total viable count, and texture profile. JAM 2011 ADSA-ASAS Joint Annual Meeting (10-14 July) – New Orleans, Louisiana, USA. The abstract number is 606

Ayyash, M. M. and N. P. Shah **(2010).** The effect of NaCl/KCl substitution on Halloumi cheese during storage: Chemical composition, proteolysis, texture profile, and microstructure. JAM 2010 ADSA®-PSA-AMPA-CSAS-ASAS Joint Annual Meeting (11-15 July) - Denver, Colorado, USA. The abstract number 791

Poster presentation

Ayyash, M. M., and N. P. Shah **(2010).** The Effect of NaCl Substitution with KCl on Akawi Cheese: Chemical Composition, Proteolysis, ACE-inhibitory Activity, Probiotic survival, Texture Profile and Sensory Properties The 43rd annual Australian Food Science & Technology (AIFST) Convention, 25-27th July 2010 at the Sebel Albert Park, Melbourne, VIC, Australia

Ayyash, M. M., F. Sherkat, and N. P. Shah **(2012).** The Effect of NaCl Substitution with KCl on Akawi Cheese: Chemical Composition, Proteolysis, ACE-inhibitory Activity, Probiotic survival, Texture Profile and Sensory Properties. The 45th Annual AIFST Convention, 15 – 18th of July 2012 at Adelaide Convention Center, Adelaide, SA, Australia

VII. Table of Contents

VIII. List of Tables

IX. List of Figures

X. List of Abbreviation

ACE = angiotensin-I-converting enzyme

AOAC = Association of Official Analytical Chemists

ANOVA = analysis of variance

BSA = bovine serum albumin

CFU = colony forming unit

CFE = cell free extract

CFS = cell free supernatant

CN = casein

FAA = free amino acids

g = gram

h = hour

HHL = hippuryl-histidiyl-leucine

RP-HPLC = reverse phase-high performance liquid chromatography

L = litre

LAB = lactic acid bacteria

mg = milligram

min = minute

mL = millilitre

mm = millimetre

mo = month

MRS = DeMan Rogosa Sharpe

NSLAB = non-starter lactic acid bacteria

PAGE = polyacrylamide gel electrophoresis

PTA-SN = phosphotungstic acid soluble nitrogen

s = second

SN = soluble nitrogen

TCA = trichloroacetic acid

TFA = trifluoroacetic acid

TN = total nitrogen

WSE = water soluble extract

WSN = water soluble nitrogen

v/v = volume per volume

v/w = volume per weight

α = alpha

β = beta

κ = kappa

μg = microgram

ºC = degree Celsius

1. Chapter 1: Introduction

Salt (sodium chloride; NaCl) is a common additive in the food industry. Salt plays a vital role during food processing including preservation of food and contribution to flavour, in addition to its effect on proteolysis, water activity, and texture profile (Reddy and Marth, 1991; Guinee, 2004a; Guinee and Fox, 2004). Although addition of salt plays an essential role during food processing, it is currently considered as a main risk factor for disorders related to osteoporosis, kidney stones and hypertension (Buemi et al., 2002; Kotchen, 2005; Massey, 2005; Heaney, 2006). The World Health Organization (WHO) has recommended food manufacturers to reduce salt content in their products (WHO, 2007). Meat and poultry products, dairy products, bakery products, and other food items contribute to the daily sodium intake by particular percentages which vary based on country and community food habits. (Anonymous, 2003; Desmond, 2006). For instance, bread, cereals and grains contributes by 19.5% and 34.6% in USA and UK, respectively (Anderson et al., 2010). In Australia, the Food Standards Australia New Zealand (FSANZ) found that bakery product, meat products, and cereals products contributed by 24%, 21%, and 17% in the daily sodium intake, respectively (Anonymous, 2011).

It has been reported that cheeses contribute to daily sodium intake by 7.8% in UK, 9.2% in France, 8.2% in USA and 5% in Australia (Anonymous, 2003; Meneton et al., 2009; Anderson et al., 2010). Dairy scientists across the world have embarked on projects to reduce salt content in cheese as a part of the worldwide attempt to reduce sodium intake (WHO, 2007). Thus, there has been an increased interest in reducing salt in cheeses without adversely affecting their quality and safety. Numerous attempts have been made

to achieve low salt cheeses using different techniques including simple salt reduction and partial salt replacement in various cheeses (Guinee and O'Kennedy, 2007).

Several attempts have been made to reduce salt without any preservatives or salt replacement. Schroeder et al. (1988b) prepared Cheddar cheese with salt content ranging from 0% to 1.75% and investigated its effects on chemical composition, proteolysis, sensory properties, and microbial growth over 7 months of storage. Cheeses containing 0.88% to 1.75% salt showed no significant difference in term of overall acceptability compared with control Cheddar cheese which had 2.5% salt. However, overall proteolysis and microbial growth increased with salt reduction which also has been found to increase bitterness and decrease shelf-life in cheeses. Therefore, partial salt substitution especially with KCl is considered as it did not significantly affect cheese quality compared with the control (Guinee, 2004b).

Potassium chloride is a potential candidate salt that can be used in partial substitution for NaCl. Increased intake of potassium has been reported to have a protective effect on people with hypertension (Fregly, 1981; Reddy and Marth, 1991). NaCl/KCl mixtures have been used successfully in various cheeses without any adverse effects on cheese quality (Fitzgerald and Buckley, 1985; Reddy and Marth, 1993a; 1995). Several studies have been carried out to investigate the effect of NaCl replacement with KCl on the characteristics of Cheddar cheese (Reddy and Marth, 1993a; b; Grummer et al., 2012), Feta cheese (Katsiari et al., 1997; 2000a), Kefalograviera cheese (Katsiari et al., 2001b), and Fynbo cheese (Zorrilla and Rubiolo, 1994; Zorrilla et al., 1996). These studies have reported that the characteristics (chemical composition, proteolysis and texture profile) of cheeses salted with NaCl/KCl mixture were similar to control.

Several types of cheeses, such as Mediterranean cheeses, are characterized as high-salt cheeses which need further investigation to reduce their salt content. These

Mediterranean cheeses like all other cheeses vary according to manufacturing process and ripening conditions. A few varieties of the Mediterranean cheeses have become highly popular around the world such as Mozzarella and Feta cheeses. A major aspect of most Mediterranean cheeses is that they contain high salt content such as Feta (5.0%), Halloumi (3.0 – 5.0%), Nabulsi (8.8%) and Akawi (5.0%) cheeses. Although the salt content of Mozzarella cheese is not high (2.0%), its enormous consumption volume especially the low-moisture Mozzarella cheese (LMMC) as an ingredient in different food items (pizza, pasta…etc.) warrants any attempt to reduce its salt content. Therefore the aims of this project were to:

1. Investigate the impact of salt substitution with KCl on Halloumi cheese characteristics. This is discussed in Chapters 3, 4, and 5.
2. Investigate the effect of salt replacement with KCl on Nabulsi cheese properties which has been addressed in Chapter 6.
3. Evaluate the effect of salt substitution on low-moisture Mozzarella cheese properties during storage. This is discussed in Chapters 7, 8, and 9.
4. Investigate the effect of salt substitution on Akawi cheese characteristics which is addressed in Chapter 10.
5. Investigate the effect of full and partial substitution of salt with KCl on proteinase activities of lactic acid bacteria (*L. bulagaricus* and *S. thermophilus*) and probiotic bacteria (*L. acidophilus* and *L. casei*). The salt replacement was performed at different pH levels (5.0, 5.5, and 6.0) and different salt concentrations (5% and 10%). These are discussed in Chapters 11 and 12.

This study consists of five main parts:

The first part aimed to investigate the effect of salt substitution with KCl on Halloumi cheese characteristics during storage. Four Halloumi cheese batches were prepared and

brined in four different salt treatments; only NaCl, 3NaCl:1KCl, 1NaCl:1KCl, 1NaCl:3KCl, at same concentration (18%) and stored at 4°C for 56 days. Samples were collected on days 0, 14, 28, 42 and 56 of storage and subjected for the following analyses: chemical composition, proteolysis determination, organic acid profile, minerals (Na^{+}, K^{+}, and Ca^{+2}) content, texture profile, and microstructure evaluation. The outcomes of this part are fully described in chapters 3, 4, and 5 and published in peer-reviewed international journals (Ayyash and Shah, 2010; 2011a; Ayyash et al., 2011).

The second part aimed to investigate the effect of NaCl substitution with KCl on Nabulsi cheese properties during storage. Nabulsi cheese was made and stored in 4 different brine solutions at 18% (w/v), including NaCl only (A; control); 3NaCl:1KCl (w/w; B); 1NaCl:1KCl (w/w; C); and 1NaCl:3KCl (w/w; D). Chemical composition, proteolysis, total viable count, and texture profile analysis were assessed at monthly intervals for 5 months. The findings of this part can be found in chapter 6 and is published in peer-reviewed journal (Ayyash and Shah, 2011b).

The third part aimed to study the effect of NaCl replacement with KCl on low-moisture Mozzarella cheese (LMMC) characteristics. LMMC samples were collected on 0, 9, 18 and 27 day of storage at 4°C. Chemical composition, proteolysis parameters, organic acid profile, lactic acid bacteria growth, minerals (Na^{+}, K^{+}, Ca^{+2}, and P^{+4}) concentration, soluble Ca^{+2}, texture profile, sensory properties and microstructure of cheese samples were assessed. The results of this part are presented in chapters 7, 8, and 9 and are published in peer-reviewed international journals (Ayyash and Shah, 2011d; c; Ayyash et al., 2013a).

The fourth part of the study was designed to investigate the effect of partial substitution of NaCl with KCl on Akawi cheese with probiotic bacteria during 30 days of storage at 4ºC. Chemical composition, the survival of probiotic and lactic acid bacteria, the

proteolytic activity, texture profile analysis were analysed and sensory attributes of the cheese samples were evaluated analysis was carried out in order to determine the effects of salt substitution. The findings of this part are covered in chapter 10 and published in referred international journal (Ayyash et al., 2012a).

The fifth part aimed to examine the effect of full and partial NaCl substitution with KCl on proteinase activities of cell-free extract and cell-free supernatant of four cheese cultures; two lactic acid bacteria (*S. thermophiles* and *L. bulgaricus*) and two probiotic bacteria (*L. acidophilus* and *L. casei*) which were grown in MRS broth. The MRS broths were separately mixed with 4 salt treatments (NaCl only, 1NaCl:1KCl, 1NaCl:3KCl, and KCl only) at 2 different concentrations (5% and 10%) and incubated at 37°C for 22 h. The cell pellets were used to prepare proteinase of cell-free extract, while the cell-free supernatants were used as source of extracellular proteinases. The proteolytic activities and protein contents of both fractions were determined. The supernatants after incubation of both fractions with 3 milk caseins (α-, β-, κ-casein) were subjected to angiotensin-converting-enzyme inhibitory (ACE-inhibitory) activity and proteolytic activity by ortho-phthalaldehyde (OPA) method. The outcomes of this part are covered in chapters 11 and 12 and published in peer-reviewed international journals (Ayyash et al., 2012b; 2013b).

2. Chapter 2: Literature Review[1]

2.1.Salt definition and history

Salt or sodium chloride is an inorganic compound that consists of Na^+ and Cl^- which form a halide salt bound by an ionic bond. Sodium chloride dissolves readily in polar solvents such as water whereas it is not soluble in organic solvents. It is a vital compound in human life and food (Reddy and Marth, 1991; Durack et al., 2008). Sea and rock salts are the main sources of salt production in the world.

Salt is mentioned in the Bible as "essence of life" and in the Holy Quran as salt. It has been added to food since 3,000 B.C. (Binkerd and Kolari, 1975). A part of Roman soldiers' salary was paid as salt. Hence, the word "salary" is taken from salarium or allowance of salt (Woodin, 1981; Durack et al., 2008). Salt was isolated by Sir Humphrey Davy using electrolysis technique in 1807 (Ensminger, 1994).

2.2.Importance of salt in human life

The importance of salt was well known from ancient times however it was proven as a distinct compound experimentally by Osborne and Mendel in 1918. The human body contain about 0.2% of sodium with 50% in extracellular fluids, 40% in skeleton and 10% within the cells (Ensminger, 1994; Otten et al., 2006). Sodium plays major role in maintaining volume and osmotic balance in extracellular fluids. It has an essential function in electric activity of muscle and the nerve system. The amount of sodium in the body is regulated by kidney and sweat glands (Wahlqvist, 2011). An imbalance in sodium level in the body leads to serious health issues. Higher sodium intake is directly correlated with hypertension, kidney stones and osteoporosis (Massey, 2005; Heaney,

[1]A major part of this literature review has been accepted as book chapter: **Ayyash M. M**., F. Sherkat and NP Shah, Sodium chloride substitution of cheese. In Handbook of Cheese in Health: Production, Nutrition and Medical Sciences.

2006; Hollenberg, 2006). Sodium deficiency symptoms are weight loss, diarrhoea, nausea, and headache (Ensminger, 1994).

2.3.Industrial uses of salt

Salt is involved in several industrial processes including; textile and dyeing, metal processing, rubber manufacturing, pharmaceuticals, pigment manufacture, detergent production, etc. (Ensminger, 1994).

2.4.Salt in food

Foods naturally contain sodium at different concentrations; however, it is added deliberately to food products during processing. Table 1 presents examples of the sodium content in food products in Australia and New Zealand (NUTTAB, 2010). Salt plays various roles in food and is added for different purposes. For instance, salt is added as a preservative to reduce the water activity (a_w) in canned meat products (Taormina, 2010). Salt is added during pickle production in order to control the microbial growth and thus regulating the fermentation process. In bakery products, salt enhances the overall flavour and strengthens gluten network in dough (Reddy and Marth, 1991).

Table 1: Sodium content of food products

Food items	Sodium content (mg)/100g
Food Ingredients and additives: Herbs, seasonings and spices	3 – 27,380
Beverages: Alcoholic & non-alcoholic (mg/100mL)	1 - 370
Cereals and cereal products	1 – 9,747
Dairy and dairy products	2 – 3,728
Fruits and fruit products	1 - 116
Vegetables and vegetable products	1 – 2,070
Meats and meat products	22 – 3,700
Snack foods	1 – 8,330
Confectionery and sweet spreads	1 - 700

Retrieved from NUTTAB, 2010

2.5.Salt in cheese

Salting is an important step during cheese production. For instance, Nabulsi cheese must be kept with salt (brine or dry salt) overnight in order to improve texture and increase the shelf life (Ibrahim and O'Sullivan, 1998; Yamani et al., 1998). Salt affects the microbial growth and activity, moisture content and water activity (a_w), proteolysis, texture and microstructure of cheeses (Guinee, 2004a; Guinee and Fox, 2004). Table 2 shows the salt content, moisture and salt-in-moisture contents of various types of cheeses. Nabulsi and Domiate cheeses have higher salt content 8.8% and 6.0 %, respectively. Feta, Blue and Romano-type cheeses are considered as salted cheeses and may increase the amount of daily salt intake.

Table 2: Salt content of various cheese types[1]

Cheese type	% Salt	% Moisture	S/M[2]
Nabulsi	8.8	45.2	19.0
Domiate	6.0	55.1	10.9
Feta	4.5	63.3	5.7
Blue	4.5	42.2	10.5
Romano-type	4.1	30.0	13.8
Halloumi	3.0	46.6	6.4
Camembert	2.5	52.2	4.8
Gouda	2.4	39.0	4.9
Cheddar	1.7	37.3	4.1
Mozzarella	1.4	46.3	3.1
Emmenthal	0.7	38.7	2.0

[1] (Guinee and Fox, 2004; Toufeili and Ozer, 2006).
[2] Salt-in-moisture

2.5.1. Salting methods of cheese

Three main techniques are used for salting cheeses. Every single technique has advantages and disadvantages which affect the cheese quality.

a) **Brining**: Cheese loaves are immersed in brine solution at a particular step during cheese manufacturing process. The advantage of this method is that salt is distributed evenly on cheese loaf surface. However, this method requires large containers to keep the cheese loaves in brine. Also it consumes quite large

amount of water to prepare the brine. Brining may result in zone variation of salt content in cheese mass (Guinee et al., 2000). For example, Halloumi cheese is brined in solution (water or whey) containing 10 to 12% salt for 30 to 45 days (Papademas, 2006). Nabulsi cheese is stored in 21% brine solution for 1 year after moulded curd pieces been boiled in brine solution for 10 min (Abd El-Salam and Alichanidis, 2004).

b) **Dry salting**: Milled curd pieces are salted by adding fine salt crystals directly on the surface of pieces. This method facilitates salting process which could be carried out inside the cheese vat. However, possible uneven distribution of salt in cheese would be the main disadvantage of dry salting (Sutherland, 2003). For instance, Cheddar is dry-salted after milling.

c) **Surface salt rubbing**: Mould ripened cheese surface is rubbed with salt slurry or dry salt (Guinee, 2004a). e.g. blue vein cheese

2.5.2. Salt absorption and movement in cheese

Salt as an ionic substance that instantly dissolves in water and dissociates into two ions Na^+ and Cl^- surrounded by H_2O molecules (Chang and Cruickshank, 2005).

$$NaCl \rightarrow Na^+ + Cl^-$$

When cheese is salted there is a movement of Na^+ and Cl^- from brine or dry salt into cheese and water out from the cheese. Two opposite fluxes occur when cheese is subjected to brine or dry salting methods. Na^+ and Cl^- ions diffuse into cheese matrix toward cheese centre simultaneously; water in cheese migrates from the cheese centre to the surface (Figure 1). Salt diffusion into cheese varies based on the cheese block size and distance between cheese block centre and surface.

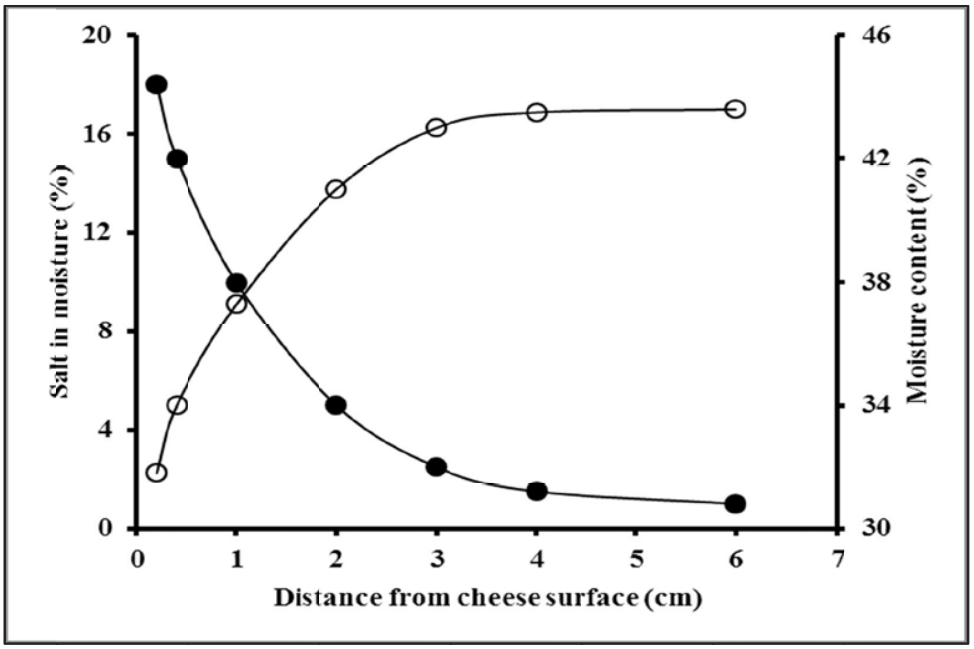

Figure 1: Moisture content (o) and salt-in-moisture (●) in a typical semi-hard cheese as a function of distance from the salting surface, from Sutherland (2003)

Geurts et al. (1974; 1980) concluded that the diffusion of NaCl into cheese and migration of H_2O out of cheese is an impeded diffusion process. The rate of NaCl diffusion in cheese matrix (cm^2) per day called coefficient of diffusion (D^*; cm^2/day) varies between 0.1 to 0.45 cm^2 / day (Guinee, 2004a). Cheese matrix consists of various components (protein, fat and ash) that act as barrier in front of NaCl ions and H_2O movements. Several factors affect the NaCl and water movements (Guinee, 2004a; Guinee and Fox, 2004):

a) Fat globules and protein aggregates are barriers that obstruct the Na^+ and water movements inside the cheese matrix. Thus, salt and water will take other tortuous paths to circumvent these barriers and thereby travel in lengthy paths.

b) Water migrates in larger amounts out of the cheese compared with low flux of Na^+ and Cl^-. Thus, the mechanical force of outward water flux could slow down the inward Na^+ and Cl^- flux.

c) The complex cheese matrix exerts a sieve effect on the diffusion of Na^+ and Cl^- into cheese and water out of cheese.

d) The soluble solids content in aqueous phase of cheese compared with pure water. Therefore the Na diffusion in cheese moisture would be slower compared with pure water.

2.5.3. Factors controlling the salt absorption

Several factors affect the salt absorption in cheese during brining or dry salting (Sutherland, 2003; Guinee, 2004a; Guinee and Fox, 2004):

2.5.3.1. Salting time

The amount of salt that is absorbed during salting process increases with the duration of salting time. However, the salting time is limited by the establishment of osmotic pressure equilibrium.

2.5.3.2. Brine concentration

The salt diffusion inside cheese matrix correlates positively with the brine concentration up to a certain limit. The higher salt concentration will increase the rate of Na^+ movement. It has been reported that an increase in brine concentration from 5% to 25% will increase salt absorption. The coefficient of diffusion (D^*) decreased sharply when salt concentration > 25% was used with brine-salted Gouda cheese (Geurts et al., 1980) and Romano cheese slices (Guinee and Fox, 1983). The reduced salt absorption may be due to dehydration of the surface layer of cheese which slow down salt movement and thereby decreases salt migration rate inward the cheese (Melilli et al., 2003).

2.5.3.3. Cheese geometry

The ratio between surface area to volume (S/V) and shape of the cheese loaf affect the salt absorption. An increase in S/V ratio will increase the salt absorption. Guinee and Fox (1986a) found that the rectangular shape of Romano-type cheese absorbed more salt than the cylindrical shape.

2.5.3.4. pH of curd and brine solution

Geurts et al. (1980) found that salt uptake in Gouda cheese at pH 5.7 was lower than that at pH 4.7. The higher salt uptake at pH 4.7 coincides with lower water loss from curds at the same pH which may be attributed to higher lactate level in low pH cheeses. A higher lactate level in aqueous phase of cheese would reduce the net loss of water (Guinee, 2004a). The effect of brine pH on salt absorption during brining needs more investigation, however it would be possible that decreasing the brine pH to about 4.7 would increase the precipitation of water soluble proteins which in turn decreases the salt uptake (Guinee and Fox, 2004).

2.5.3.5. Temperature of curd and brine

Breene et al. (1965) reported that the salt uptake of Cheddar curd at 42°C was the highest compared with those at 27, 32, and 38°C. The increase in brine temperature from 5 to 20°C increased the NaCl mobility and thereby increased the salt uptake in Gouda cheese (Geurts et al., 1974), Romano-type cheese (Guinee and Fox, 1986b) and Turkish white cheese (Turhan and Kaletunç, 1992). Any increase in the temperature of curd or brine solution would increase salt uptake in the curd. Higher temperature of brine solution may increase the pore size of the protein matrix and facilitate the true diffusion (Guinee and Fox, 2004).

2.5.3.6. Initial salt-in-moisture level of curd and pre-salting

The initial level of salt-in-moisture in curd can be controlled by dry-salting the curd before brining which is called pre-salting step. A higher initial salt-in-moisture (S/M) in curd will increase salt absorption and the final S/M level by reducing water in the final cheese. However, an excessive dry pre-salting decreases the amount of salt diffusion during brining. For instance, Melilli et al. (2003) pre-salted Ragusano curd by dry salt at 4% (w/w) level before plasticization in 4% (w/v) brine followed by ripening in 18% (w/v) brine up to 24 days at 18°C. An excessive pre-salting of curd pieces shrinks protein network in the outer layer of the curd pieces which in turn decreases the moisture in the outer layer and thereby lowers salt diffusion. Therefore, the salt uptake in Ragusano cheese was lower during ripening.

2.5.3.7. Initial moisture content in curd

The diffusion of salt during dry salting or brining methods associates positively with the increase in initial moisture content of the curd. Thus, the salt content of cheese with high initial moisture content will be higher than the cheese with low initial moisture (Guinee and Fox, 2004). Geurts et al. (1974) reported that salt absorption increased in the high initial moisture Gouda-type and Edam cheeses during brining.

2.5.4. Salt impact and role in cheese

Salt is traditionally added to cheese during processing for preservative and flavour reasons. However, salt affects directly or indirectly several other properties of cheese. The microbial growth, proteolysis, water activity, and moisture content are major parameters affected by salt addition. The level of influence on cheese properties is correlated with salt concentration in cheese (Sutherland, 2003; Guinee, 2004a; Guinee and Fox, 2004):

2.5.4.1. Effect of salt on moisture content

The increase in salt concentration decreases moisture content in cheese. This trend can be clearly observed during cheese storage in brine solution and dry salting methods. As been mentioned, cheese water migrate outward of the cheese loaf whereas salt diffuses inward gradually (Sutherland, 2003).

2.5.4.2. Effect of salt on water activity (a_w)

The curd water activity is affected by solutes dissolved in its moisture. Salt diffuses directly into cheese moisture which in turn reduces its water activity. In early stage of ripening, salt is the main factor affecting the water activity. Salt binds free water in cheese and decreases the water activity.

2.5.4.3. Effect of salt on microbial growth

Salt pays a vital role in microbial growth in cheeses. An increase in salt concentration decreases the microbial growth, both the starter and the non-starter cultures. The addition of salt to the cheese curd decreases water activity and increases the pH values which in turn reduce the microbial activity (Guinee, 2004a).

2.5.4.4. Effect of salt on enzyme activity and proteolysis

Cheese enzymes are categorized into three main groups; coagulants, indigenous milk enzymes, and microbial enzymes. The influence of salt on these enzymes is varied and related to salt concentration (Sutherland, 2003; Guinee and Fox, 2004):

a) **Coagulants**: these enzymes are mainly responsible on the primary proteolysis in cheese during coagulation and the first stage of ripening. The hydrolysis of α-casein is the main target for these enzymes (Fox and McSweeney, 1996; Upadhyay et al., 2004). It has been reported that salt concentration around 1.0%

to 6.0% simulates α-casein hydrolysis. However, further increase in salt concentration up to 20% inhibits α-casein degradation which implies inhibition in coagulant activity.

b) **Indigenous milk enzymes:** plasmin is the major indigenous enzyme that contributes to proteolysis of β-casein during cheese ripening. Any increase in salt concentration beyond 2% (w/w) starts to inhibit plasmin activity (Noomen, 1978).

c) **Microbial enzymes**: Reducing water activity at high salt concentrations may adversely affect the microbial activity. Also and as a result of the salt effect on pH, the microbial activity may be adversely affected during ripening.

2.5.4.5. Effect of salt on texture profile and microstructure of cheese

The impact of salt on texture profile and microstructure of cheese is a result of its effects on moisture content, proteolysis, and casein hydration. Significant differences in microstructure can be observed between salted and unsalted cheeses. Soft, pasty, weak, and adhesive are the main characteristics of unsalted cheese. While, salted cheese is characterized with crumbliness, dryness and hardness. Cheese microstructure is greatly affected by salt addition at low level (*ca.* < 6.0%). For instance, an increase in salt-in-moisture content from 0.25 to 3.5% in Mozzarella cheese produces more homogenous and uniform structure.

2.6.Salt related with health issues

The National Health and Medical Research Council (Anonymous, 2003) in Australia has developed a nutrient reference values for Australia and New Zealand including recommended dietary intake (RDI) for all nutrients. The RDI for sodium at different life stages and genders are presented in Table 3. These RDIs are similar to those published

in USA (Otten et al., 2006). Table 3 shows that RDI of NaCl is the same for both men and women at 1.18 to 2.36 g per day.

Table 3: The recommended daily intake of sodium (mg/day) and the equivalent amount of salt (g/day) for different life stages and genders[1]

Age (years)	Sodium mg/day		NaCl g/day	
Children & Adolescents	Recommended	Upper Level	Recommended	Upper Level
1 - 3	200 – 400	1000	0.51 – 1.02	2.56 (9 – 17 mmol)
4 - 8	300 – 600	1400	0.76 – 1.53	3.59 (13 – 26 mmol)
9 - 13	400 – 800	2000	1.02 – 2.05	5.13 (17 – 34 mmol)
14 - 18	460 – 920	2300	1.18 – 2.36	5.89 (20 – 40 mmol)
Adults (+18)				
Men	460 – 920	2300	1.18 – 2.36	5.89 (20 – 40 mmol)
Women	460 – 920	2300	1.18 – 2.36	5.89 (20 – 40 mmol)

[1] Nutrient Reference Values for Australia and New Zealand (endorsed by the NHMRC on 9 September 2005)

Several studies and worldwide organisations reports showed that the actual daily intake of sodium is greater than the RDI (Elliott and Brown, 2006; WHO, 2007). Elliott and Brown (2006) reported that the daily sodium intake is in excess of 100 to 200 mmol per day especially in Asian communities. The UK dietary and nutritional survey of adults showed that the daily salt intake was 11 g in men and 8.1 g in women which is nearly 5 times than of RDI (Hoare et al., 2003). Excessive salt intake is highly correlated with various chronic diseases:

a) Salt and hypertension

Hypertension is a term that is used to describe a high blood pressure disease. According to the seventh Joint National Committee (JNC 7), normal individuals have blood pressure of *ca* 120/80 mmHg while individuals with ≥ 140/90 mmHg blood pressure are classified as hypertensive. Individuals with blood pressure greater than 120/80 but lower than 140/90 mmHg are considered as pre-hypertension (Chobanian et al., 2003). Epidemiological studies, treatment trials, and animal studies have provided evidences on the positive association of sodium intake with hypertension. Thus, an excessive

intake of sodium is highly correlated with hypertension (Kotchen, 2005; He and MacGregor, 2007; Penner et al., 2007).

b) Salt and cardiovascular diseases (CVD)

Cardiovascular diseases (e.g. heart attack, stroke) are major death causes in the world. World Health Organisation (WHO) reported that 30% of total death incidences in 2005 were due to CVD. Moreover, the report showed that this level will increase by 17% in 2006 to 2015 (WHO, 2005). Numerous epidemiological studies have shown that hypertension is a major risk factor that causes CVD (Alderman, 2006; He and MacGregor, 2007; Penner et al., 2007). Thus, increased salt intake is positively correlated with CVDs.

c) Salt and other harmful health issues

Several studies have shown positive association between kidney stones and increased salt intake (Massey, 1995; Lin et al., 2003; Massey, 2005). Stomach cancer is the second death cause compared to other cancer types. Ecological studies have shown significantly positive correlation between salt intake and stomach cancer (Joossens et al., 1996; He and MacGregor, 2007).

Therefore, dietary salt reduction is considered a worldwide necessity. Numerous health organisations and epidemiological studies recommend reducing salt intake in order to reduce the risk of chronic diseases especially hypertension (Elliott and Brown, 2006; WHO, 2007).

2.7.Salt reduction approaches

Over the last decade, more than 250 patents were registered for salt reduction in different food products (Toldrá and Barat, 2009). In UK, the Food Standard Agency (FSA) cooperated with UK food industry to reduce salt in food products and targeted different salt reduction levels for different food items (Angus, 2007). Several

approaches have been applied in order to reduce salt content in foods during manufacturing without adversely affecting food safety and quality.

2.7.1. Simple salt reduction

This approach is aimed at gradually reducing the amount of salt that is added to food during processing. Uncontrolled salt reduction may have adverse effects on food properties. Low taste, high microbial growth and weak texture are the main characteristics of low-salt products (Reddy and Marth, 1991; McMahon, 2010). Several studies have been carried out to investigate the lowest salt concentration that can be applied without affecting food safety. However, risk of pathogens growth and low taste are burdens on food industries.

2.7.2. Salt reduction combined with food additives

In respect to safety issues related to simple reduction of salt in food, several researchers proposed to add natural food preservatives to low salt products (Toldrá and Barat, 2009). Nisin, lactoferrin, and other natural antimicrobials have been suggested to be added in food products in order to prevent or supress food pathogens and increase the product shelf-life (Taormina, 2010). Thus, the natural antimicrobials would compensate the reduced salt effect on pathogens. However, the type of antimicrobial, targeted pathogen and food matrix are main factors that affect the efficacy of a particular antimicrobial agent (Betts et al., 2007; Durack et al., 2008). Taste enhancers such as monosodium glutamate (MSG), alapyridaine, glycine and others are added to food products in order to enhance the flavour and mask the lack of saltiness (Kilcast and den Ridder, 2007; Durack et al., 2008). Nonetheless, food additives generally are not preferred by consumers.

2.7.3. Salt substitution with other salts

Salt replacement with other salts (potassium chloride, calcium chloride and magnesium chloride) may be considered as the most successful approach among salt reduction strategies. Several researchers reported that salt replacement had similar effect on food properties compared with control made with NaCl (Kilcast and den Ridder, 2007). Potassium chloride (KCl) was successfully used to substitute NaCl up to 50% in various foods (Guinee, 2004b; Kilcast and den Ridder, 2007). Epidemiological studies showed that excessive intake of potassium had no adverse effect on hypertensive patients (Geleijnse et al., 2003; Geleijnse et al., 2007). The main drawback associated with KCl is the bitterness which can be noticed at high KCl concentration.

2.8.Attempts to reduce salt in various foods

Numerous studies and industrial attempts have been carried out in order to reduce salt in various foods using different salt reduction approaches. Meat and poultry products, dairy products, bakery products, and other food items contribute to the daily sodium intake by particular percentages which vary based on country and community food habits (Anonymous, 2003; Desmond, 2006). For instance, bread, cereals and grains contributes by 19.5% and 34.6% in USA and UK, respectively (Anderson et al., 2010). In Australia, the Food Standards Australia New Zealand (FSANZ) has estimated the sources of sodium in the Australians` diet. The FSANZ found that bread product, meat products, cereals products, and other food items contributed by 24%, 21%, 17% and 24% in the daily sodium intake, respectively (Anonymous, 2011). The Food Standard Agency in UK aimed to reduce salt content in meat and poultry products by 40% to 50% by 2006. However, it seemed that this goal was not achievable and thereby the Food Standard Agency extended the period to 2010 in order to achieve the target. Three

salt reduction approaches mentioned above were applied on different meat products in order to maintain quality and safety (Desmond, 2006).

2.9.Salt reduction in cheese

Since 2002, cheese production has been increasing by ~ 2% per annum. The worldwide cheese consumption is high especially in the developed countries with about 12.5 Kg per capita per annum (Fox and McSweeney, 2004). In Australia, the total cheese production in 2010 reached 338000 tonnes while the cheese consumption was 12.7 Kg per capita per annum (Anonymous, 2012). It has been reported that cheeses contribute to daily sodium intake by 7.8% in UK, 9.2% in France, 8.2% in USA and 5% in Australia (Anonymous, 2003; Meneton et al., 2009; Anderson et al., 2010). The approximately high cheese consumption in these countries that contribution to the daily sodium intake encouraged dairy scientists to embark on projects to reduce salt content in cheese as a part of the worldwide attempt to reduce sodium intake (WHO, 2007). Several issues are associated with low-salt cheese production (McMahon, 2010).

2.9.1 Simple salt reduction in cheese

Several attempts have made to reduce salt without any preservatives or salt replacement. Schroeder et al. (1988b) prepared Cheddar cheese with salt content ranging from 0% to 1.75% and investigated the effects on chemical composition, proteolysis parameters, sensory properties, and microbial growth over 7 months of storage. Cheeses containing 0.88% to 1.75% salt showed no significant difference in term of overall acceptability compared with control Cheddar cheese. However, overall proteolysis and microbial growth increased with NaCl reduction. Wyatt (1983) evaluated the overall acceptability of low-salt Cottage cheese with 25, 50, and 75% less salt and found a significant drop in the overall acceptability above 50% reduction. However,, concerns on microbial safety,

bitterness development, soft texture and abnormal fermentation during storage are main issues that could not be overcome with simple salt reduction (Guinee and O'Kennedy, 2007; McMahon, 2010).

The overall acceptability of low-salt cottage cheese with 25, 50, and 75% less salt was examined by Wyatt (1983). A significant drop in the overall acceptability above 50% reduction was reported. Schroeder et al. (1988a) investigated the effect of salt reduction ranging from 1.44% to 0% (w/w) on sensory, microbiological and chemical properties of Cheddar cheese. . The authors found that proteolysis, growth of lactic acid bacteria and water activity increased in low-slat cheeses over 7 months of storage period. The overall acceptability of Cheddar cheeses salted between 0.88 to 1.44% was similar. The low salt Cheddar cheese (at 0.73%) received normal acceptability scores (Schroeder et al., 1988a). Kelly et al. (1996) reported that the rate of proteolysis in Cheddar cheese made with different salt levels (0 to 3.3%, w/w) had an inverse relationship with salt concentration. The higher the salt concentration was the lower the proteolysis rate. Kristiansen et al. (1999) examined proteolysis in Danbo-type cheese brined at four different levels of NaCl (0.06, 2.6, 4.3 and 6.4%) for 96 hours at 16ºC. The authors found that cheeses brined at higher salt concentration had lower proteolysis compared with other cheeses. Physio-chemical properties of Feta cheese brined at 8, 15, and 18% NaCl was investigated by Prasad and Alvarez (1999) during the storage period of 63 days. The authors found that Feta cheeses brined in higher salt concentration became harder (Prasad and 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) for two weeks. Cheeses kept in 20% and 25% brines showed higher hardness and a change in colour compared to those in lower salt concentration. The texture changes of Dil cheese

brined at two salt concentrations (3 and 6% NaCl) during storage for 3 months were examined by Kilic and Isin (2004). At high salt concentration, Dil cheeses showed harder texture compared with cheeses kept at lower salt concentrations. Guven et al. (2006) prepared Beyaz cheese (Turkish white-brined cheese) followed by brining at 12, 14, 16 or 18% NaCl and ripening at 7°C for 9 weeks. Beyaz cheeses brined in 12 and 14% NaCl concentration showed higher overall acceptability and flavour scores compared with those kept at 16 and 18% brine. The authors demonstrated that all cheeses were affected by storage period in terms of dry matter, pH values, protein content and hardness. Hamid et al. (2008) made Sudanese white cheese and stored at 7ºC for 8 months in different brine solutions (4 - 6% salt concentrations). The authors reported that the microbial growth and proteolysis in cheeses brined in 4% salt concentration was higher than those brined at cheeses with 6% salt content. In contrary, the fat and crude protein contents in cheeses kept at 6% brine were higher with significantly better sensory characteristics than those kept in 4% brine (Hamid et al., 2008).

2.9.2 NaCl substitution with other salts

Recently, replacement of NaCl with other salts such as potassium chloride (KCl), calcium chloride ($CaCl_2$), and magnesium chloride ($MgCl_2$) or with patented salt mixture has been reported (Guinee and Fox, 2004; Guinee and O'Kennedy, 2007; Durack et al., 2008). Lefier et al. (1987) salted Gruyere cheese with NaCl/$MgCl_2$ mixture which showed acceptable taste with slight bitterness and soft body. Martens et al. (1976) lowered salt content of Gouda cheese from 650 to 72 mg/100g and replaced it with 283 mg KCl /100g and reported acceptable sensory properties and good quality.

Reddy and Marth (1993a) successfully prepared Cheddar cheese with NaCl/KCl mixture with acceptable organoleptic properties similar to control cheese made with

only NaCl, and no significant differences in chemical composition, microbiological and texture properties. Katsiari et al. (1997; 2000a; b) investigated the effect of partial substitution of NaCl with KCl at different levels (3NaCl:1KCl, 1NaCl:1KCl, and 1NaCl:3KCl) on Feta cheese characteristics during 180 day of storage and found no significant differences in all sensory attributes compared with control. However, cheese salted with 3NaCl:1KCl scored somewhat higher than other salt mixtures. This study also showed no significant differences in physio-chemical and microbiological parameters between experimental Feta cheeses.

Similar results were reported in a study examining the effects of partial salt replacement with KCl on Greek Kefalograviera cheese (Katsiari et al., 1998; 2001b; a). Kefalograviera cheeses salted with 3NaCl:1KCl, 1NaCl:1KCl and 1NaCl:3KCl had similar sensory scores with no significant differences compared with control.

Guven and Karaca (2001) prepared white cheese and stored in different brines containing 1NaCl:1$CaCl_2$; 1NaCl:1KCl; 1NaCl:1$MgCl_2$; or 1NaCl:0.33$CaCl_2$:0.33KCl:0.33$MgCl_2$ for 12 weeks. They reported no significant differences in proteolysis indices and acidity between experimental cheeses at the same storage period. However, this study did not investigate the organoleptic properties of the cheeses stored in different brines.

Fynbo (a semi-hard Danish cheese) was prepared by Laborda and Rubiolo (1999) with NaCl/KCl mixture who then examined proteolysis indices and sensory properties. They found that the proteolysis indices did not differ significantly between Fynbo cheese salted with NaCl/KCl mixture and the control made only with NaCl at the same ripening temperature and storage time. The sensory quality of Fynbo cheese salted with NaCl/KCl mixture was found to the acceptable (Zorrilla and Rubiolo, 1999). Kamleh et al. (2012) have concluded that salt substitution with KCl had no significant effect on

overall acceptability of Halloumi cheese and therefore salt substitution could be successfully used in Halloumi cheese manufacture.

On the base of these studies, we may conclude that partial replacement of NaCl with NaCl/KCl mixtures was successful without adverse effect on cheese characteristics compared with simple salt reduction. Furthermore, KCl was found to be the most acceptable salt for partial NaCl replacement among other salts. KCl intake has not been linked to development of hypertension and cardiovascular diseases (Buemi et al., 2002; Geleijnse et al., 2007). Several drawbacks can be noticed in the previous studies which need further investigations:

1) The impact of salt replacement should be studied on several cheeses characterized by high salt contents such as Halloumi, Akawi and Nabulsi cheeses. Due to the unique characteristics of every individual cheese and the differences in manufacturing processes, the effect of salt substitution with KCl should be investigated in each individual cheese.

2) Although the microbial growth has been reported to be affected by salt substitution, the effect on the proteolytic enzymes of the starter cultures during cheese ripening need to be further explored since these enzymes have great role in cheese ripening which in turn affects the cheese quality and flavour.

2.10. Mediterranean cheeses

It is commonly believed that the Mediterranean region is the origin where cheese was first evolved (Fox and McSweeney, 2004). A few Mediterranean cheeses types have become highly popular around the world such as Mozzarella, Feta, and Halloumi cheeses. Relatively, Mediterranean cheeses contain relatively higher salt content such as Feta (5.0%), Halloumi (3.0 – 5.0%), Nabulsi (8.8%), Akawi (5.0%) and Mozzarella (2.0 to 3.0%) cheeses. The contribution of Mozzarella cheese to daily sodium intake is

considerable due to high consumption of Mozzarella cheese (especially the low-moisture Mozzarella cheese; LMMC) as ingredient in different food items such as pizza, pasta…etc (Kindstedt et al., 2004). Therefore, any attempt to reduce its salt content would be greatly beneficial.

2.10.1. Low-moisture Mozzarella cheese

Mozzarella cheese is an Italian traditional pasta-filata cheese which is traditionally made from buffalo milk. Currently, Mozzarella cheese is globally produced from bovine milk at about 100 tonnes or more per day (Kindstedt et al., 2004). Because of its functional properties, Mozzarella cheese especially its low-moisture variety (LMMC) is commonly used as pizza topping. LMMC`s moisture content is around 45 - 52% and its fat-in-dry matter 45% (Jana and Upadhay, 1991). Stretchability, meltability, browning, elasticity and oiling off are major functional properties of LMMC (Kindstedt, 2004). There are no precise definitions for these attribute, however a general definition was presented by Kindstedt (1995). Stretchability is the ability of the cooked cheese strands to remain together as a cohesive mass while being pulled. Meltability is defined as the ease with which cheese flows or spreads upon heating and loss of integrity of the individual cheese shreds (Rowney et al., 1999). Browning occurs during pizza cooking at high temperature as a result of decomposition of sugars and Maillard reaction between amine groups and reducing sugars (lactose and galactose) (Matzdorf et al., 1994; Mukherjee and Hutkins, 1994). Oiling-off is defined as releasing of fat from cheese shreds during pizza baking at a high temperature. However, excessive fat release results in pools of oil on the surface of pizza which is considered as defect. Therefore, a moderate oiling-off is a desirable characteristic which improve the mouthfeel and glossy appearance (Wang and Sun, 2004).

A general LMMC production process is presented in Figure 2. Bovine milk is pasteurized at 72°C for 15 seconds after the milk has been standardized. The pasteurized milk is tempered at around 37 - 40°C followed by starter culture addition and left for 30-45 minutes. Rennet is added at ~ 32°C and set for 35-40 minutes until curd is formed. The curd is then cut into small cubes and cooked at temperature 35 - 40°C for 10-25 minutes and then whey is drained. Curd is aggregated together forming block to begin cheddaring step which ends at pH 5.3-5.2. Curd blocks are cut into small pieces and immersed into hot water 60 - 85°C for few minutes. A gentle stretching and folding motion is made until smooth texture with white plastic mass is formed. The hot plastic mass is transferred into stainless steel molds. After cooling in chilled water, the formed blocks are floated in cold brine solution (around 4% NaCl) for 2-12 hour followed by packing and storing at low temperatures (Kosikowski and Mistry, 1997).

Several factors impact on the functional properties of LMMC which have been extensively studied. Proteolysis, moisture content, fat content, calcium content, pH, and salt content are the major factors (Kindstedt, 1995; Guinee et al., 2002). Salt content of LMMC varies (~ 1.4% to 2.5%; w/w) depend on salting method, brine concentration and time. Several studies have been carried out in order to investigate the effect of salt concentration and salting methods on Mozzarella cheese properties.

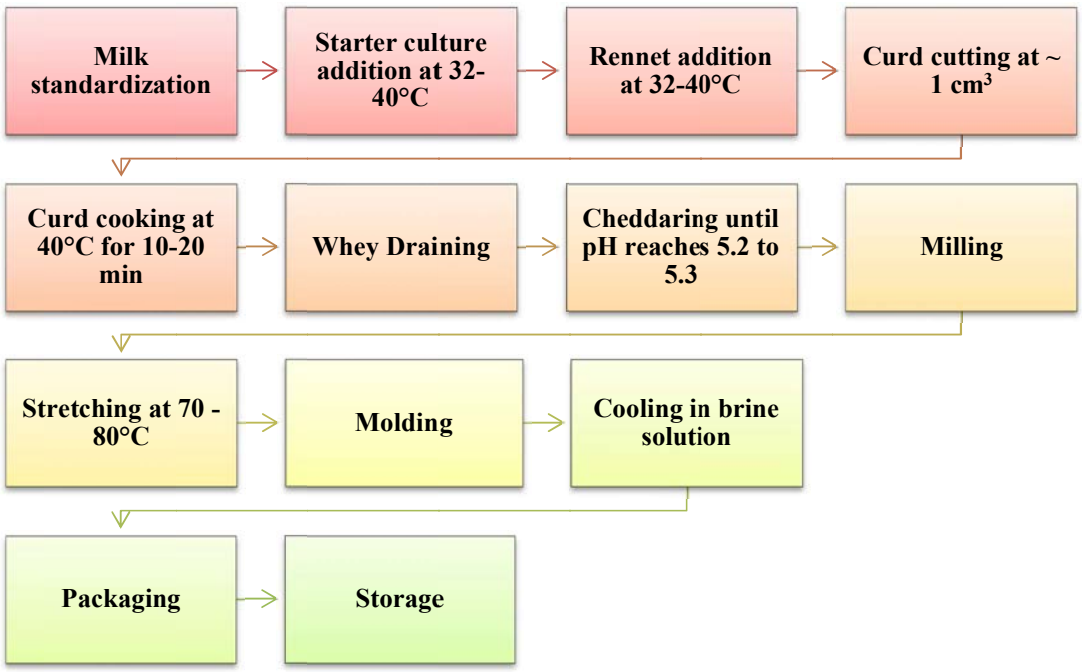

Figure 2: Block diagram of low-moisture Mozzarella cheese production

Paulson et al. (1998) studied the effect of different salt concentrations and salting methods on non-fat Mozzarella cheese properties. The authors found that moisture content in dry-salted cheese by 1% was higher compared with 0% and 0.5%. A different microstructure was observed between unsalted and salted Mozzarella cheeses. Unsalted cheeses had numerous pocket filled with whey and larger fissures than salted cheeses (Paulson et al., 1998). Guinee et al. (2000) investigated the effects of salting method (dry or brine) on LMMC`s chemical composition, proteolysis and functional properties during storage. The authors concluded that LMMC salted with dry method had higher moisture content, lower protein and calcium, was less hard and gave higher actual yield. Brine salting of LMMC is time consuming and produces zonal variation throughout the cheese mass (Guinee et al., 2000).

Because of the high consumption of LMMC as pizza topping around the world which in turn may increase the total daily sodium intake, an attempt to produce low-salt LMMC would fall within WHO recommendations to reduce salt in food product. To our knowledge, there is no information available in terms of the effect of NaCl substitution with KCl on LMMC characteristics during storage.

2.10.2. Halloumi cheese

Halloumi cheese one of the most famous semi-hard Mediterranean cheeses. It is traditionally produced in Cyprus; at the present, it is produced in significant amounts throughout the world. It is believed that the cheese was introduced to Cyprus by Arab mercenaries from Syria and Palestine (Papademas, 2006). Since 1999 the Halloumi cheese export has increased significantly throughout the world. Traditionally, Halloumi cheese was produced from sheep and goat milk, however and because of the seasonal production of goat and sheep milks, cow`s milk is used to produce Halloumi cheese (Papademas and Robinson, 1998). Figure 3 shows the steps of traditional Halloumi cheese production. Briefly, raw milk is tempered to 37^{o}C, and starter culture (i.e. yogurt) is added. After 30 min of ripening, coagulant is added and set for 35 min. The curd is cut into 1 cm^{3} cubes, let stand for 10 min followed by stirring for 15 min at 37^{o}C. The curd is transferred into cheesecloth and whey is drained. The curd is pressed under a pressure of 0.35 – 0.45 MPa for about 1 h to fuse the curd pieces and expel whey. Pressed curd is cut into various retail blocks and heated in deproteinized whey at 90 – 95^{o}C for at least 30 min with continuous stirring. Afterwards, blocks are dry-salted or immersed into brine solutions. Cheese blocks are vacuum-packaged or kept into containers with brined whey solution.

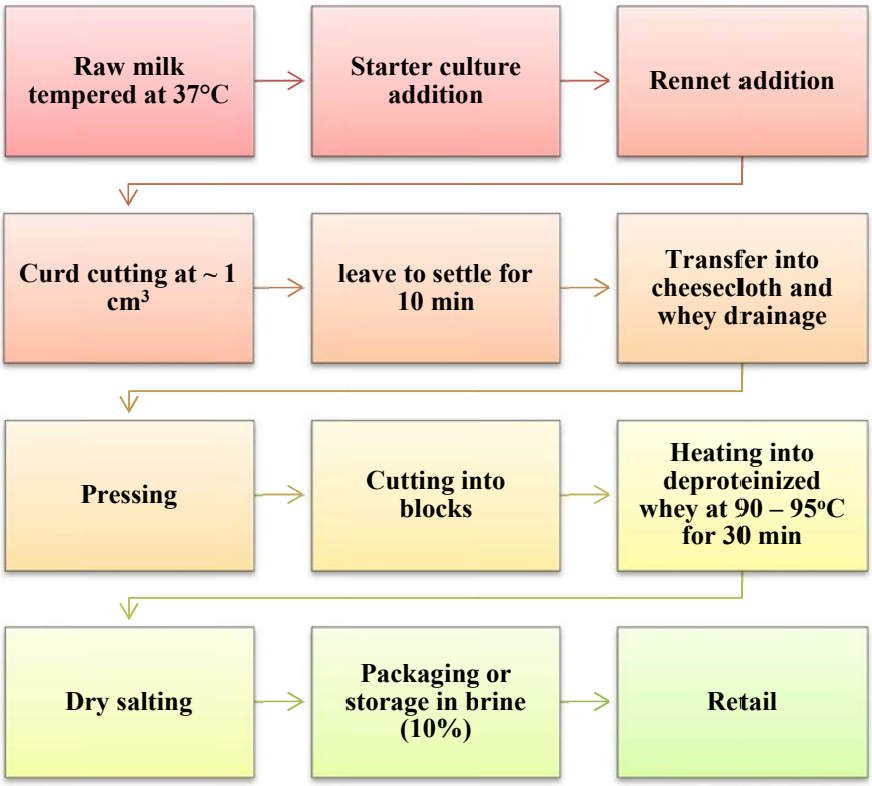

Figure 3: Block diagram of traditional Halloumi cheese production

Salt content of Halloumi cheese varies from 2.7 up to 4.5 g/100 g which is considered as high salt content. Usually Halloumi cheeses are kept in high salt brine around 10% in various retail containers. Several studies have been carried out to investigate the influence of milk source, fat content, rennet concentration, ripening and brining conditions on Halloumi cheese characteristics (Kaminarides et al., 2000; Papademas and Robinson, 2000; Guley and Akbulut, 2004; Raphaelides et al., 2006; Kaminarides et al., 2007; Theophilou and Wilbey, 2007).

Atasever et al. (2003) examined the impact of different salting methods (dry salting or brining) on Halloumi cheese properties and found that dry salted cheeses had lower moisture, ash and salt contents and higher acidity value and fat content compared with

brine salted cheeses. However, the coliform, total viable count and yeast and mould were lower in brine salted cheeses.

According to Halloumi cheese manufacturing process, it contains high salt concentration which makes Halloumi cheese a hazardous food that hypertensive people should avoid. At present, there is no details are available about salt reduction in Halloumi cheese by substitution with KCl.

2.10.3. Nabulsi cheese

Nabulsi cheese is one of the most popular cheeses in Middle Eastern countries such as Jordan, Palestine, Lebanon and Syria. Traditionally it is produced from sheep or goat milk or mixture of both. Therefore, production of Nabulsi cheese is greatly increased in spring season. The high salt content of Nabulsi cheese allows it to stay safe and be able to consume for one year (Abd El-Salam and Alichanidis, 2004). This cheese is classified as unripened, semi-hard cheese with moisture content 45 to 55% and salt content around 8.8%. The manufacturing steps of Nabulsi cheese is illustrated in Figure 4.

Raw or pasteurized milk is tempered to ~32 - 35 °C followed by rennet addition and setting for 30 to 60 min. Coagulated milk is cut into ~ 1 cm^3 cubes and settled for 10-15 min and then whey is drained. Curd is transferred into cheesecloth for continued whey drainage. Afterwards, curd is pressed under 0.4 MPa per cm^2 for ~ 2 h followed by cutting into small blocks (~ 4 × 4 × 1.5 cm) and then sprinkled with salt or immersed in a brine solution (18 to 21%) overnight. Cheese blocks are boiled in a brine solution (18 to 21%) for 5 to 10 min and cooled at room temperature. Cooled blocks are stored in metal or plastic containers filled with a brine solution (18 to 21%) (Toufeili and Ozer, 2006).

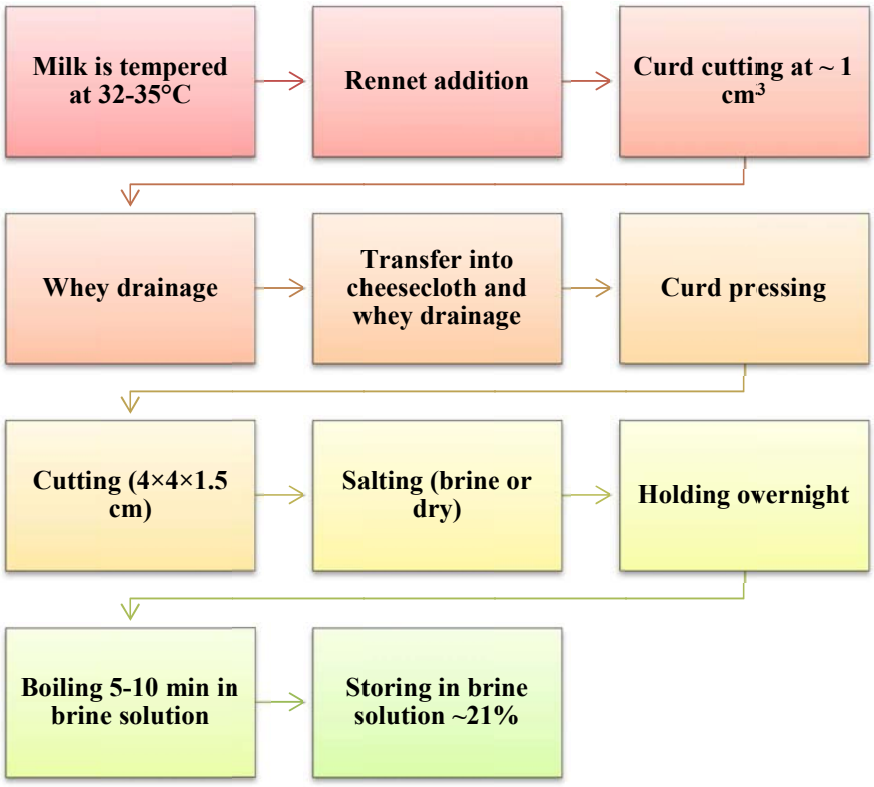

Figure 4: Block diagram of Nabulsi cheese production

Nabulsi cheese quality varies according to milk source, milk seasonality, salt concentration and boiling process. High salt concentration and boiling process of Nabulsi cheese play major roles in terms of cheese safety. However, its high salt content prevents people with hypertension and pre-hypertension condition to consume this cheese. To present, no attempt has been reported to reduce salt content in Nabulsi cheese by replacement with KCl.

2.10.4. Akawi cheese

This cheese is popular in Middle Eastern countries (Syria, Lebanon, Jordan, and Palestine) and has recently become well known in the Gulf countries (Saudi Arabia,

Kuwait, and United Arab Emirates). The manufacturing steps are illustrated in Figure 5. Traditionally, Akawi cheese is produced from pasteurized bovine or ovine milk (or a mixture of both). For cheese making, the milk is pasteurized, cooled to about 35 ºC followed by starter culture addition (1.5%). After 1 h, rennet is added to coagulate milk within an hour. The curd is then cut in ~ 1 cm^3, whey drained and curd pieces are wrapped into cheesecloth in small portions (150 to 250 g) followed by pressing for about 1 h and brining in approximately 10 – 15% brine solution at 4ºC. In general, Akawi cheese contains about 51.0% moisture, 21.6% fat, 22.5% protein and 5% ash. Storage of Akawi cheese in brine solution is a critical step for maintaining its quality and safety during 1 month of storage (Abd El-Salam and Alichanidis, 2004).

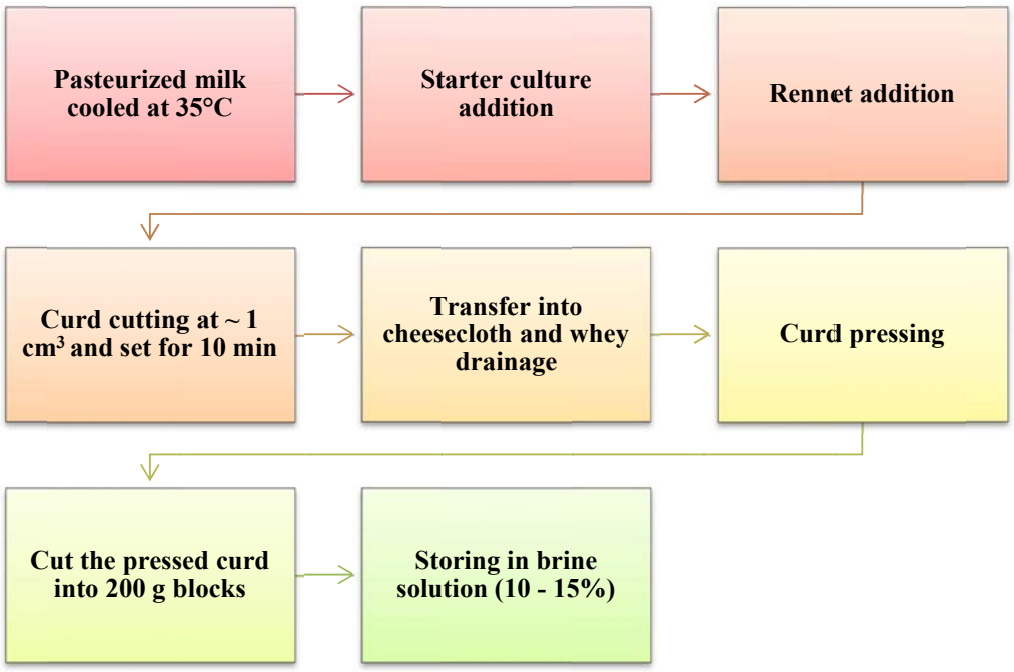

Figure 5: Block diagram of the Akawi cheese manufacturing

People in Middle Eastern countries consume Akawi cheese at breakfast and with dinner meals. However, due to the high salt content of this cheese which is a result of brining in high NaCl solution (~ 15%), hypertensive people are unable to consume it. Therefore,

a valuable knowledge and great health benefits would be achieved when produce Akawi cheese with low salt content by substitution with KCl.

3. Chapter 3: Effect of partial substitution of NaCl with KCl on Halloumi cheese during storage: Chemical composition, lactic bacterial count, and organic acids production

Introduction

Chapter three investigate the effect of NaCl substitution with KCl on chemical composition, lactic acid bacteria population and organic acids production in Halloumi cheese. The paper entitled Effect of partial substitution of NaCl with KCl on Halloumi cheese during storage: Chemical composition, lactic bacterial count, and organic acids production by Ayyash M.M and N.P Shah was published in the peer reviewed Journal of Food Science 75(6):C525-C529

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

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

Declaration by [candidate name]: Mutamed Ayyash **Signature:** [redacted] **Date:** 11/9/12

Paper Title:

Effect of partial substitution of NaCl with KCl on Halloumi cheese during storage: Chemical composition, lactic bacterial count, and organic acids production

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

Name	Contribution %	Nature of Contribution
Mutamed Ayyash	75	Design and perform the experiment Perform the samples analysis
		Evaluate the analytical data Perform the statistical analysis by SAS
		Prepare the major part of the manuscript
Nagendra Shah	25	Contribute to writing manuscript and submission to Journal

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
5. The original data is stored at the following location(s):

Location(s):
School of Biomedical and Health Sciences, Faculty of Health, Engineering and Science, Victoria University - Werribee campus, Victoria, Australia

and will be held for at least five years from the date indicated below:

		Date
Signature 1	[redacted]	11/09/12
Signature 2		11/09/12
Signature 3		
Signature 4		

Ayyash, M.M. and Shah, N.P. (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, 75: C525-C529. doi:10.1111/j.1750-3841.2010.01691.x

4. Chapter 4: Effect of Partial Substitution of NaCl with KCl on Proteolysis of Halloumi Cheese.

Introduction

Chapter four investigates the effect of partial substitution of NaCl with KCl on proteolysis of Halloumi cheese during storage for 56 days at 4^{o}C. A paper entitled Effect of Partial Substitution of NaCl with KCl on Proteolysis of Halloumi Cheese by Ayyash M.M and N.P Shah was published in the peer reviewed Journal of Food Science 76(1):C31-C37

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

Declaration by [candidate name]: Mutamed Ayyash **Signature:** **Date:** 11/9/12

Paper Title:

Effect of Partial Substitution of NaCl with KCl on Proteolysis of Halloumi Cheese

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

Name	Contribution %	Nature of Contribution
Mutamed Ayyash	75	Design and perform the experiment Perform the samples analysis
		Evaluate the analytical data Perform the statistical analysis by SAS
		Prepare the major part of the manuscript
Nagendra Shah	25	Contribute to writing manuscript and submission to Journal

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DECLARATION BY CO-AUTHORS

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1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to a) granting bodies, b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit; and
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Signature 1		11/9/12
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Signature 3		
Signature 4		

Ayyash, M.M. and Shah, N.P. (2011), Effect of Partial Substitution of NaCl with KCl on Proteolysis of Halloumi Cheese. Journal of Food Science, 76: C31-C37. doi:10.1111/j.1750-3841.2010.01901.x

5. Chapter 5: The effect of sodium chloride substitution with potassium chloride on texture profile and microstructure of Halloumi cheese.

Introduction

Chapter five investigates the effect of partial substitution of NaCl with KCl on texture profile and microstructure of Halloumi cheese during storage at 4°C for 56 days. Sensory evaluation of Halloumi cheese is presented in Appendix A. A paper entitled the effect of sodium chloride substitution with potassium chloride on texture profile and microstructure of Halloumi cheese by Ayyash M.M., F. Sherkat, R. Williams, Phil Frances and N.P Shah was published in the peer reviewed Journal of Dairy Science 94(1):37-42. The microstructure images in this chapter are enlarged in Appendix C, pages 166-169.

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Declaration by [candidate name]: Mutamed Ayyash **Signature:** [redacted] **Date:** 11/9/12

Paper Title:

The effect of sodium chloride substitution with potassium chloride on texture profile and microstructure of Halloumi cheese

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

Name	Contribution %	Nature of Contribution
Mutamed Ayyash	70	Design and perform the experiment Perform the samples analysis
		Evaluate the analytical data Perform the statistical analysis by SAS Prepare the major part of the manuscript
Nagendra Shah	10	Contribute to writing manuscript and submission to Journal
Frank Sherkat	10	Contribute to writing manuscript
Roderick Williams	5	Contribute to writing a texture profile method part of the manuscript
Phil Frances	5	Contribute to writing a microstructure method part of the manuscript

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
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and will be held for at least five years from the date indicated below:

		Date
Signature 1	[redacted]	11/9/12
Signature 2		11/09/12
Signature 3		11/09/12
Signature 4		
Signature 5		20/9/12.

J. Dairy Sci. 94:37–42
doi:10.3168/jds.2010-3407

The effect of sodium chloride substitution with potassium chloride on texture profile and microstructure of Halloumi cheese

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ABSTRACT

The effect of partial substitution of NaCl with KCl on texture profile and microstructure of Halloumi cheese was investigated. Four batches of Halloumi cheese were made and kept in 4 different brine solutions (18%, wt/wt), including A) NaCl only, B) 3NaCl:1KCl, C) 1NaCl:1KCl, and D) 1NaCl:3KCl and then stored at 4°C for 56 d. The texture profile was analyzed using an Instron universal machine, whereas an environmental scanning electron microscope was used to investigate the effect of NaCl substitution on the microstructure of cheeses. No significant difference was found in hardness, cohesiveness, adhesiveness, and gumminess among experimental cheeses at the same storage day. Hardness, cohesiveness, and gumminess decreased significantly during storage period with the same salt treatment, whereas adhesiveness significantly increased. Environmental scanning electron microscope micrographs showed a compact and closed texture for cheeses at the same storage period. The microstructure of all cheeses became more closed and compact with storage period. Calcium content negatively correlated with hardness and Na and K contents during storage with the same salt treatment.
Key words: Halloumi cheese, texture profile, environmental scanning electron microscope, microstructure

INTRODUCTION

Halloumi cheese is a traditional Cyprus cheese made from ovine, caprine, and bovine milk (Anifantakis and Kaminarides, 1983). Recently, Halloumi cheese has become popular in the Middle Eastern countries (Kaminarides et al., 2007) and has gained an international acceptance in the European Union and the United States (Papademas and Robinson, 2001; Tamime, 2006). Normally, its color varies from whitish to yellowish, depending on the milk source (Robinson, 1991). Heating Halloumi pieces at 90 to 95°C in heat-deproteinated whey for 30 min is considered a distinctive processing step during cheese manufacturing. Halloumi cheese is usually stored in 12 to 18% brine solution at 4°C (Abd El-Salam and Alichanidis, 2004). Although salt influences cheese characteristics, including proteolysis, lipolysis, texture profile, and flavor, and traditionally has been used as a preservative (Guinee and Fox, 2004), it causes serious health issues for consumers. A high positive association between NaCl intake and hypertension has been reported (Forshee, 2008). Similarly, a positive correlation has been found between salt intake and osteoporosis and kidney stones (Massey and Whiting, 1995; Massey, 2005; Heaney, 2006). The dietary Na intake of NaCl among people in developed countries is 10 to 35 times higher than the recommended daily intake of 2.5 g of sodium (Shank et al., 1982; Kaplan, 2000; Guinee, 2004). Hence, there has been a trend to decrease NaCl content in food (Reddy and Marth, 1991; Guinee, 2004); however, a reduction in salt adversely affects the characteristics of cheeses and other foods (Petik, 1987; Katsiari and Voutsinas, 1994a; Johnson et al., 2009). Numerous studies have been carried out to decrease Na content in cheeses by substituting NaCl with KCl (Zorrilla and Rubiolo, 1994; Katsiari et al., 1998; Sihufe et al., 2006). An excess of dietary intake of KCl has no undesirable effect on people with Na-induced hypertension (Lemann et al., 1993).

In general, texture profile and microstructure of cheeses are important parameters. Salting of cheeses affects the texture profile and microstructure directly or indirectly. Several studies have investigated the effect of partial substitution of NaCl with KCl on the texture profile of cheeses. Fitzgerald and Buckley (1985) reported that the texture profile of Cheddar cheese made with a NaCl and KCl mixture was similar compared with that of the control (only NaCl). Katsiari et al. (1997, 1998) reported similar results for Feta and Kefalograviera cheeses, respectively. However, information

Received May 5, 2010.
Accepted October 12, 2010.
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is lacking on the texture profile and microstructure of Halloumi cheese as a result of reduction in the salt level. In this study, our objectives were to investigate the effect of partial substitution of NaCl with KCl on the texture profile and microstructure of Halloumi cheese during storage at 4°C for 56 d.

MATERIALS AND METHODS

Cheesemaking

Homogenized and pasteurized full fat bovine milk (3% fat) was purchased from a local dairy plant (Melbourne, Victoria, Australia). Halloumi cheese was manufactured according to Alichanidis and Polychroniadou (2008) with some modifications. In brief, milk (20 L) was tempered to 37°C, and starter culture consisting of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* was added according to manufacturer instructions (Chr. Hansen, Bayswater, Victoria, Australia). After 30 min of ripening, 5 mL of chymosin (Chy-Max, Chr. Hansen) diluted in 100 mL of distilled water was added to milk, followed by mixing for 2 min. The milk coagulated in 35 min and the curd was cut into 1 cm^3 cubes and let stand for 10 min, followed by stirring for 15 min at 37°C. The curd was transferred into cheesecloth and whey was drained. The curd was pressed for 60 min; each 1 kg of curd was pressed with a 60-kg weight. Pressed curd was cut into 6 × 6 × 3-cm loaves and heated in heat-deproteinized whey at 90 to 95°C for 30 min. Cheeses were randomly distributed into 4 different brine solutions (18%, wt/wt) made from different NaCl:KCl ratios as follows: NaCl only (A; control); 3NaCl:1KCl (B); 1NaCl:1KCl (C); and 1NaCl:3KCl (D). Cheeses in brine solutions were stored at 4°C and samples were taken for determination of texture profile, microstructure, and Na, K, and Ca contents at 0, 14, 28, 42, and 56 d of storage. Experimental cheeses were replicated 4 times.

Chemical Composition

Moisture was determined by the oven-drying method at 102°C, fat by the Babcock method, protein by the Kjeldahl method, and ash by the muffle furnace method according to the AOAC methods (AOAC International, 1995). For pH measurement, grated cheese (20 g) was macerated with 20 mL of distilled water, and the pH of the resultant slurry was measured using a calibrated digital pH meter (MeterLab, Pacific Laboratory Products, Blackburn, Victoria, Australia). Analyses were carried out in quadruplicate.

Texture Profile Analysis

Texture profile (hardness, cohesiveness, adhesiveness, and gumminess) was analyzed according to Bhaskaracharya and Shah (1999) with some modifications. Cylinders of 25 × 20-mm cheeses were cut from Halloumi cheese blocks at the center. Specimens were kept in a refrigerator at 4°C overnight, followed by determination of texture profile. Hardness, cohesiveness, adhesiveness, and gumminess were measured using an Instron universal testing machine (model 5564; Instron Ltd., London, UK) based on the principle described by Pons and Fiszman (1996). The samples were compressed to 50% of their heights using a 100-N load cell with a flat plunger and the crosshead movement was adjusted to 50 mm/min. Double compression was achieved and the data were collected using Merline software. Analyses were carried out in quadruplicate.

Microstructure by Environmental Scanning Electron Microscopy

Experimental cheese specimens were imaged by FEI Quanta environmental scanning electron microscopy (**ESEM**; Philips Electron Optics, Eindhoven, the Netherlands) using ESEM mode. Images were taken at accelerating voltage at 30 kV under 0.47 kPa pressure and 1,200 × magnification at 4°C. Specimens were not conductivity coated before imaging.

Determination of Na, K, and Ca Contents by Multitype Inductively Coupled Plasma Atomic Emission Spectrometry

Sodium, potassium, and calcium contents in cheeses were analyzed by multitype inductively coupled plasma atomic emission spectrometry (ICPE-9000; Shimadzu Scientific Instruments (Oceania) Pty Ltd, Rydalmere, NSW, Australia) according to Cichoscki et al. (2002) with some modifications. In brief, after the ash determination of cheese samples, an aliquot (approximately 0.5 g) was dissolved with 10 mL of 1*N* HNO_3 and kept frozen (−20°C) until analyzed with the ICPE-9000. Samples were diluted 100 times, followed by filtration using 0.45 μm micro-syringes (Millex, Millipore, Bedford, MA) before direct injection into the ICPE-9000. The ICPE-9000 consisted of an ASC-6100 autosampler, hydride generator HVG-ICP, hydrofluoric acid sample injection system HFS-2, low-temperature thermostatic chamber NCB-1200, and software package ICPE-9000. To calculate Na, K, and Ca concentrations in samples, a standard curve consisting of the 3 elements was pre-

Table 1. Moisture, protein, fat, ash contents (% on wet basis), and pH value of Halloumi cheeses kept in brine solutions with 4 levels of NaCl and NaCl/KCl mixtures at 0 d of storage[1]

Salt treatment[2]	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	pH
A	51.50 ± 0.8[a]	21.41 ± 0.59[a]	21.75 ± 1.67[a]	4.55 ± 0.50[a]	5.27 ± 0.04[b]
B	50.75 ± 0.27[a]	20.64 ± 0.29[a]	22.80 ± 1.49[a]	4.53 ± 0.36[a]	5.33 ± 0.03[ab]
C	50.48 ± 0.60[a]	20.13 ± 0.44[a]	21.30 ± 1.28[a]	3.86 ± 0.33[a]	5.38 ± 0.07[ab]
D	50.21 ± 0.49[a]	20.11 ± 0.88[a]	19.33 ± 0.98[a]	4.48 ± 0.66[a]	5.48 ± 0.12[a]

[a,b]Means in each column with the same letter did not differ significantly ($P > 0.05$).
[1]Results are expressed as mean values ± SE of 4 trials.
[2]A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt).

pared at 1, 10, 20, 30, and 40 mg/kg. Analyses were carried out in quadruplicate.

Statistical Analysis

One-way ANOVA was used to test differences among 4 experimental cheeses for each salt treatment or sampling day and significance was tested at $P < 0.05$. Pearson correlation was measured at $P < 0.05$ between all variables. Data were analyzed using SAS software version 9.2 (SAS Institute, 2008).

RESULTS AND DISCUSSION

Chemical Composition

Moisture, protein, fat, and ash contents and the pH value of experimental cheeses at 0 d are shown in Table 1. As shown in the table, salt treatment did not affect ($P > 0.05$) the chemical composition of Halloumi cheese at 0 d of storage. Moisture, protein, ash, and fat contents showed no significant ($P > 0.05$) differences among experimental cheeses. This suggests that KCl may have the same effect as NaCl on Halloumi cheese. These findings are in accordance with those of Katsiari et al. (1997, 1998) and Reddy and Marth (1993) for Feta, Kefalograviera, and Cheddar cheeses, respectively.

Texture Profile Analysis

Hardness, cohesiveness, adhesiveness, and gumminess results of Halloumi cheeses kept in NaCl only (A) and NaCl and KCl mixtures (B, C, and D) are presented in Table 2. No significant ($P > 0.05$) difference was found in hardness, cohesiveness, adhesiveness, and

Table 2. Texture profile of Halloumi cheeses kept in 4 levels of NaCl and KCl during storage at 4°C[1]

Storage time	Salt treatment[2]	Hardness	Cohesiveness	Adhesiveness	Gumminess
d 0	A	28.200 ± 1.774[a]	0.324 ± 0.013[a]	0.146 ± 0.009[a]	9.211 ± 0.984[a]
	B	32.378 ± 1.266[a]	0.345 ± 0.048[a]	0.113 ± 0.010[a]	11.150 ± 1.515[a]
	C	27.840 ± 2.693[a]	0.379 ± 0.018[a]	0.167 ± 0.060[a]	10.580 ± 1.228[a]
	D	27.540 ± 3.304[a]	0.375 ± 0.046[a]	0.174 ± 0.061[a]	10.509 ± 2.031[a]
d 14	A	49.733 ± 8.882[a]	0.309 ± 0.030[a]	0.149 ± 0.062[a]	15.312 ± 1.042[a]
	B	43.785 ± 5.904[a]	0.265 ± 0.047[a]	0.233 ± 0.056[a]	11.411 ± 2.145[a]
	C	43.612 ± 4.775[a]	0.250 ± 0.026[a]	0.129 ± 0.060[a]	10.938 ± 1.505[a]
	D	41.410 ± 2.162[a]	0.241 ± 0.045[a]	0.157 ± 0.083[a]	10.113 ± 2.060[a]
d 28	A	40.160 ± 8.813[a]	0.343 ± 0.022[a]	0.113 ± 0.037[a]	8.343 ± 3.927[a]
	B	25.908 ± 7.528[a]	0.286 ± 0.020[b]	0.126 ± 0.038[a]	7.129 ± 1.650[a]
	C	29.158 ± 5.403[a]	0.229 ± 0.026[b]	0.135 ± 0.053[a]	7.054 ± 1.828[a]
	D	26.612 ± 4.783[a]	0.280 ± 0.056[ab]	0.168 ± 0.050[a]	7.758 ± 2.100[a]
d 42	A	25.759 ± 7.401[a]	0.244 ± 0.044[a]	0.436 ± 0.190[a]	6.044 ± 0.691[a]
	B	17.357 ± 4.092[a]	0.203 ± 0.024[a]	0.199 ± 0.133[a]	3.642 ± 1.017[a]
	C	16.462 ± 3.192[a]	0.238 ± 0.053[a]	0.372 ± 0.095[a]	3.895 ± 0.939[a]
	D	19.505 ± 4.488[a]	0.209 ± 0.034[a]	0.284 ± 0.137[a]	3.868 ± 0.737[a]
d 56	A	22.443 ± 7.256[a]	0.310 ± 0.062[a]	0.269 ± 0.129[a]	4.493 ± 0.701[a]
	B	14.411 ± 1.023[a]	0.266 ± 0.064[a]	0.344 ± 0.091[a]	3.993 ± 2.163[a]
	C	13.315 ± 2.775[a]	0.302 ± 0.029[a]	0.260 ± 0.119[a]	3.912 ± 0.644[a]
	D	10.640 ± 2.374[a]	0.261 ± 0.025[a]	0.426 ± 0.065[a]	2.762 ± 0.570[a]

[a,b]Means in each column and at the same storage time with the same letter did not differ significantly ($P > 0.05$).
[1]Results are expressed as mean values ± SE of 4 trials.
[2]A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt).

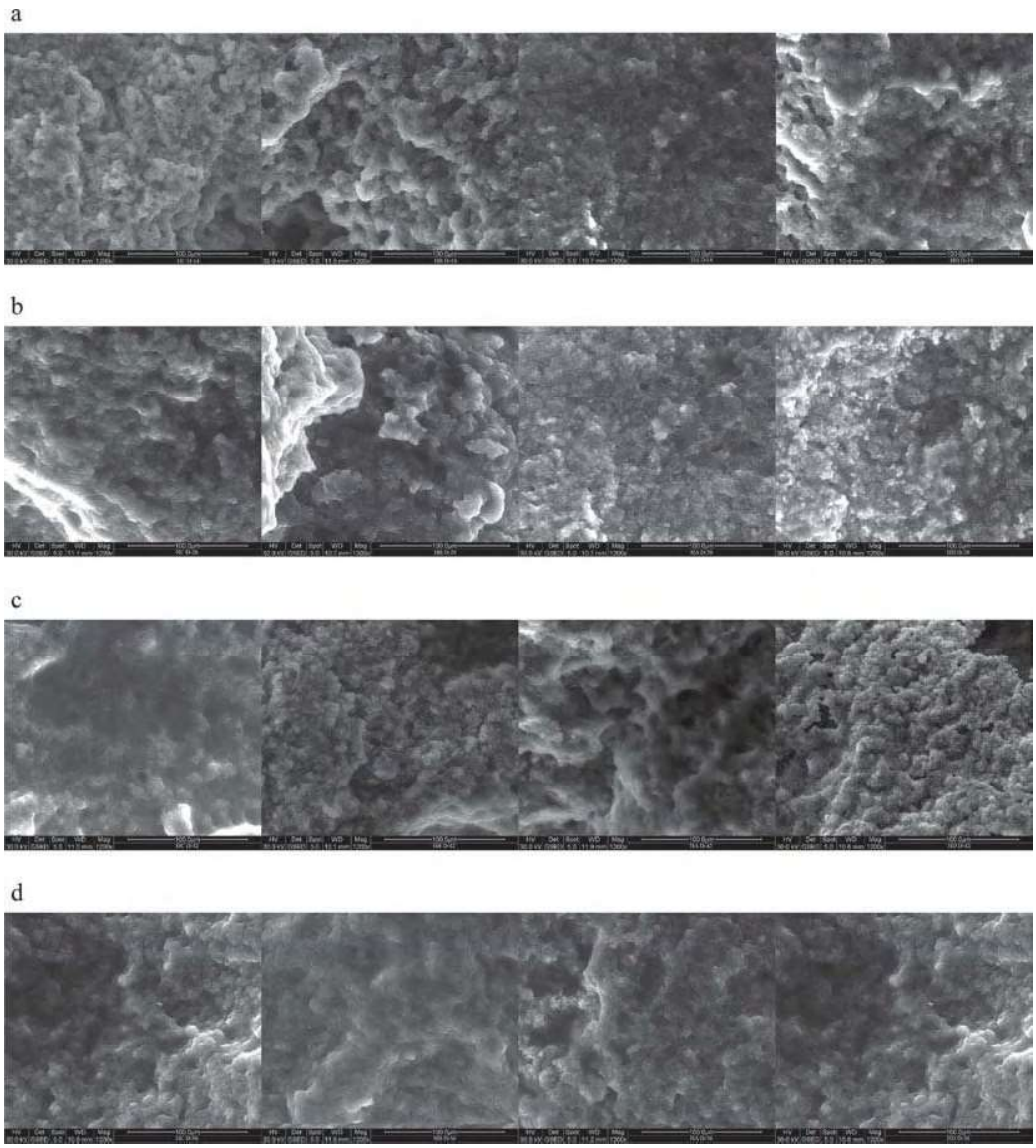

Figure 1. Environmental scanning electron micrograph of Halloumi cheese made with NaCl only (A; control); 3NaCl:1KCl (B; wt/wt); 1NaCl:1KCl (C; wt/wt); and 1NaCl:3KCl (D; wt/wt) at 14 (a), 28 (b), 42 (c), and 56 (d) d of storage. Treatments A to D are shown from left to right, respectively.

gumminess among experimental cheeses at the same storage period. This suggests that KCl may have the same role in Halloumi cheese as does NaCl, up to 75% replacement (Taylor, 1983). These observations are in agreement with those of Katsiari et al. (1997, 1998) and Fitzgerald and Buckley (1985) for Feta, Kefalograviera, and Cheddar cheeses, respectively.

For the same salt treatment, ANOVA showed that hardness and gumminess decreased significantly ($P < 0.05$) after 2 wk of storage. These findings are in accordance with those of Katsiari and Voutsinas (1994b), Katsiari et al. (1998), and Zaki (1990) for Kefalograviera and Domiati cheeses. This trend may be attributed to 2 reasons. First, the decrease in Ca of 65% (from approximately 0.43 mg/100g at d 0 to approximately 0.15 mg/100 g at d 56) during storage, led to a reduction in cross-linkages between caseins. The Ca decrease, attributed to Ca ion diffusion in brine solution, may cause a decrease in colloidal Ca, which, in turn, increases cheese softening. Second, the increase in proteoylsis (unpublished data) during storage hydrolyzed protein and softened the cheese matrix (Guinee et al., 2002; Joshi et al., 2003).

The ANOVA showed that adhesiveness increased ($P < 0.05$), whereas cohesiveness decreased ($P < 0.05$) at the end of the storage period compared with the

Table 3. Pearson correlations of Na, K, and Ca with the texture profile for the same salt treatment[1]

Element	Salt treatment[2]	Hardness	Cohesiveness	Adhesiveness	Gumminess
Na	A	−0.1793	−0.3245	0.7377	−0.3226
		(0.5225)	(0.2381)	(0.0017)	(0.2410)
	B	−0.6375	−0.4530	0.4252	−0.7591
		(0.0044)	(0.0591)	(0.0786)	(0.0003)
	C	−0.6023	−0.3467	0.5011	−0.7479
		(0.0050)	(0.1342)	(0.0244)	(0.0001)
	D	−0.5909	−0.5929	0.4058	−0.7539
		(0.0204)	(0.0198)	(0.1334)	(0.0012)
K	A	−0.5076	−0.3171	0.6234	−0.6179
		(0.0534)	(0.2494)	(0.0130)	(0.0141)
	B	0.0908	−0.3151	0.3251	−0.1211
		(0.7201)	(0.2029)	(0.1880)	(0.6320)
	C	−0.6743	−0.2793	0.5082	−0.7781
		(0.0011)	(0.2330)	(0.0221)	(<0.0001)
	D	−0.6663	−0.4842	0.4065	−0.7132
		(0.0067)	(0.0674)	(0.1327)	(0.0028)
Ca	A	−0.0870	−0.0231	−0.5906	−0.0948
		(0.7579)	(0.9348)	(0.0205)	(0.7369)
	B	0.3984	0.5347	−0.4468	0.6485
		(0.1015)	(0.0222)	(0.0630)	(0.0036)
	C	0.3914	0.4859	−0.2720	0.6202
		(0.0879)	(0.0298)	(0.2460)	(0.0035)
	D	0.2565	0.6464	−0.2092	0.5528
		(0.3562)	(0.0092)	(0.4542)	(0.0326)

[1]Results are expressed as correlation coefficients (P-values in parentheses).

[2]Salt treatment: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt).

adhesiveness and cohesiveness at d 0 for the same salt treatment. These observations are in agreement with those of Bhaskaracharya and Shah (2000), which were similar for Mozzarella cheese. The increase in adhesiveness may be due to the increase in proteolysis during storage, which, in turn, increased small peptides in the serum phase and, thus, increased adhesiveness (Bhaskaracharya and Shah, 2000). The decrease ($P < 0.05$) in cohesiveness may be due to the decrease in Ca and increase in proteolysis (Bhaskaracharya and Shah, 2000).

Microstructure

The ESEM images of experimental cheeses at 14, 28, 42, and 56 d storage are shown in Figure 1 (A, B, C, and D, respectively). Micrographs show compact and closed structures with small voids in all experimental cheeses. This suggests that microstructure of Halloumi cheeses kept in various NaCl and KCl mixtures did not differ compared with that of the control. However, voids disappeared slightly with prolonged storage and this was more obvious in cheeses stored between d 14 and 56 (Figure 1A and 1D, respectively) as compared with those stored between periods close together, such as d 42 and 56 (Figure 1C and 1D, respectively). This may be attributed to a decrease in moisture content (Ayyash and Shah, 2010) and an increase in proteolysis. It has been reported that proteolysis in cheese produces small peptides that are soluble in the serum phase of cheese (Fox and McSweeney, 1996; Upadhyay et al., 2004). Therefore, we believe that the increase in small peptides in Halloumi cheeses and the decrease in moisture content increased the compactness of Halloumi cheese texture.

Correlations

Pearson correlation coefficients between Na, K, and Ca with texture profile parameters are presented in Table 3. In general, Ca had a positive correlation with hardness, cohesiveness, and gumminess, whereas a negative correlation was shown with adhesiveness for the NaCl and KCl mixture treatments. This supports the observation that the decrease in hardness can be attributed to the decrease in Ca content, as mentioned earlier. Hardness, cohesiveness, and gumminess correlated negatively with K and Na, whereas adhesiveness showed a positive correlation with K and Na. The negative correlation of Na and K versus the positive correlation of Ca with hardness, cohesiveness, and gumminess may be due to Na or K-Ca ion exchange. Guinee and Fox (2004) reported that the addition of NaCl led to Na-Ca ion exchange with paracasein. In our study, ion

exchange may have occurred between Ca and Na or K; therefore, the correlations of these elements with texture profile parameters were contradictory.

CONCLUSIONS

The partial substitution of NaCl with KCl appears to result in a similar texture profile and microstructure of Halloumi cheese compared with those of the control (only NaCl). Hardness, cohesiveness, adhesiveness, and gumminess of Halloumi cheeses kept in NaCl/KCl brine solution were similar to those stored in NaCl only. Calcium played a major role in influencing the texture profile.

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6. Chapter 6: The effect of substituting NaCl with KCl on Nabulsi cheese: Chemical composition, total viable count, and texture profile

Introduction

Chapter six covers the effect of NaCl substitution with KCl on chemical composition, microbiological quality, proteolysis and physical properties of Nabulsi cheese during storage at room temperature for 5 months. Sensory evaluation of Nabulsi cheese is presented in Appendix A. A paper entitled the effect of substituting NaCl with KCl on Nabulsi cheese: Chemical composition, total viable count, and texture profile by Ayyash M.M and N.P Shah was published in the peer reviewed Journal of Dairy Science 94(6):2741-2751. The microstructure images in this chapter are enlarged in Appendix C, pages 170-171.

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

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

Declaration by [candidate name]: Mutamed Ayyash

Signature:

Date: 11/09/2012

Paper Title:

The effect of substituting NaCl with KCl on Nabulsi cheese: Chemical composition, total viable count, and texture profile

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

Name	Contribution %	Nature of Contribution
Mutamed Ayyash	75	Design and perform the experiment perform the samples analysis
		evaluate the analytical data Perform the statistical analysis by SAS
		Prepare the major part of the manuscript
Nagendra Shah	25	Contribute to writing manuscript and submission to Journal

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The undersigned certify that:

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2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
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J. Dairy Sci. 94:2741–2751
doi:10.3168/jds.2010-3976

The effect of substituting NaCl with KCl on Nabulsi cheese: Chemical composition, total viable count, and texture profile

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ABSTRACT

The effect of substituting NaCl with KCl on Nabulsi cheese characteristics was investigated. Nabulsi cheese was made and stored in 4 different brine solutions at 18%, including NaCl only (A; control); 3NaCl:1KCl (wt/wt; B); 1NaCl:1KCl (wt/wt; C); and 1NaCl:3KCl (wt/wt; D). Chemical composition, proteolysis, total viable count, and texture profile analysis were assessed at monthly intervals for 5 mo. No significant effect was found among experimental cheeses in terms of chemical composition or texture profile. Proteolytic activities were higher in cheeses kept in brine solutions that contained higher KCl (B, C, and D) compared with the control. At the end of the storage period, water-soluble nitrogen in Nabulsi cheeses stored in B, C, and D was higher than that in the control cheese (A). In addition, total viable count increased significantly after 1 mo of storage for all salt treatments. Hardness and gumminess generally decreased significantly during storage within the same salt treatment.
Key words: Nabulsi cheese, proteolysis, texture profile, microstructure

INTRODUCTION

White brined cheeses are traditional cheeses of the Middle Eastern region: Halloumi cheese is popular in Cyprus and Lebanon, Domiati cheese in Egypt, and Nabulsi cheese in Jordan, Syria, and Palestine (Yamani et al., 1998). Nabulsi cheese is traditionally produced from sheep or goat milk, or both, during the spring and summer seasons. However, due to currently high demand, Nabulsi cheese is also produced from cow milk (Abu-Alruz et al., 2009b) or mixture a mixture of cow and sheep milk. It is classified as a semi-hard cheese with high salt content and produced without addition of starter culture (Abd El-Salam and Alichanidis, 2004; Abu-Alruz et al., 2009b). A brief procedure of Nabulsi cheese production is as follows: pasteurized milk is tempered to approximately 35°C and rennet is added. After 30 to 60 min, the curd is cut and settled for 10 to 15 min, and then transferred into cheesecloth to drain whey; the curd is then pressed at 0.4 MPa, and the cheese is cut into small blocks (~4 × 4 × 1.5 cm) and sprinkled with salt or immersed in a brine solution (18 to 21%) overnight. The cheese blocks are then boiled in a brine solution (18 to 21%) for 5 to 10 min, cooled, and stored in metal or plastic jars filled with a brine solution (Ibrahim and O'Sullivan, 1998; Abd El-Salam and Alichanidis, 2004; Tamime, 2006). Salt addition is a critical step during cheese making to maintain the quality and safety of Nabulsi cheese during 6 to 12 mo of storage. Nabulsi cheese is consumed as a part of breakfast or eaten as a snack and it is an essential component of the Middle Eastern sweets such as Kunafa (Yamani et al., 1998). Nabulsi cheese manufacturers intentionally increase the salt content of the cheese to promote water loss and to preserve cheese during prolonged storage.

Middle Eastern people may avoid consumption of Nabulsi cheese because of the high salt content. A positive correlation has been found between high sodium (Na) intake and hypertension (Kotchen, 2005), osteoporosis (Heaney, 2006), kidney stones (Massey, 2005), and cardiovascular diseases (Penner et al., 2007). The World Health Organization has recommended decreasing salt in all food types to reduce health problems associated with high intake of salt (World Health Organization, 2007). It has been reported that cheeses contribute to approximately 11 to 20% of the total sodium dietary intake (Guinee, 2004a,b; Tamime, 2006). Consequently, and because of high cheese consumption in Middle Eastern countries, Nabulsi cheese contributes to a considerable amount of the total dietary Na intake for consumers in the Middle East. Hence, it is important to reduce salt content in Nabulsi cheese rather than decreasing its consumption. Several studies have been carried out to improve the manufacturing procedure (Ibrahim and O'Sullivan, 1998), stretchability and meltability (Abu-Alruz et al., 2009b; Mazahreh et al., 2009), and the microbiological quality of Nabulsi cheese (Humied et al., 1990). This study is the first attempt aimed at reducing the salt content (NaCl) in Nabulsi

Received October 31, 2010.
Accepted February 24, 2011.
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cheese by substituting NaCl with KCl. In particular, the aim of this study was to investigate the effect of substituting NaCl with KCl on chemical composition, microbiological quality, proteolysis, and physical properties of Nabulsi cheese during storage at room temperature for 5 mo.

MATERIALS AND METHODS

Cheese Making

Nabulsi cheese was manufactured according to Abu-Alruz et al. (2009a) with some modifications as shown in Figure 1. Full-fat homogenized and pasteurized bovine milk was obtained from the local market (Melbourne, VIC, Australia). Forty liters of milk was tempered to 37°C, and diluted chymosin (Chy-Max, Chr. Hansen, Bayswater, VIC, Australia) was added according to the manufacturer's instruction. After 35 min, coagulated curd was cut into 1-cm^3 cubes and left for 10 min. Then, the curd was transferred into cheesecloth and left for 15 min to drain the whey. The curd was pressed (each 1-kg weight of curd was pressed with 60 kg of weight) for 90 min and cut into 4 × 4 × 1.5 cm pieces, divided randomly and equally, and kept in 4 brine solutions (prepared using Milli-Q water; the pH of the brine solution was not adjusted) at 21%, including only NaCl (**A**, control), 3NaCl:1KCl (**B**), 1NaCl:1KCl (**C**), and 1NaCl:3KCl (**D**) and dipped for 72 h at room temperature (~21°C). Thereafter, each cheese portion was boiled for 15 min in the same brine solutions until the cheese temperature reached approximately 85°C, placed in clean and sanitized plastic containers, and cooled (~40°C). Finally, each batch of cheese was transferred into 4 fresh brine solutions at 18% in separate plastic containers and stored for 5 mo at room temperature (~21°C). Samples (~100 g each) were taken at monthly intervals to determine chemical composition, Na, K, Ca, and P contents, and enumeration of total viable count (**TVC**). All analyses were carried out in duplicate and all experiments were replicated 3 times.

Chemical Composition

Chemical composition was determined according to AOAC methods (AOAC, 1995); moisture was determined by oven-drying method at 102°C, fat by the Babcock method, protein by the Kjeldahl method, and ash by the muffle furnace method. For pH measurement, 20 g of grated cheese was macerated in 20 mL of distilled water, and the pH of the resultant slurry was measured using a calibrated digital pH meter (MeterLab, Pacific Laboratory Products, Melbourne, VIC, Australia).

TVC

Total viable count was enumerated according to an ISO/IDF method (ISO/IDF, 2002). Briefly, 11 g of grated cheese was blended with 99 mL of sterilized peptone and water solution (0.1% peptone) for 2 min using a Stomacher-400 laboratory blender (Seward Medical, London, UK). Appropriate dilutions were made using 0.1% peptone and water solution, and enumeration was carried out using tryptone soy agar (Oxoid Ltd., West Heidelberg, VIC, Australia). Plates were aerobically incubated at 37°C for 48 h.

Assessment of Proteolysis

The water-soluble extracts (**WSE**) of the cheese samples were prepared according to Kuchroo and Fox (1982). The nitrogen in the extract was estimated by the Kjeldahl method (AOAC, 1995). Twelve percent TCA-soluble nitrogen (**TCA-SN**) and 5% phosphotungstic acid (**PTA-SN**) were determined according to Ayyash and Shah (2010b). The TCA-SN was estimated in 9 mL of filtrate obtained after precipitation of filtered WSE of cheese with 24% TCA (Sigma-Aldrich, St. Louis, MO). The extent of secondary proteolysis (PTA-SN) was assayed similarly to TCA-SN using 9 mL of filtrate obtained after precipitation of filtered WSE of cheese with 10% phosphotungstic acid (Sigma-Aldrich).

Determination of Na, K, Ca, and P Contents

Contents of Na, K, Ca, and P in cheeses were determined by multitype inductively coupled plasma atomic emission spectrometry [ICPE-9000, Shimadzu Scientific Instruments (Oceania) Pty Ltd., Rydalmere, NSW, Australia] according to Ayyash and Shah (2010a). After the ash determination of cheese samples, an aliquot (~0.5 g) was dissolved in 10 mL of 1 N HNO_3 and kept frozen (−20°C) until analyzed using the ICPE-9000. All samples were diluted 100 times and then filtered using a Millex 0.45-μm filter (Millipore, Bedford, MA) before directly injecting into the ICPE-9000. The ICPE-9000 consisted of an ASC-6100 autosampler, a hydride generator HVG-ICP, a hydrofluoric acid sample injection system HFS-2, a low-temperature thermostatic chamber NCB-1200, and a software package ICPE-9000. To calculate Na, K, Ca, and P concentrations in samples, a standard curve consisting of the 4 elements was prepared at 1, 10, 20, 30, and 40 μg/mL.

Texture Profile Analysis

Texture profile (hardness, cohesiveness, adhesiveness, and gumminess) was analyzed according to Bhaska-

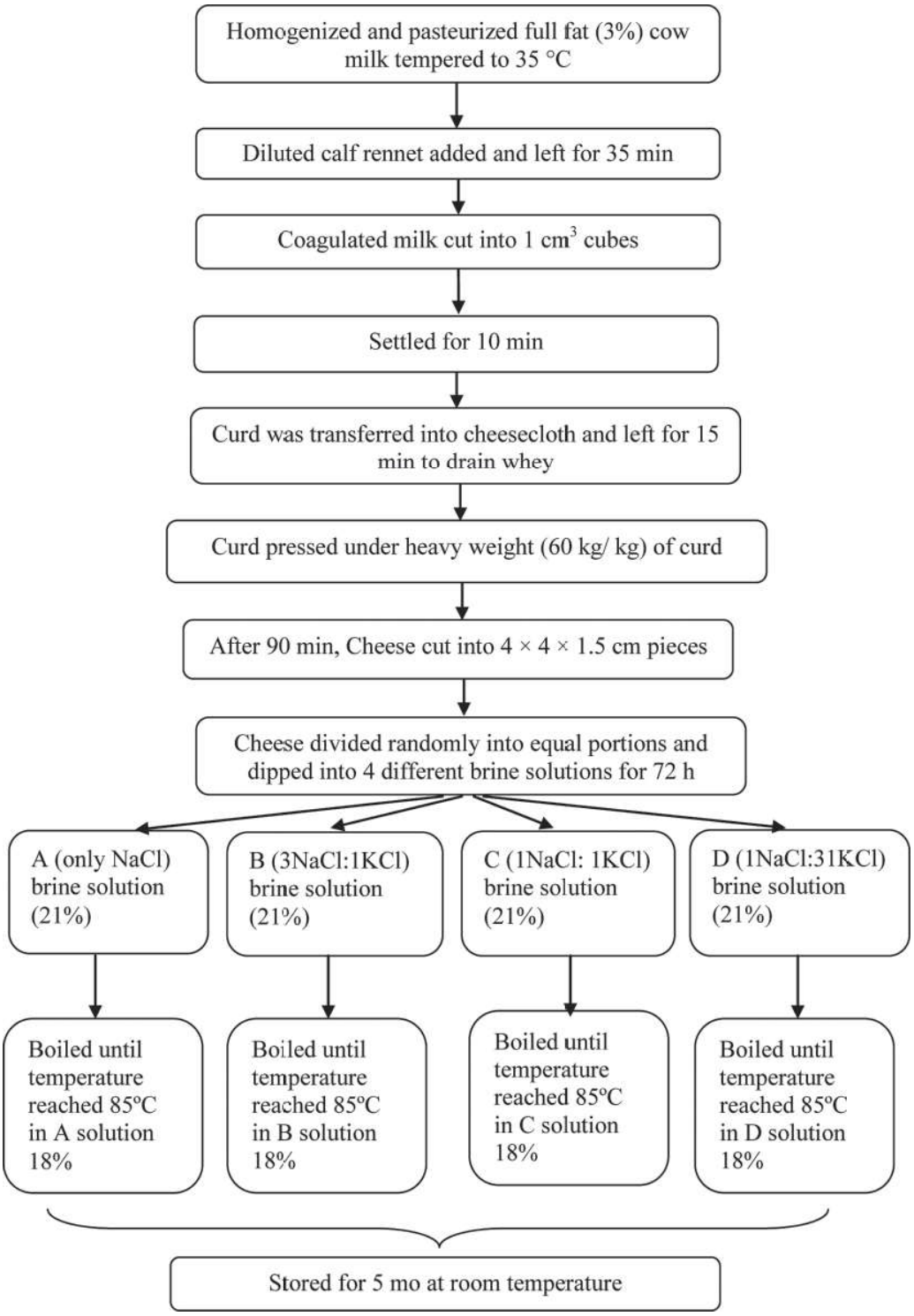

Figure 1. Procedure for manufacture of Nabulsi cheese.

racharya and Shah (1999) with some modifications. Cylinders (25 × 15 mm, diameter × height) of cheeses were cut from the center of the Nabulsi cheese blocks. Specimens were kept in a refrigerator at 4°C overnight followed by determination of texture profile. Hardness, cohesiveness, adhesiveness, and gumminess were measured using an Instron universal testing machine (model 5564, Instron Ltd., London, UK) based on the principle

described by Pons and Fiszman (1996). Each sample was compressed to 50% of its height using a 100-N load cell with a flat plunger, and the crosshead movement was adjusted to 50 mm/min. A double compression was achieved and the data were collected using Merline software (Instron Pty Ltd. Melbourne, VIC, Australia). Analyses were carried out in duplicate.

Microstructure by Environmental Scanning Electron Microscopy

Experimental cheese specimens were imaged by using FEI Quanta environmental scanning electron microscopy (**ESEM**; Philips Electron Optics, Eindhoven, the Netherlands) using ESEM mode. Images were taken at accelerating voltage at 30 kV under 3.5 Torr of pressure and 1,200 × magnification at 4°C. Specimens were not conductivity coated before imaging. Images were visually examined to determine differences among batches.

Statistical Analysis

One-way ANOVA was used to test differences among the 4 experimental cheeses (A, B, C, and D) at each salt treatment or sampling day, and significance was tested at $P < 0.05$. Pearson correlation was measured at $P < 0.05$ between all measured variables in the same salt treatment. Two-way ANOVA was carried out to investigate the effect of salt treatment and storage period interaction for all measured variables. Data were analyzed using SAS software version 9.2 (SAS Institute, 2008).

RESULTS AND DISCUSSION

Chemical Composition

Moisture, protein, fat, and ash contents and pH values of Nabulsi cheeses kept in 4 brine solution are presented in Table 1. In general, the moisture, protein, ash, and pH decreased significantly ($P < 0.05$) between 0 and 5 mo of storage within a salt treatment. However, storage period had no significant ($P > 0.05$) effect on the fat content (Table 1). The reduction in moisture content occurred due to the migration of moisture from cheese loaves as reported by Geurts et al. (1980) and Guinee and Fox (2004). The decrease in protein content may be attributed to the proteolytic activity during storage, which resulted in an increase in WSN and TCA-SN in experimental cheeses (Table 2). These findings agree with those of Ayyash and Shah (2010a), who reported that moisture and intact protein content of Halloumi cheese decreased ($P < 0.05$) during storage.

The decreases in ash content and pH are in contradiction with the results of Ayyash and Shah (2010a) and Katsiari et al. (1997, 1998). This may be attributed to the manufacturing process of Nabulsi cheese, which differs from those of Halloumi, Feta, and Kefalograviera cheeses. In this study, cheese loaves were dipped into brine solution at 21% for 72 h before boiling and storage. This step increased the salt content in cheeses, which was reflected in the ash content and pH value of cheese loaves before boiling and storing in brine solutions at a lower brine concentration of 18%. During storage, cheese loaves lost excessive salt to brine solution to reach an equilibrium, which in turn affected the ash content of cheeses. The decrease in pH values during storage may be due to microbial growth, especially that of nonstarter lactic acid bacteria, which may ferment lactose and produce organic acids in cheeses with decreased pH values.

In general, ANOVA showed no significant ($P < 0.05$) differences in chemical composition between experimental Nabulsi cheeses at the same storage times (Table 1). Occasional differences were observed between salt treatments in terms of moisture, protein, fat, and ash contents (Table 1). We assumed that these differences were due to variations in cheese loaves and did not relate to salt treatment.

As seen in Table 1, pH values of cheeses kept in the D brine solution were significantly higher compared with A (control) at 0 mo of storage. At 1 and 2 mo of storage, pH values of Nabulsi cheeses kept in D and C treatments were significantly higher compared with those of cheeses in the A and B treatments. This suggests that when KCl became a part of the brine solution, the pH values increased. This is due to the nature of KCl solutions, which have higher pH value (~0.3) compared with those of NaCl. This finding is in accordance with those of other researchers (Fitzgerald and Buckley, 1985; Katsiari et al., 1998; Ayyash and Shah, 2010a), who reported a slight increase in pH values of Kefalograviera, Halloumi, and Cheddar cheeses, respectively, made with NaCl-KCl mixtures. However, for the rest of the storage period, the pH values of cheeses kept in NaCl-KCl mixtures became significantly lower compared with the control cheeses (Table 1). This may be attributed to higher microbial activity in those cheeses because of high pH, which is more conducive to microbial growth (Ayyash and Shah, 2010a).

Assessment of Proteolysis and TVC

Water-soluble N, TCA-SN, PTA-SN, and TVC of Nabulsi cheeses kept in 4 brine solutions are presented in Table 2. Analysis of variance showed that TVC in-

Table 1. Moisture, protein, fat, and ash contents, and pH values of Nabulsi cheeses stored in 4 levels of NaCl and KCl during storage for 5 mo at room temperature[1]

Storage (mo)	Salt treatment[2]	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	pH
0	A	59.12 ± 1.32^{a}	25.21 ± 0.36^{a}	22.37 ± 0.94^{a}	10.88 ± 0.28^{a}	6.41 ± 0.02^{b}
	B	58.71 ± 1.18^{a}	24.76 ± 0.27^{a}	23.57 ± 0.23^{a}	10.12 ± 0.29^{a}	6.52 ± 0.03^{b}
	C	58.02 ± 0.69^{ab}	24.68 ± 1.63^{a}	23.93 ± 1.37^{a}	10.77 ± 0.17^{a}	6.50 ± 0.07^{b}
	D	55.25 ± 0.56^{b}	25.49 ± 0.58^{a}	23.50 ± 1.35^{a}	10.19 ± 0.20^{a}	6.65 ± 0.00^{a}
1	A	50.81 ± 0.34^{a}	21.81 ± 0.52^{a}	21.00 ± 0.06^{a}	10.22 ± 0.18^{a}	6.38 ± 0.09^{c}
	B	49.83 ± 0.85^{ab}	21.79 ± 0.25^{a}	21.00 ± 0.59^{a}	10.11 ± 0.21^{a}	6.55 ± 0.03^{b}
	C	50.01 ± 0.72^{ab}	21.79 ± 0.41^{a}	22.37 ± 0.47^{a}	10.06 ± 0.18^{a}	6.70 ± 0.01^{a}
	D	48.66 ± 0.25^{b}	21.58 ± 0.60^{a}	20.93 ± 0.78^{a}	9.92 ± 0.10^{a}	6.78 ± 0.01^{a}
2	A	51.67 ± 0.31^{a}	22.79 ± 0.66^{a}	22.20 ± 0.40^{a}	9.43 ± 0.66^{a}	7.00 ± 0.06^{b}
	B	50.80 ± 0.10^{ab}	22.26 ± 0.57^{a}	20.60 ± 0.32^{b}	9.74 ± 0.13^{a}	7.03 ± 0.04^{b}
	C	51.80 ± 0.47^{a}	23.49 ± 0.40^{a}	21.93 ± 0.61^{ab}	10.07 ± 0.25^{a}	7.20 ± 0.02^{a}
	D	49.44 ± 0.70^{b}	22.72 ± 0.98^{a}	22.63 ± 0.37^{a}	10.70 ± 0.18^{a}	7.23 ± 0.01^{a}
3	A	52.76 ± 1.45^{a}	23.19 ± 0.95^{a}	23.33 ± 0.27^{a}	9.98 ± 0.15^{a}	6.52 ± 0.03^{a}
	B	51.35 ± 0.41^{a}	21.74 ± 1.59^{a}	22.53 ± 0.32^{ab}	9.83 ± 0.10^{a}	6.41 ± 0.01^{b}
	C	52.59 ± 0.81^{a}	22.79 ± 0.52^{a}	22.70 ± 0.23^{ab}	10.04 ± 0.03^{a}	6.53 ± 0.00^{a}
	D	52.06 ± 0.49^{a}	22.12 ± 0.26^{a}	21.77 ± 0.64^{b}	9.78 ± 0.09^{a}	6.34 ± 0.01^{c}
4	A	51.54 ± 0.57^{ab}	23.17 ± 0.28^{a}	22.37 ± 0.13^{a}	9.90 ± 0.23^{a}	6.34 ± 0.00^{a}
	B	49.92 ± 0.36^{bc}	22.78 ± 0.78^{a}	22.50 ± 0.58^{a}	10.04 ± 0.02^{a}	6.10 ± 0.01^{d}
	C	51.87 ± 0.51^{a}	23.29 ± 0.25^{a}	23.33 ± 0.17^{a}	10.29 ± 0.05^{a}	6.26 ± 0.01^{b}
	D	49.46 ± 0.60^{c}	21.05 ± 0.39^{b}	22.33 ± 0.44^{a}	10.33 ± 0.19^{a}	6.20 ± 0.01^{c}
5	A	51.78 ± 0.39^{a}	22.72 ± 0.48^{ab}	21.97 ± 0.74^{a}	9.88 ± 0.16^{b}	6.49 ± 0.03^{a}
	B	49.72 ± 0.60^{ab}	22.03 ± 0.11^{bc}	21.07 ± 0.30^{ab}	10.10 ± 0.01^{ab}	6.16 ± 0.02^{d}
	C	50.93 ± 0.24^{a}	23.65 ± 0.13^{a}	21.73 ± 0.15^{a}	10.11 ± 0.02^{ab}	6.36 ± 0.01^{b}
	D	47.29 ± 1.43^{b}	21.38 ± 0.59^{c}	20.37 ± 0.19^{b}	10.22 ± 0.10^{a}	6.27 ± 0.01^{c}

[a–d]Means in each column and at the same storage time with same letter did not differ significantly ($P > 0.05$).

[1]Mean values ± SE of 3 trials.

[2]Salt treatment: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt).

creased significantly ($P < 0.05$) from 0 to 3 mo of storage, whereas WSN, TCA-SN, and PTA-SN increased significantly ($P < 0.05$) in all experimental cheeses during 5 mo of storage. It was noticed that the WSN, TCA-SN, and PTA-SN contents in all experimental cheeses were lower in respect to the long storage period. This may due to the high heat treatment applied to cheeses during manufacturing. Heating experimental cheeses also inactivated the chymosin residues; chymosin is considered as a primary proteolytic agent in cheeses (Fox and McSweeney, 1996; Upadhyay et al., 2004). Thus, we assumed that plasmin (a heat-stable indigenous enzyme) remained in cheeses and acted as a primary proteolytic agent instead of chymosin. The activity of plasmin is slower than that of chymosin (Farkye and Fox, 1991; Fox and McSweeney, 1996; Upadhyay et al., 2004). Hence, the increase in WSN and TCA-SN was slower.

As shown in Table 2, in general, significant ($P < 0.05$) differences were observed among experimental cheeses in WSN at the same time of storage. Those differences were observed clearly in the latter part (3, 4, and 5 mo) of the storage period. It was apparent that WSN in cheeses stored in treatment D was higher ($P < 0.05$) compared with that in the other experimental cheeses at 4 and 5 mo of storage. This may suggest that plasmin activity increased in parallel with the increase in KCl concentration. This finding is in accordance with Ayyash and Shah (2010b), who showed higher WSN values in experimental Halloumi cheeses stored in 1NaCl:1KCl and in 1NaCl:3KCl compared with control cheese during storage.

No significant ($P > 0.05$) differences in TCA-SN and PTA-SN were observed among experimental cheeses in the early part of the storage period (0, 1, and 2 mo). However, a significant ($P < 0.05$) difference was obvious at mo 5 of storage, where cheese in the D treatment showed higher ($P < 0.05$) TCA-SN compared with other cheeses. That may be attributed to the higher WSN of cheese in D treatment that, in turn, provided substrates (large and medium peptides) for spoilage microorganisms and finally increased TCA-SN. Upadhyay et al. (2004) reported that enzymes from starter culture and nonstarter bacteria hydrolyze peptides resulting from primary (chymosin) enzymatic activity. Significant ($P < 0.05$) differences were observed between experimental cheeses in TVC during mo 1 and 2. This suggests that NaCl substitution with KCl has a similar effect on TVC of Nabulsi cheese. This finding in agreement with those of Ayyash and Shah (2010b).

Water-soluble N, TCA-SN, and PTA-SN were inversely correlated with Ca and P contents in all salting treatments. Pearson correlations (r) of proteolysis variables ranged from −0.44 to −0.68 for Ca and from

Table 2. Water-soluble N (WSN), TCA-soluble N (TCA-SN), phosphotungstic acid-soluble N (PTA-SN), and total viable count (TVC) of Nabulsi cheeses stored in 4 levels of NaCl and KCl during storage for 5 mo at room temperature[1]

Storage (mo)	Salt treatment[2]	Parameter[3]			
		WSN	TCA-SN	PTA-SN	TVC
0	A	1.02 ± 0.02^{b}	0.79 ± 0.04^{a}	0.39 ± 0.02^{a}	ND[4]
	B	1.32 ± 0.12^{a}	0.81 ± 0.03^{a}	0.33 ± 0.04^{a}	ND
	C	1.09 ± 0.09^{ab}	0.87 ± 0.04^{a}	0.39 ± 0.01^{a}	ND
	D	1.10 ± 0.07^{ab}	0.83 ± 0.01^{a}	0.33 ± 0.01^{a}	ND
1	A	1.04 ± 0.10^{a}	0.34 ± 0.05^{a}	0.16 ± 0.05^{a}	4.56 ± 0.01^{a}
	B	1.02 ± 0.01^{a}	0.45 ± 0.06^{a}	0.17 ± 0.03^{a}	4.11 ± 0.18^{b}
	C	1.12 ± 0.08^{a}	0.38 ± 0.01^{a}	0.16 ± 0.00^{a}	4.37 ± 0.10^{ab}
	D	0.96 ± 0.02^{a}	0.39 ± 0.06^{a}	0.20 ± 0.04^{a}	4.34 ± 0.12^{ab}
2	A	1.26 ± 0.06^{b}	0.91 ± 0.04^{a}	0.34 ± 0.02^{a}	5.29 ± 0.10^{a}
	B	1.54 ± 0.09^{ab}	1.14 ± 0.27^{a}	0.29 ± 0.04^{a}	5.10 ± 0.10^{ab}
	C	1.53 ± 0.05^{ab}	0.75 ± 0.06^{a}	0.27 ± 0.03^{a}	4.79 ± 0.01^{b}
	D	1.57 ± 0.14^{a}	0.89 ± 0.06^{a}	0.31 ± 0.05^{a}	4.93 ± 0.14^{b}
3	A	2.18 ± 0.21^{a}	3.43 ± 0.44^{a}	1.20 ± 0.12^{a}	6.06 ± 0.18^{a}
	B	1.60 ± 0.18^{b}	1.10 ± 0.13^{b}	0.46 ± 0.06^{b}	5.62 ± 0.14^{a}
	C	1.43 ± 0.06^{b}	0.89 ± 0.05^{b}	0.27 ± 0.01^{b}	5.61 ± 0.17^{a}
	D	1.86 ± 0.06^{ab}	1.33 ± 0.14^{b}	0.26 ± 0.02^{b}	5.57 ± 0.16^{a}
4	A	1.78 ± 0.06^{b}	1.10 ± 0.55^{a}	0.77 ± 0.04^{a}	5.22 ± 0.04^{a}
	B	1.85 ± 0.02^{b}	1.16 ± 0.06^{a}	0.33 ± 0.02^{b}	5.00 ± 0.26^{a}
	C	1.94 ± 0.06^{b}	1.34 ± 0.13^{a}	0.37 ± 0.07^{b}	4.99 ± 0.17^{a}
	D	2.81 ± 0.07^{a}	2.09 ± 0.40^{a}	0.37 ± 0.19^{b}	4.80 ± 0.31^{a}
5	A	3.81 ± 0.15^{c}	4.38 ± 0.28^{b}	2.44 ± 0.06^{a}	5.20 ± 0.05^{a}
	B	4.63 ± 0.23^{b}	3.61 ± 0.12^{c}	1.15 ± 0.05^{c}	5.05 ± 0.24^{a}
	C	4.05 ± 0.14^{bc}	3.03 ± 0.05^{d}	0.69 ± 0.35^{c}	5.02 ± 0.16^{a}
	D	6.86 ± 0.35^{a}	5.31 ± 0.09^{a}	1.78 ± 0.10^{b}	4.93 ± 0.38^{a}

[a–d]Means in each column and at the same storage time with same letter did not differ significantly ($P > 0.05$).

[1]Mean values ± SE of 3 trials.

[2]Salt treatment: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt).

[3]WSN = water-soluble N as a percentage of total N; TCA-SN = 12% TCA-soluble N as a percentage of total N; PTA-SN = 5% phosphotungstic acid-soluble N as a percentage of total N; TVC = total viable count ($\log_{10}$ cfu/g).

[4]ND = not detected.

−0.23 to −0.69 for P. This supports the suggestion that a reduction in colloidal calcium in cheeses increased proteolytic activity. In addition, TVC inversely was correlated with Ca and P in all salting treatments; r-values ranged from −0.65 to −0.79 for Ca and from −0.66 to −0.79 for P. This may be attributed to proteolysis: when Ca and P contents decreased during storage, proteolytic activity increased and then provided primary nutrients essential for microbial growth.

Texture Profile Analysis

Hardness, cohesiveness, adhesiveness, and gumminess of experimental cheeses are shown in Table 3. According to the ANOVA, adhesiveness did not change significantly ($P > 0.05$), whereas cohesiveness increased ($P < 0.05$) and hardness decreased ($P < 0.05$) during storage. This may due to increased proteolytic activity that reduced the protein network; hence, hardness decreased while cohesiveness and adhesiveness increased (Lawrence et al., 1987; Johnson and Lucey, 2006). In addition, reductions in Ca and P contents during storage may have decreased cross-linkage between caseins, resulting in decreased hardness (Guinee et al., 2002; Joshi et al., 2003).

Although a slight difference in texture profile was observed between experimental cheeses at specific sampling times, no significant ($P > 0.05$) difference was observed among experimental cheeses during most of the storage period. This may be attributed to natural differences between cheeses loaves and not related to salting treatments. These findings are in accordance with those of Ayyash and Shah (2011) and Katsiari et al. (1997, 1998), who reported no significant ($P > 0.05$) differences between experimental cheeses in textural profile of Feta, Kefalograviera, and Halloumi cheeses.

Hardness and gumminess were positively correlated ($P < 0.05$) with Ca (r-values from 0.64 to 0.81 and 0.17 to 0.62, respectively) and P (from 0.63 to 0.80 and 0.37 to 0.53, respectively); however, cohesiveness and adhesiveness were correlated negatively. This provides further explanation as to why calcium reduction improved softening of Nabulsi cheeses.

Table 3. Hardness (N), cohesiveness, adhesiveness (J/m^3), and gumminess (N) of Nabulsi cheeses stored in 4 levels of NaCl and KCl during storage for 5 mo at room temperature[1]

Storage (mo)	Salt treatment[2]	Hardness	Cohesiveness	Adhesiveness	Gumminess
0	A	178.0 ± 35.1^{a}	2.66 ± 0.54^{b}	0.03 ± 0.01^{b}	505.3 ± 67.8^{a}
	B	188.9 ± 7.2^{a}	3.86 ± 0.37^{a}	0.12 ± 0.02^{a}	722.9 ± 47.3^{a}
	C	200.6 ± 12.1^{a}	3.14 ± 0.08^{ab}	0.03 ± 0.02^{b}	631.5 ± 50.4^{a}
	D	176.5 ± 22.4^{a}	3.26 ± 0.14^{ab}	0.04 ± 0.03^{ab}	569.3 ± 54.0^{a}
1	A	209.3 ± 43.3^{a}	5.70 ± 0.35^{a}	0.09 ± 0.02^{b}	1,207.7 ± 99.9^{a}
	B	143.1 ± 4.3^{ab}	4.74 ± 0.05^{b}	0.17 ± 0.04^{ab}	678.0 ± 20.7^{b}
	C	140.0 ± 7.2^{ab}	4.90 ± 0.21^{b}	0.15 ± 0.03^{ab}	687.7 ± 57.4^{b}
	D	137.2 ± 0.06^{b}	4.57 ± 0.09^{b}	0.20 ± 0.01^{a}	627.2 ± 12.0^{b}
2	A	137.7 ± 12.9^{a}	5.08 ± 0.23^{a}	0.10 ± 0.05^{b}	703.9 ± 91.5^{a}
	B	109.8 ± 3.0^{bc}	5.01 ± 0.43^{a}	0.15 ± 0.04^{ab}	552.3 ± 59.4^{ab}
	C	125.1 ± 8.2^{ab}	4.38 ± 0.19^{a}	0.06 ± 0.03^{b}	550.7 ± 56.1^{ab}
	D	88.6 ± 3.8^{c}	4.46 ± 0.09^{a}	0.27 ± 0.04^{a}	395.0 ± 18.2^{b}
3	A	103.9 ± 6.7^{b}	6.65 ± 0.06^{a}	0.06 ± 0.13^{a}	691.9 ± 51.3^{a}
	B	131.7 ± 8.6^{a}	5.15 ± 0.22^{b}	0.03 ± 0.04^{a}	679.9 ± 63.2^{a}
	C	124.1 ± 1.8^{ab}	5.13 ± 0.31^{b}	0.13 ± 0.05^{a}	636.3 ± 42.9^{a}
	D	106.0 ± 11.9^{ab}	5.28 ± 0.44^{b}	0.31 ± 0.18^{a}	549.3 ± 22.6^{a}
4	A	118.4 ± 8.0^{a}	5.76 ± 0.11^{a}	0.03 ± 0.03^{a}	683.7 ± 60.3^{a}
	B	111.7 ± 7.2^{a}	4.33 ± 0.18^{c}	0.17 ± 0.08^{a}	483.9 ± 7.0^{b}
	C	112.3 ± 7.3^{a}	5.07 ± 0.34^{b}	0.11 ± 0.06^{a}	568.8 ± 56.1^{ab}
	D	101.7 ± 6.1^{a}	5.20 ± 0.11^{ab}	0.09 ± 0.05^{a}	528.7 ± 37.8^{ab}
5	A	133.0 ± 28.6^{a}	6.77 ± 0.11^{a}	0.08 ± 0.08^{a}	906.0 ± 25.8^{a}
	B	128.4 ± 7.6^{a}	5.30 ± 0.34^{b}	0.13 ± 0.08^{a}	684.2 ± 74.6^{ab}
	C	112.6 ± 3.5^{a}	5.24 ± 0.09^{b}	0.12 ± 0.06^{a}	591.0 ± 27.5^{ab}
	D	89.6 ± 2.6^{a}	5.25 ± 0.30^{b}	0.21 ± 0.09^{a}	472.0 ± 38.6^{b}

$^{a-c}$Means in each column and at the same storage time with same letter did not differ significantly ($P > 0.05$).

[1]Mean values ± SE of 3 trials.

[2]Salt treatment: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt).

Na, K, Ca, and P

The concentrations (mg/100 g of cheese) of Na, K, Ca, and P in the 4 experimental cheeses during storage are presented in Figures 2, 3, 4, and 5, respectively. The content of Na varied ($P < 0.05$) with brine solution and storage time (Figure 2). The difference in Na content in cheeses kept in 4 brine solutions was significant ($P < 0.05$): Na contents in Nabulsi cheeses ranked in the order A > B > C > D, which was in line with the amount of Na in cheeses. Analysis of variance showed that Na contents increased ($P < 0.05$) in experimental

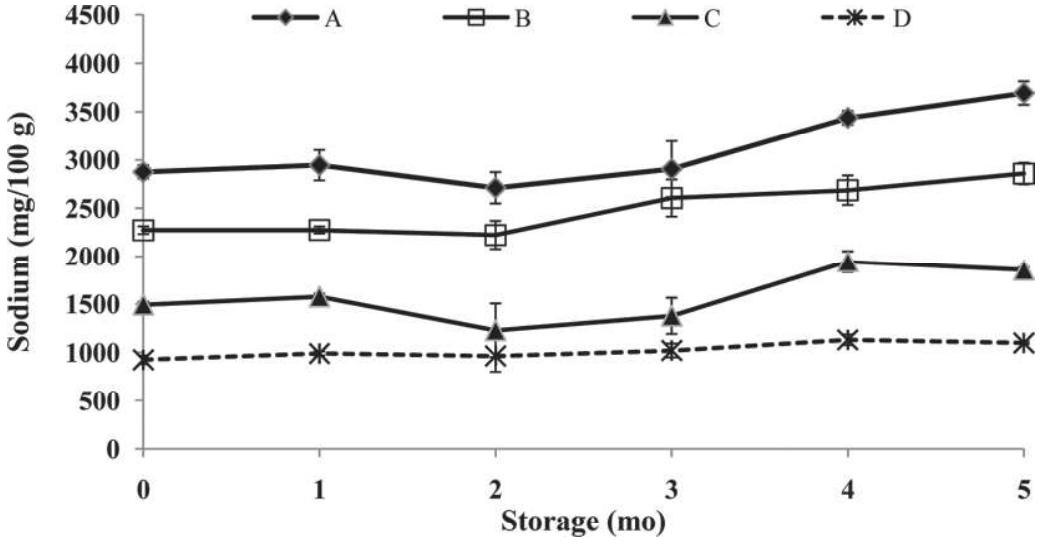

Figure 2. Sodium contents of Nabulsi cheeses kept with 4 levels of NaCl and KCl: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt), during storage for 5 mo at room temperature.

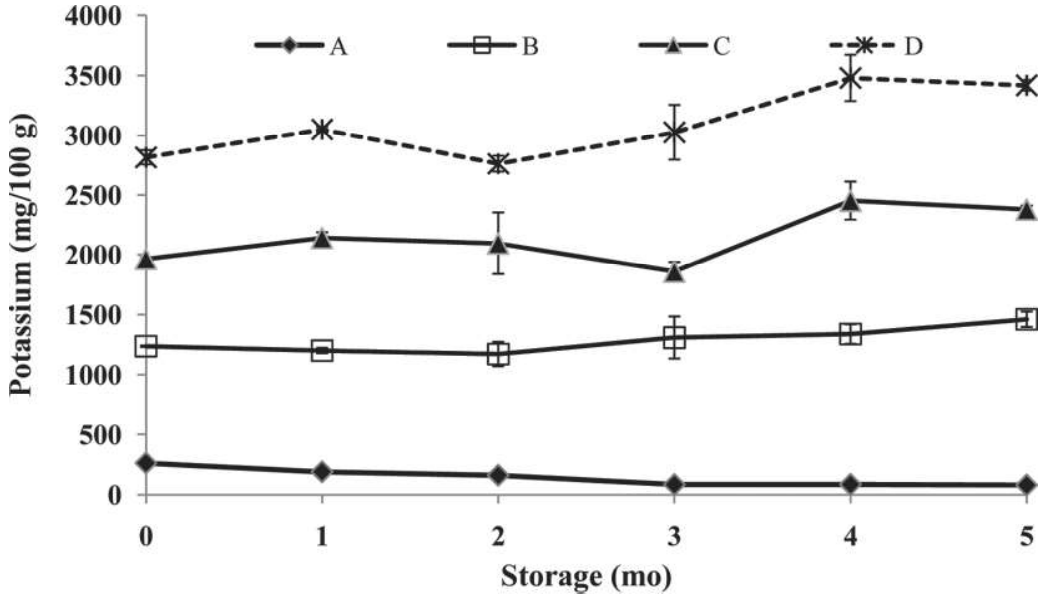

Figure 3. Potassium contents of Nabulsi cheeses kept with 4 levels of NaCl and KCl: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt), during storage for 5 mo at room temperature.

cheeses during storage for all salt treatments except for D. This may be due to Na migration from high concentration (brine solution) to lower concentration in cheese loaves (Geurts et al., 1974). The Na content in Nabulsi cheeses stored in brine solution D increased ($P > 0.05$) during storage, which may be due to the high penetration factor of K compared with Na and the lower Na than K concentration in brine solution D. Zorrilla and Rubiolo (1994) reported that KCl concentrations in the inner rings of each slice of Fynbo cheese were greater than NaCl concentrations. These findings agree with those of other researchers (Katsiari et al., 1997, 1998;

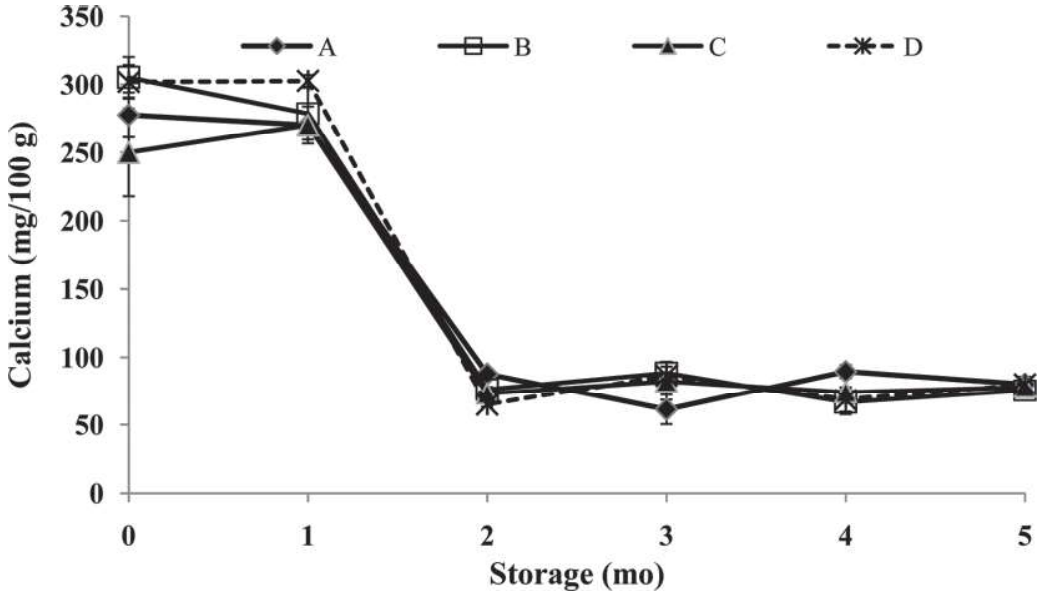

Figure 4. Calcium contents of Nabulsi cheeses kept with 4 levels of NaCl and KCl: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt), during storage for 5 mo at room temperature.

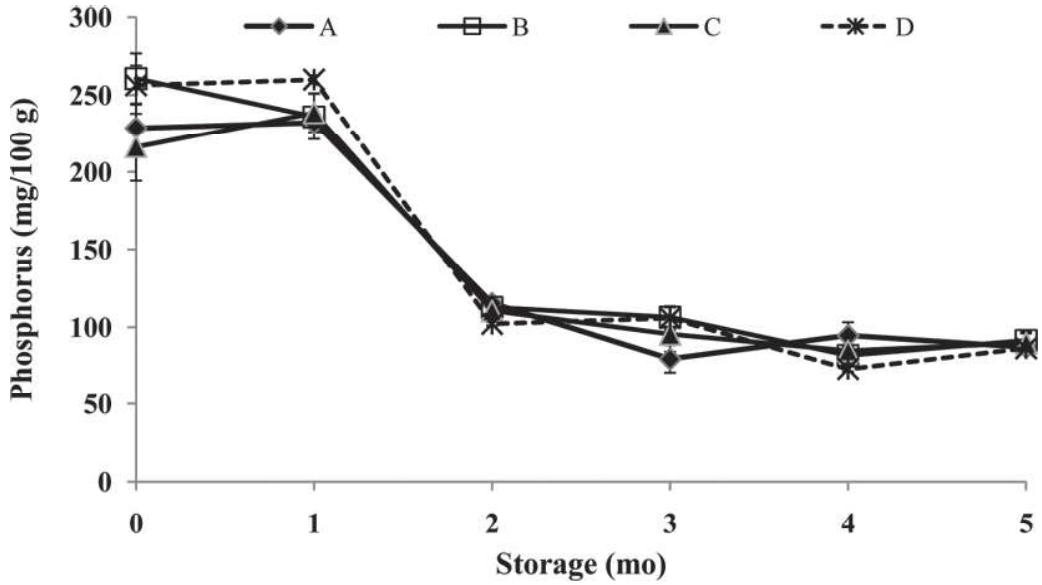

Figure 5. Phosphorus contents of Nabulsi cheeses kept with 4 levels of NaCl and KCl: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt), during storage for 5 mo at room temperature.

Ayyash and Shah, 2010a), who reported that Na contents increased during storage in Feta, Kefalograviera, and Halloumi cheeses.

The content of K varied ($P < 0.05$) depending on the brine solution and storage time (Figure 3). The differences in K content among experimental cheeses kept in 4 brine solutions were significant ($P < 0.05$): K contents in Nabulsi cheeses ranked in the order D > C > B > A, according to brine solutions. Analysis of variance showed that K contents increased ($P <$

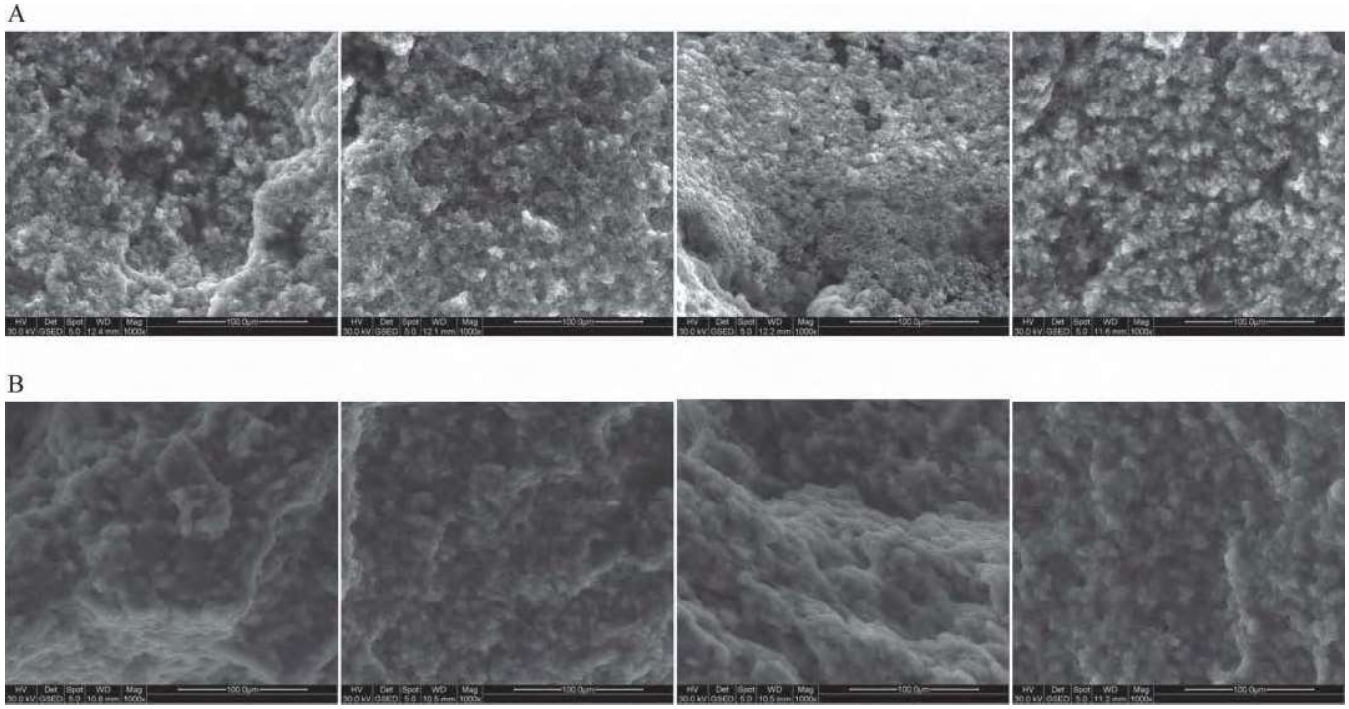

Figure 6. Environmental scanning electron micrograph of Nabulsi cheeses kept with 4 levels of NaCl and KCl: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt), at 0 mo (panel A) and 5 mo (panel B). The individual micrographs correspond to treatments A, B, C, and D (from left to right).

0.05) in experimental cheeses during storage for all salt treatments except for A. The K contents of Nabulsi cheeses kept in brine solution A decreased ($P > 0.05$) during storage. This trend may be attributed to the high K content that already existed in cheeses for treatment A; hence, K migrated from cheeses to the brine to reach equilibrium between cheeses and brine solution.

Analysis of variance showed that Ca and P contents in experimental cheese decreased ($P < 0.05$) during storage within a salt treatment. This may be attributed to migration of Ca and P with moisture migration from cheese to brine solution (Ayyash and Shah, 2010a). Pearson correlation values between Ca and P ranged from 0.96 to 0.98. No significant ($P > 0.05$) differences were observed in Ca and P contents between experimental cheeses at the same storage time. These findings are in accordance with Ayyash and Shah (2010a), who reported a similar trend for Ca in Halloumi cheese kept in 4 brine solutions.

Microstructure

The ESEM images of experimental cheeses at 0 and 5 mo of storage are shown in Figure 6 (panels A and B, respectively). Micrographs show compact, rough, and closed structures with small voids in all experimental cheeses. Microstructure of Nabulsi cheeses kept in various NaCl and KCl mixtures did not appear to differ compared with control cheeses. After 5 mo of storage, all experimental cheeses showed smooth texture compared with cheeses at 0 mo. This may be related to a reduction in Ca content during storage, resulting in a smooth and more homogeneous cheese microstructure (Joshi et al., 2004) and an increase in proteolytic activity during storage, which in turn increased intermediate products that could hold water and close voids in protein network.

CONCLUSIONS

Storage of Nabulsi cheeses in brine solutions partially substituted with KCl at 3NaCl:1KCl, 1NaCl:1KCl, or 1NaCl:3KCl did not significantly affect chemical composition or texture profile characteristics. Proteolytic activity of Nabulsi cheeses kept in brine solutions with a higher amount of KCl was greater compared with that in control cheeses. Penetration of KCl was greater than that of NaCl in the brine solution. Storage time significantly affected the moisture, ash, protein, proteolysis (WSN and TCA-SN), Ca, P, total viable count, hardness, and gumminess of Nabulsi cheeses within a salt treatment.

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7. Chapter 7: The effect of substitution of NaCl with KCl on chemical composition and functional properties of low-moisture Mozzarella cheese

Introduction

Chapter seven examines the effect of NaCl substitution with KCl on chemical composition, organic acids profile and functional properties of low-moisture Mozzarella cheese. A paper entitled the effect of substitution of NaCl with KCl on chemical composition and functional properties of low-moisture Mozzarella cheese by Ayyash M.M and N.P Shah was published in the peer reviewed Journal of Dairy Science 94(8):3761-3768

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

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

Declaration by [candidate name]: Mutamed Ayyash

Signature: [redacted] 11/9/12

Paper Title:

The effect of substitution of NaCl with KCl on chemical composition and functional properties of low-moisture Mozzarella cheese

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

Name	Contribution %	Nature of Contribution
Mutamed Ayyash	75	Design and perform the experiment Perform the samples analysis
		Evaluate the analytical data Perform the statistical analysis by SAS
		Prepare the major part of the manuscript
Nagendra Shah	25	Contribute to writing manuscript and submission to Journal

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

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J. Dairy Sci. 94:3761–3768
doi:10.3168/jds.2010-4103

The effect of substitution of NaCl with KCl on chemical composition and functional properties of low-moisture Mozzarella cheese

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ABSTRACT

The effect of NaCl substitution with KCl on chemical composition, organic acids profile, soluble calcium, and functionality of low-moisture Mozzarella cheese (LMMC) was investigated. Functionality (meltability and browning), organic acids profile, and chemical composition were determined. Chemical composition showed no significant difference between experimental cheeses at same storage period, and same salt treatment. Meltability of LMMC salted with 3NaCl:1KCl, 1NaCl:1KCl, and 1NaCl:3KCl was higher compared with only NaCl (control). The amount of soluble Ca and P increased significantly during storage, with no significant difference between salt treatments. Organic acids profile did not differ between salt treatments at the same storage time.
Key words: NaCl substitution, chemical composition, functional property, low-moisture Mozzarella cheese

INTRODUCTION

Salt (NaCl) is traditionally used as a preservative and is added to cheeses to control bacterial growth and enzyme activity, and to improve flavor (Guinee and Fox, 2004; Doyle and Glass, 2010). However, an increased level of NaCl in foods leads to health problems (Fitzgerald and Buckley, 1985). The recommended daily intake for sodium is 2.4 g, equivalent to 110 mmol of Na or 6.0 g of NaCl (Kaplan, 2000); the daily sodium intake in developed countries is 10 to 35 times higher than the recommended daily intake (Dillon, 1987). Salt contributes to hypertension, which in turn, causes cardiovascular disease. Positive correlations between salt and osteoporosis (Heaney, 2006) and kidney stones have been reported. Salt content in cheeses varies from 0.7% in Swiss-type cheese to about 8% in brined cheeses (Sihufe et al., 2003; Massey, 2005; Heaney, 2006). The World Health Organization has recommended that food manufacturers reduce the salt content in their products (World Health Organization, 2007). Dairy products, especially cheese, contribute 11 to 20% of total sodium intake (Guinee, 2004a,b; Tamime, 2006). Therefore, there is increased interest worldwide in producing cheeses with low sodium content (Demott, 1985; McMahon, 2010). However, when salt concentration in cheeses is reduced, proteolysis, water activity, acidity, and bitterness increase, firmness decreases (Katsiari et al., 1998), and irregular fermentation occurs (Johnson et al., 2009). Accordingly, substitution of NaCl with other salts has been considered as an alternative technique to reduce sodium in cheeses. Potassium chloride (KCl) has been recognized as a potential salt to substitute NaCl (Petik, 1987). A mixture of NaCl and KCl has been successfully used in various cheeses without any adverse effects on cheese quality (Reddy and Marth, 1991), including Halloumi cheese (Ayyash and Shah, 2010), Kefalograviera cheese (Katsiari et al., 1997, 2000), and Cheddar cheese (Katsiari et al., 2001); however, no information is available on low-moisture Mozzarella cheese (**LMMC**). Low-moisture Mozzarella cheese is one of the most popular cheeses in the world used for pizza manufacturing. Production of LMMC has been increasing rapidly over the last 2 decades (Kindstedt, 2002; Kindstedt et al., 2004). Hence, producing low-salt LMMC will be in line with worldwide demands to reduce salt in food. The aim of this study was to examine the effect of NaCl substitution with KCl on chemical composition, organic acids profile, and functional properties of LMMC.

MATERIALS AND METHODS

Cheese Making

Full-fat (3% fat) homogenized and pasteurized bovine milk was purchased from a local dairy plant (Melbourne, Victoria, Australia). Low-moisture Mozzarella cheese was manufactured according to Feeney et al. (2001) with some modifications (Figure 1). Forty liters of milk was tempered to 40°C and inoculated with TCC-4 direct-in-vat starter culture (Chr. Hansen, Bayswater, Victoria, Australia) consisting of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp.

Received December 17, 2010.
Accepted April 2, 2011.
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bulgaricus, which was added according to the manufacturer's instructions. After 40 min, chymosin (Chr. Hansen) diluted (1:20) with distilled water was added according to the manufacturer's instructions, and milk coagulated in 35 min. Curd was cut to 1-cm cubes and cooked at 40°C with a continuous agitation for 15 min. Whey was drained and manually pressed to release additional whey from the curd. The curd was cheddared and milled until the pH of the slabs reached 5.3 to 5.2. The milled curd was dry-salted at 46 g/kg using 4 combinations of NaCl and KCl: NaCl only (**A**; control), 3NaCl:1KCl (**B**), 1NaCl:1KCl (**C**), and 1NaCl:3KCl (**D**) and allowed to mellow for 20 min. The salted curd was plasticized in 4% brine solutions (A, B, C, and D) at 80°C for 5 min. The plasticized curd was molded in circular molds (~2.5 kg per block). Cheese blocks were kept in a refrigerator until the block temperature reached 15°C. The larger circular blocks were cut radially into small blocks (500 g per block) and then each block was vacuum-packaged into barrier bags using a Multivac A300/16 machine (Multivac Sepp Haggenmuller KG, Wolfertschwenden, Germany) followed by storage at 4°C for 27 d. All experimental cheeses were made in triplicate.

Sampling

Samples of LMMC from all salt treatments were taken at 0, 9, 18, and 27 d of storage. The whole (500 g) block was wiped with a paper towel and shredded, and then subsamples were taken for the following analyses.

Chemical Composition

Moisture was determined by the oven-drying method at 102°C, protein by the Kjeldahl method, fat by the Babcock method, and ash using the muffle furnace method according to AOAC (1995). For pH measurement, grated cheese (40 g) was macerated with 40 mL of distilled water, and the pH of the resultant slurry was measured using a calibrated digital pH meter (MeterLab, Pacific Laboratory Products, Blackburn, Victoria, Australia).

Organic Acids Analysis

Lactic, citric, and acetic acids were determined using HPLC according to Ayyash and Shah (2010). Briefly, grated cheese samples (5 g) were taken from shredded cheese, blended with 25 mL of 0.009 *N* sulfuric acid and 70 μL of 15.5 *N* nitric acid, and homogenized using an Ultraturrax homogenizer (Janke & Kunkel K.G., Staufen i. Breisgau, Germany) at 20,000 rpm. After standing for 1 h in a 50°C water bath, the slurry was centrifuged for 20 min at 4,000 × *g* at 4°C. The soluble fraction (1.5 mL) located between the upper layer (fat) and the precipitate (casein) was further centrifuged (14,000 × *g*, 10 min) using a bench-top centrifuge (RT7, Sorvall, Newtown, CT). The supernatant was filtered using a 0.45-μm filter (Millex, Millipore, Bedford, MA) and aliquots of approximately 1 mL from each sample were stored in HPLC vials at −20°C until analyzed. The HPLC system consisted of a Varian 9012 solvent delivery system, a Varian 9100 auto-sampler, a Varian 9050 variable wavelength UV/visible tunable absorbance detector and a 730 data module (Varian Inc., Palo Alto, CA). An Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad Laboratories, Richmond, CA), was used. Sulfuric acid (0.009 *N*), filtered through a 0.45-μm membrane filter (Millex, Millipore), was used as a mobile phase at a flow rate of 0.6 mL/min. The detection device was an UV-visible detector set at 220 nm with running time of 15 min.

Determination of Na, K, Ca, and P Contents by Multitype Inductively Coupled Plasma Atomic Emission Spectrometry

Contents of Na, K, Ca, and P in cheeses were determined by multitype inductively coupled plasma atomic emission spectrometry [ICPE-9000, Shimadzu Scientific Instruments (Oceania) Pty Ltd., Rydalmere, New South Wales, Australia] according to Ayyash and Shah (2010), and cheese samples were prepared according to Cortez et al. (2008). Grated cheeses (5 g) from shredded samples were digested in a mixture of HNO_3 and $HClO_4$ (5:1; Merck Pty. Ltd., Kilsyth, Victoria, Australia) on a hot plate until the digests were clear. The clear digests were filtered with a 0.45-μm filter and analyzed using the ICPE-9000. The ICPE-9000 consisted of an ASC-6100 autosampler, a hydride generator HVG-ICP, a hydrofluoric acid sample injection system HFS-2, a low-temperature thermostatic chamber NCB-1200, and the software package ICPE-9000. To calculate Na, K, Ca, and P concentrations in samples, a standard curve consisting of the 4 elements was prepared at 1, 10, 20, 30, and 40 μg/mL.

Soluble Ca and P

Soluble Ca expressed as percentage of total Ca in experimental LMMC was analyzed according to Metzger et al. (2001) with some modifications. Briefly, grated cheese (10 g) was taken from the shredded cheese lot and homogenized with 90 mL of MilliQ water (Millex) using an Ultraturrax homogenizer (Janke & Kunkel K.G.) at 10,000 rpm for 3 min. The cheese slurry was centrifuged at 4,000 × *g* for 20 min and then the supernatant

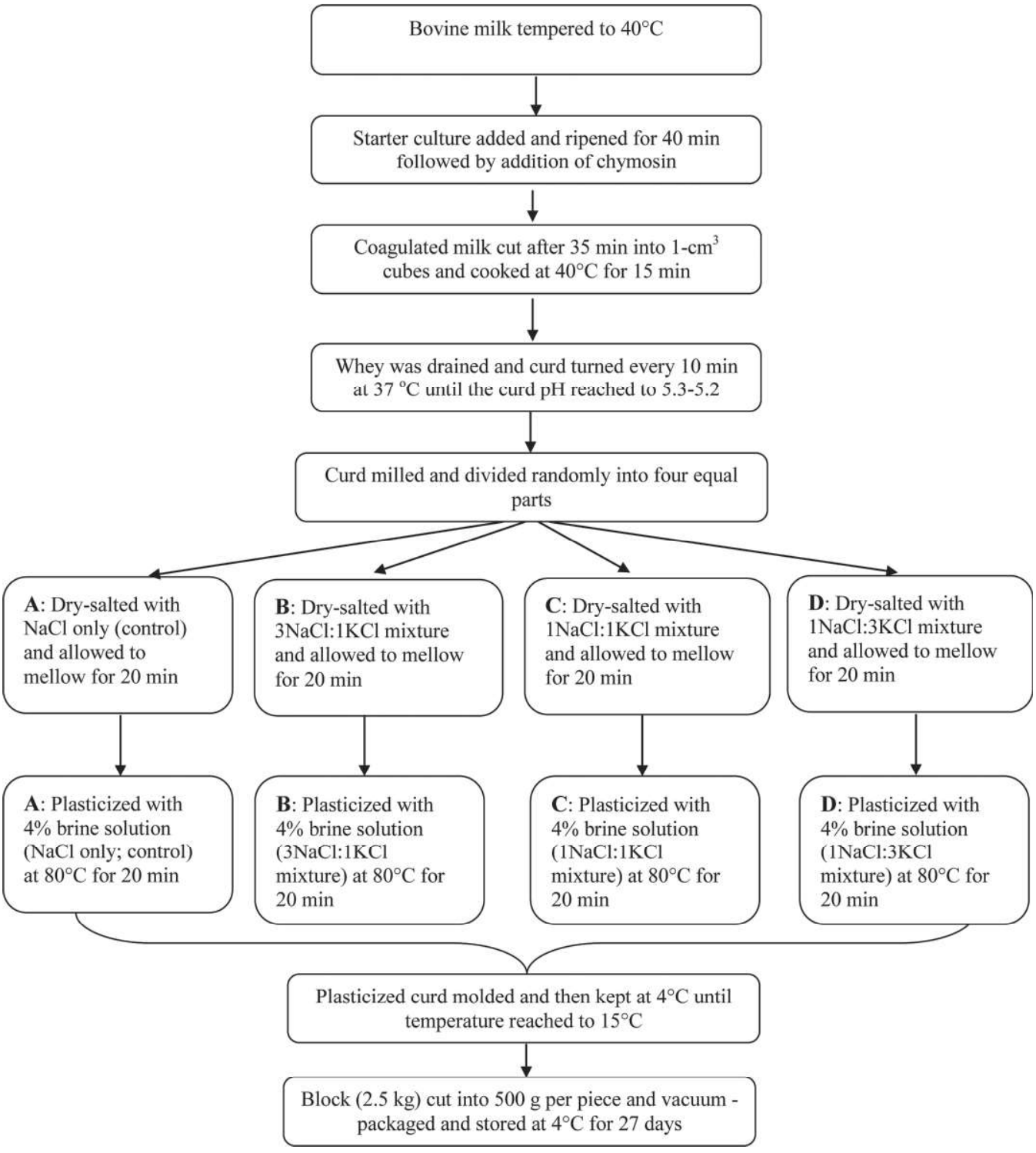

Figure 1. Manufacturing and salting procedure for low-moisture Mozzarella cheese.

was filtered using a Whatman #41 filter (Whatman International Ltd., Maidstone, UK). The filtrate was refiltered with a 0.45-μm filter (Millex), and soluble Ca and P were analyzed using inductively coupled plasma atomic emission spectrometry as described above. The Ca and P percentage was calculated as follow:

$$\text{Percentage soluble Ca or P} = \frac{\text{Soluble Ca or P (mg/100 g)}}{\text{Total Ca or P (mg/100 g)}} \times 100.$$

Meltability

Meltability of LMMC was determined according to Zisu and Shah (2005) using 250-mm-long glass tubes with a diameter of 24 mm and a thickness of 3 mm (R.B. Instruments, Mt. Eliza, Victoria, Australia) with some modifications. A plunger was used to pack 15 g of finely grated cheese into the glass tubes that were sealed at one end with a rubber stopper. The other end was sealed with a perforated rubber stopper to enclose the sample. The length of the compressed cheese sample was measured by using a Vernier caliper. Tubes were placed horizontally into an forced-air oven preheated to 110°C and kept for 100 min. Cheeses were allowed to cool to room temperature (~22°C) and the length of the melted cheese was recorded. The difference in the initial and the final length was presented as the melt distance (cm), and percentage increase in melt was calculated as follows:

$$\text{Percentage meltability} = \frac{\text{Melt distance after melting}}{\text{Initial length before melting}} \times 100.$$

Browning

Browning of LMMC was determined according to Barbano et al. (1994). Ground cheese samples were weighed (20 g) into an aluminum pan (7 cm in diameter and 3 cm high) and allowed to temper at room temperature (20°C) before heating. The pans containing the samples were placed into a preheated, forced-air oven at 100°C for 1 h. Cheese samples were cooled to room temperature. Color was measured using Minolta Chroma-meter CR-300 (Minolta Corporation Ltd., Ramsey, NJ) which was calibrated before testing. Three color indices, L (light to dark), a (red to green), and b (yellow to blue) values, were taken for each sample in triplicate.

Statistical Analysis

One-way ANOVA was performed to investigate significant difference at level 0.05 between experimental cheeses at same storage period. Fisher's test was carried out to examine differences between means of experimental cheeses at the same storage time (least significant difference, LSD). The significance of storage period was examined within a salt treatment using LSD. Two-way ANOVA was performed to investigate significant effect of salt treatment and storage period interaction on cheese attributes. Pearson correlations were calculated to investigate a correlation between the 4 measured minerals and other variables within the same salt treatment.

RESULTS AND DISCUSSION

Chemical Composition

The effects of substitution of NaCl with KCl at different levels on chemical composition of LMMC are presented in Table 1. Moisture, protein, fat, and ash contents did not differ significantly ($P > 0.5$) between experimental cheeses at same storage time. This may suggest that substitution of NaCl with KCl does not have any effect on chemical composition of LMMC (Guinee, 2004b). These findings are in accordance with the results of Ayyash and Shah (2010) and Katsiari et al. (1997, 1998), who reported no significant ($P > 0.5$) differences in chemical composition of Halloumi, Feta, and Kefalograviera cheeses, respectively. The ANOVA showed no significant difference ($P > 0.05$) in chemical composition of experimental cheeses during storage within a salt treatment.

Organic Acids Profile and pH Value

Contents of lactic, acetic, and citric acids and pH values of LMMC made with the 4 salt treatments are shown in Table 2. In general, experimental LMMC did not differ significantly ($P > 0.05$) in terms of organic acids profile or pH value. This is in agreement with the results on Halloumi cheese of Ayyash and Shah (2010). However, cheeses from treatments C and D showed higher ($P < 0.05$) contents of lactic, citric, and acetic acids compared with those for treatments B and A. In addition, pH values of cheeses in treatments A and B were lower compared with those in C and D. This may suggest that when KCl increased and NaCl decreased in a salt treatment, the production of organic acids increased. This also agrees with results of Ayyash and Shah (2010). Although the ANOVA showed no significant ($P > 0.05$) effect of storage period on organic acids profile and pH value, a slight decrease in pH value occurred during storage.

Contents of Ca, P, K, and Na

Contents of Ca, P, K, and Na in experimental LMMC made with the 4 salt treatments are presented in Figures

Table 1. Moisture, protein, fat, and ash contents of low-moisture Mozzarella cheese made with 4 different salt treatments and stored at 4°C for 27 d[1]

Storage time (d)	Salt treatment[2]	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
0	A	50.99 ± 1.59[a]	26.81 ± 0.84[a]	21.70 ± 0.99[a]	3.10 ± 0.14[a]
	B	51.13 ± 0.60[a]	26.53 ± 1.43[a]	22.20 ± 1.19[a]	3.10 ± 0.15[a]
	C	50.53 ± 0.86[a]	27.77 ± 0.49[a]	21.20 ± 0.44[a]	3.03 ± 0.07[a]
	D	50.80 ± 0.84[a]	27.93 ± 0.13[a]	21.73 ± 0.82[a]	3.07 ± 0.16[a]
9	A	50.28 ± 0.52[a]	27.10 ± 0.35[a]	24.13 ± 0.69[a]	2.97 ± 0.11[a]
	B	49.67 ± 0.09[a]	28.02 ± 0.69[a]	21.57 ± 0.69[b]	2.81 ± 0.04[a]
	C	49.80 ± 0.63[a]	27.65 ± 0.32[a]	21.10 ± 0.61[b]	2.88 ± 0.04[a]
	D	50.59 ± 0.90[a]	27.30 ± 0.22[a]	21.05 ± 1.05[b]	3.05 ± 0.14[a]
18	A	49.08 ± 0.21[a]	26.95 ± 0.39[a]	23.55 ± 0.26[a]	2.90 ± 0.05[a]
	B	50.13 ± 0.49[a]	27.07 ± 0.05[a]	21.93 ± 0.72[a]	2.91 ± 0.03[a]
	C	50.56 ± 0.45[a]	27.12 ± 0.36[a]	23.40 ± 0.95[a]	2.92 ± 0.09[a]
	D	49.79 ± 0.69[a]	27.75 ± 0.39[a]	23.60 ± 0.70[a]	2.99 ± 0.08[a]
27	A	48.86 ± 0.45[ab]	26.81 ± 0.38[ab]	21.77 ± 0.43[b]	2.80 ± 0.05[a]
	B	48.61 ± 0.16[b]	25.66 ± 0.48[b]	21.90 ± 0.45[b]	2.91 ± 0.03[a]
	C	49.16 ± 0.71[ab]	27.13 ± 0.34[a]	23.80 ± 0.35[a]	2.80 ± 0.15[a]
	D	50.31 ± 0.27[a]	27.17 ± 0.41[a]	22.77 ± 0.45[ab]	2.96 ± 0.02[a]

[a,b]Means in each column and at the same storage period with same letter did not differ significantly ($P > 0.05$).

[1]Mean values ± SE of 3 trials.

[2]Salt treatments: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt).

2, 3, 4, and 5, respectively. Contents of K and Na did differ ($P < 0.05$) between experimental cheeses. This expected finding was due to the salt mixtures that were added during processing of LMMC. Similar results were found by Ayyash and Shah (2010) for Halloumi cheese. Contents of Na and K did not differ during storage within a salt treatment (Figures 4 and 5). Contents of Ca and P in experimental LMMC decreased slightly ($P > 0.05$) during storage, with no significant differences between cheeses salted with different salt mixtures.

Soluble Ca and P

The percentage of soluble Ca and P in experimental LMMC is presented in Figures 6 and 7, respectively. Soluble Ca and P percentages did not differ ($P > 0.05$) between experimental cheeses at the same storage time (Figures 6 and 7), indicating that NaCl and KCl had similar effects on soluble Ca and P in LMMC. Within a salt treatment, soluble Ca and P levels increased ($P < 0.05$) in all experimental cheeses during storage. This

Table 2. Lactic, acetic, and citric acids content (mg/100 g of cheese) and pH values of low-moisture Mozzarella cheese made with 4 different salt treatments and stored at 4°C for 27 d[1]

Storage time (d)	Salt treatment[2]	Lactic acid	Acetic acid	Citric acid	pH
0	A	692.88 ± 24.3[b]	159.95 ± 7.4[b]	139.46 ± 2.3[b]	5.14 ± 0.02[b]
	B	676.00 ± 27.4[ab]	174.12 ± 1.5[ab]	145.61 ± 2.7[ab]	5.18 ± 0.04[ab]
	C	726.32 ± 12.5[a]	185.37 ± 3.0[a]	154.26 ± 2.6[a]	5.18 ± 0.03[ab]
	D	692.37 ± 23.1[ab]	173.67 ± 0.4[ab]	147.69 ± 4.5[ab]	5.24 ± 0.02[a]
9	A	692.66 ± 16.3[b]	174.20 ± 6.4[a]	147.22 ± 2.1[a]	5.16 ± 0.02[a]
	B	690.99 ± 34.2[b]	177.91 ± 1.6[a]	152.18 ± 1.7[a]	5.13 ± 0.07[a]
	C	730.05 ± 8.8[ab]	180.78 ± 3.9[a]	150.70 ± 1.4[a]	5.23 ± 0.03[a]
	D	775.44 ± 31.3[a]	185.36 ± 6.9[a]	153.09 ± 3.0[a]	5.22 ± 0.02[a]
18	A	801.49 ± 25.2[a]	167.35 ± 2.4[bc]	156.66 ± 3.8[a]	5.12 ± 0.01[a]
	B	732.18 ± 36.1[a]	156.80 ± 2.3[c]	152.67 ± 7.6[a]	5.21 ± 0.01[a]
	C	832.58 ± 51.7[a]	199.15 ± 10.2[a]	159.26 ± 2.7[a]	5.20 ± 0.05[a]
	D	775.15 ± 24.7[a]	185.21 ± 6.8[ab]	153.77 ± 7.9[a]	5.20 ± 0.01[a]
27	A	781.78 ± 33.1[a]	157.45 ± 17.9[a]	164.73 ± 3.4[a]	5.13 ± 0.02[ab]
	B	675.06 ± 31.4[b]	158.12 ± 2.5[a]	165.51 ± 1.7[a]	5.12 ± 0.03[ab]
	C	736.49 ± 20.5[ab]	163.39 ± 1.4[a]	158.84 ± 3.8[a]	5.11 ± 0.04[b]
	D	734.68 ± 29.4[ab]	183.22 ± 4.9[a]	154.08 ± 7.3[a]	5.20 ± 0.02[a]

[a–c]Means in each column and at the same storage period with same letter did not differ significantly ($P > 0.05$).

[1]Mean values ± SE of 3 trials.

[2]Salt treatments: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt).

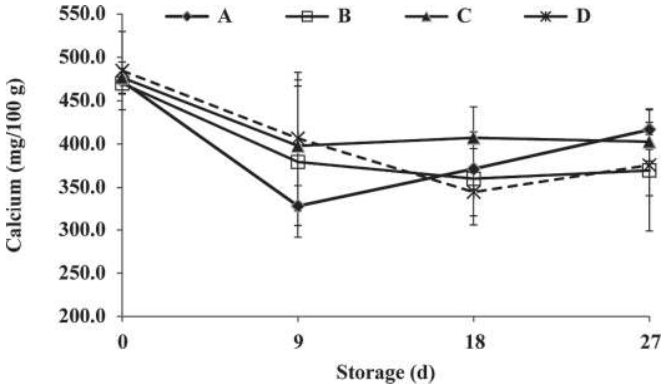

Figure 2. Calcium contents of low-moisture Mozzarella cheese salted with 4 levels of NaCl and KCl: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt), during storage at 4°C for 27 d.

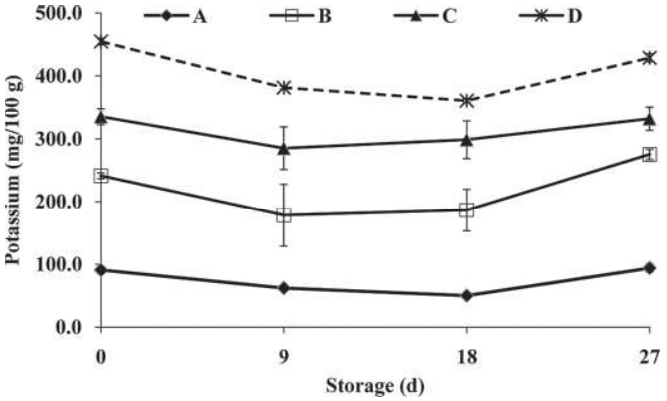

Figure 4. Potassium contents of low-moisture Mozzarella cheese salted with 4 levels of NaCl and KCl: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt), during storage at 4°C for 27 d.

is attributed to the conversion of insoluble Ca and P to soluble Ca and P during storage and is in accordance with the results of Metzger et al. (2001).

Meltability

Increase in meltability, measured in centimeters and as a percentage, of experimental LMMC during storage is shown in Figure 8A and B. Meltability did not differ ($P > 0.05$) between experimental LMMC at the same storage time. However, at the end of the storage period, LMMC in treatment A showed lower meltability compared with those in treatments B, C, and D. As shown in Figure 8, meltability increased significantly ($P < 0.05$) during storage within each salt treatment. This increase may be attributed to 2 factors: (1) an increase in proteolysis during storage, which increased meltability (Kindstedt et al., 2004; Upadhyay et al., 2004), and (2) an increase in the conversion of insoluble Ca to soluble Ca, which in turn reduced the strength between casein networks (Feeney et al., 2002; Guinee et al., 2002). Meltability correlated positively with an increase in soluble Ca during storage of LMMC within a salt treatment.

Browning

The L-values of experimental LMMC during storage are presented in Figure 9. The L-values did not differ ($P > 0.05$) between experimental LMMC at the same storage time. Analysis of variance showed no significant effect of salt treatment on browning. L-values increased ($P < 0.05$) during storage within a salt treatment; this means there was less browning at the end of stor-

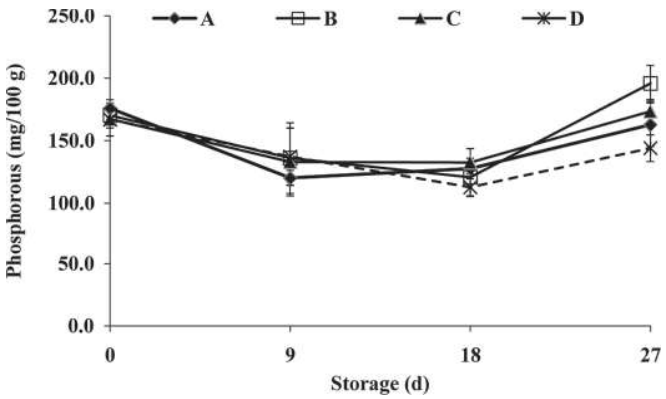

Figure 3. Phosphorous contents of low-moisture Mozzarella cheese salted with 4 levels of NaCl and KCl: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt), during storage at 4°C for 27 d.

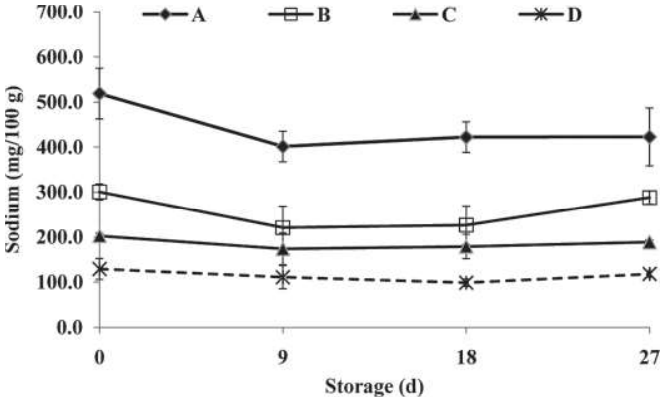

Figure 5. Sodium contents of low-moisture Mozzarella cheese salted with 4 levels of NaCl and KCl: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt), during storage at 4°C for 27 d.

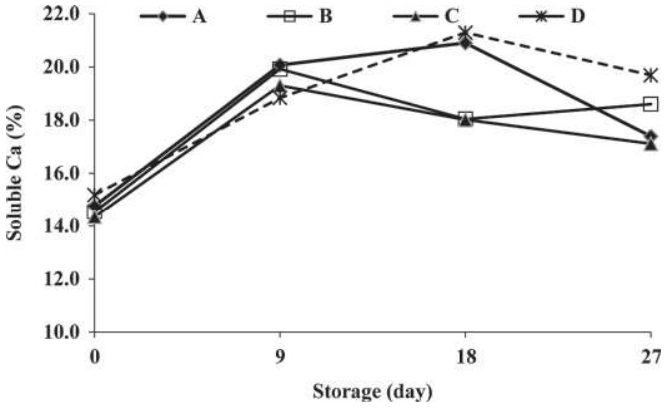

Figure 6. Soluble Ca percentage of low-moisture Mozzarella cheese salted with 4 levels of NaCl and KCl: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt), during storage at 4°C for 27 d.

age compared with that on d 0. This increase is in agreement with Osaili et al. (2010) who reported an increase in L-value of LMMC during storage. L-values correlated negatively with soluble Ca contents during storage within a salt treatment. As shown in Figure 9, LMMC of treatment A showed lower ($P < 0.05$) L-values during storage compared with other treatments. This may suggest that the presence of KCl reduced the amount of browning of LMMC during baking. Experimental LMMC salted with KCl (treatments C and D) showed lower phosphotungstic acid-soluble N compared with control (our unpublished data). Thus, browning should be lower in these cheeses compared with control cheeses. It has been postulated that browning correlates positively with proteolysis in cheeses (Mukherjee and Hutkins, 1994; Rudan and Barbano, 1998).

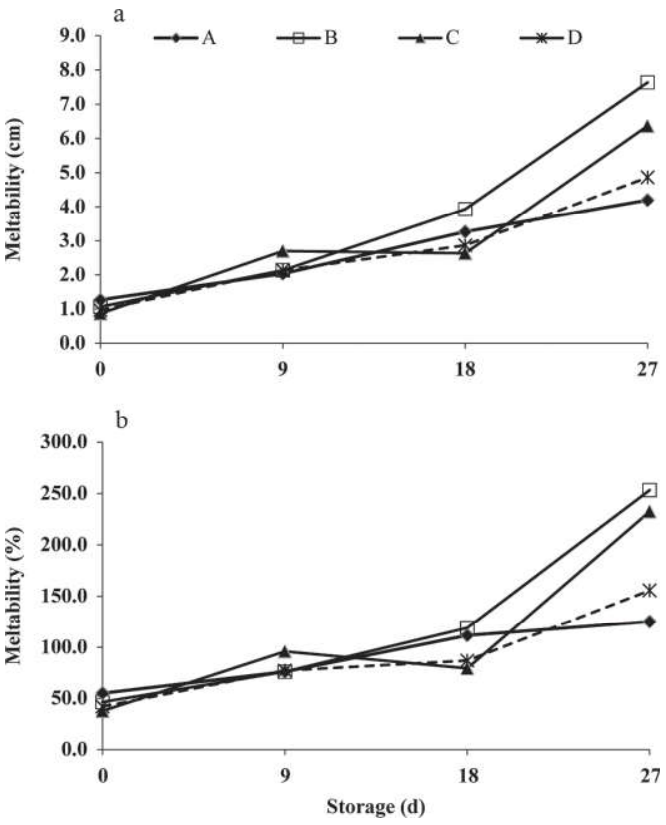

Figure 8. Meltability increase in centimeters (a) and in percentage (b) of low-moisture Mozzarella cheese salted with 4 levels of NaCl and KCl: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt), during storage at 4°C for 27 d.

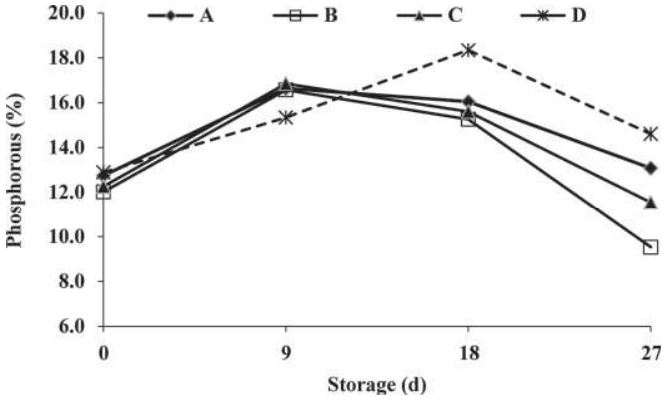

Figure 7. Soluble percentage P of low-moisture Mozzarella cheese salted with 4 levels of NaCl and KCl: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt), during storage at 4°C for 27 d.

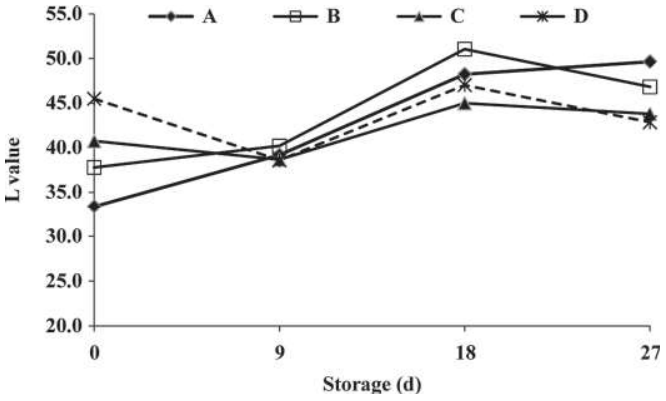

Figure 9. Lightness (L)-value of low-moisture Mozzarella cheese salted with 4 levels of NaCl and KCl: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt), during storage at 4°C for 27 d.

CONCLUSIONS

The substitution of NaCl with KCl (3:1, 1:1, 1:3) had similar effects on chemical composition, organic acids profile, and functional properties of LMMC. In addition, LMMC salted with 1NaCl:1KCl and 1NaCl:3KCl showed significantly higher meltability and browning of LMMC compared with control. The pH values of LMMC salted with 1NaCl:1KCl and 1NaCl:3KCl were, in general, higher than those of the control cheese. Browning of LMMC salted with only NaCl was lower than that of the other salt treatments.

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8. Chapter 8: Proteolysis of low-moisture Mozzarella cheese as affected by substitution of NaCl with KCl

Introduction

Chapter eight investigates the effect of partial substitution of NaCl with KCl on proteolysis, production of ACE-inhibitory peptides and lactic acid bacterial growth during ripening. A paper entitled proteolysis of low-moisture Mozzarella cheese as affected by substitution of NaCl with KCl by Ayyash M.M and N.P Shah was published in the peer reviewed Journal of Dairy Science 94(8):3769-3777.

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

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

Declaration by [candidate name]: Mutamed Ayyash **Signature:** [redacted] **Date:** 11/9/12

Paper Title:

Proteolysis of low-moisture Mozzarella cheese as affected by substitution of NaCl with KCl

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

Name	Contribution %	Nature of Contribution
Mutamed Ayyash	75	Design and perform the experiment Perform the samples analysis
		Evaluate the analytical data Perform the statistical analysis by SAS
		Prepare the major part of the manuscript
Nagendra Shah	25	Contribute to writing manuscript and submission to Journal

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
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J. Dairy Sci. 94:3769–3777
doi:10.3168/jds.2010-4104

Proteolysis of low-moisture Mozzarella cheese as affected by substitution of NaCl with KCl

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ABSTRACT

The proteolytic and ACE inhibitory activities of low-moisture Mozzarella cheese (LMMC) as affected by partial substitution of NaCl with KCl were investigated. Experimental LMMC were made and salted with 4 salt mixtures: NaCl only (control), 3NaCl:1KCl, 1NaCl:1KCl, and 1NaCl:3KCl, and then proteolytic activity and angiotensin-converting enzyme inhibitory activity were determined. Salt treatment significantly affected angiotensin-converting enzyme inhibitory activity and phosphotungstic acid-soluble N of LMMC during storage. Water-soluble N, trichloroacetic acid-soluble N, lactic acid bacteria population, and total free amino acids were unaffected during storage. Nonetheless, water-soluble N and trichloroacetic acid-soluble N increased significantly during storage within a salt treatment. Peptide profiles and urea-PAGE gels did not differ between experimental cheeses at the same storage time.

Key words: proteolysis, NaCl substitution, angiotensin-converting enzyme inhibition, low-moisture Mozzarella cheese

INTRODUCTION

Proteolysis is the major biochemical event to occur in cheese, besides glycolysis and lipolysis, that affects textural and functional properties of cheeses (Fox and McSweeney, 1996; Upadhyay et al., 2004). In general, cheeses show similar proteolysis trends; however, differences in cheese nature and manufacturing processes affect the proteolytic pattern. In general, proteolysis in rennet-coagulated cheeses starts with residual rennet activity or plasmin retained in curd after draining of whey and is called primary proteolysis. In this phase, caseins are broken down to large and small peptides. Second, other milk-indigenous enzymes and microbial enzymes (proteinases and peptidases) hydrolyze large peptide units to smaller units. Finally, bacterial enzymes from lactic acid bacteria (**LAB**) and nonstarter LAB hydrolyze small peptides into dipeptides and amino acids (Fox and McSweeney, 1996; Sousa et al., 2001; Upadhyay et al., 2004). Several factors have been found to affect proteolysis in low-moisture Mozzarella cheese (**LMMC**) that in turn affect its characteristics, including ripening temperature (Feeney et al., 2001), pH (Yun et al., 1993a,b; Yazici and Akbulut, 2007), starter culture type (Zisu and Shah, 2005, 2006), salt concentration, but not salt type (Rowney et al., 2004; Olson, 2007), manufacturing procedure (Renda et al., 1997; Osaili et al., 2010), and concentration of rennet (Dave et al., 2003). Salt (NaCl) is an important factor that affects the proteolytic pattern of LMMC during storage. Salt reduces the free water available in cheese, which consequently affects enzyme activity and growth of LAB (Guinee, 2004a; Upadhyay et al., 2004). Although NaCl addition is an essential step during LMMC production, it also causes major health hazards. A strong positive correlation has been found between NaCl and hypertension (Kotchen, 2005), osteoporosis (Heaney, 2006), and kidney stones (Massey, 2005). The World Health Organization (2007) recommended that food manufacturers reduce salt in their products. Numerous studies have reported a range of contribution of cheese to daily sodium intake (Guinee, 2004b; Fischer et al., 2009; Meneton et al., 2009; Anderson et al., 2010), which highlights the need to reduce NaCl in major cheeses such as LMMC. Currently, dairy plants produce tonnes of LMMC per day (Kindstedt et al., 2004). Nonetheless, simply reducing NaCl in cheeses can adversely affect cheese quality and safety. Decreasing the NaCl level accelerates proteolysis, produces off-flavors, and allows pathogens to grow (Guinee, 2004b; McMahon, 2010). Therefore, studies have focused on replacing NaCl with KCl, which is the most successful candidate to be used as NaCl replacer (Guinee, 2004a,b). Intake of KCl intake has not been linked to development of hypertension or cardiovascular diseases (Buemi et al., 2002; Geleijnse et al., 2007). Few studies have been carried out to investigate the effect of substitution of NaCl with KCl on proteolysis of Feta cheese (Katsiari et al., 2000), Halloumi cheese (Ayyash and Shah, 2010), and Fynbo cheese (Zorrilla et al.,

Received December 17, 2010.
Accepted April 2, 2011.
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1996). However, no information is available on proteolysis or production of angiotensin-converting enzyme (**ACE**) inhibitory peptides in LMMC during ripening. Angiotensin-I-converting enzyme (peptidyldipeptide hydrolase, EC 3.4.15.1) increases blood pressure by converting angiotensin-I to angiotensin-II, a potent vasoconstrictor, and by degrading bradykinin, a vasodilatory peptide (Johnston, 1992). Inhibition of ACE leads to a decrease in the level of the vasoconstrictory peptide, and therefore may result in an antihypertensive effect and may influence different regulatory systems involved in modulating blood pressure, immune defense, and nervous system activity (Meisel, 1998; Ong and Shah, 2008). The presence of these ACE inhibitory peptides is influenced directly by proteolysis, but only to a certain degree (Ong et al., 2007). Therefore, and because proteolysis is affected by salt substitution (Ayyash and Shah, 2010), the authors hypothesized that ACE inhibitory activity might be affected by substitution of NaCl with KCl. The objectives of this study were to investigate the effect of partial substitution of NaCl with KCl on proteolysis, production of ACE inhibitory peptides, and LAB growth during ripening.

MATERIALS AND METHODS

Cheese Making

Homogenized and pasteurized full-fat (3% fat) bovine milk was purchased from a local dairy plant (Melbourne, Victoria, Australia) and tempered to 40°C. Experimental LMMC was manufactured according to Feeney et al. (2001) with some modifications. Forty liters of milk was inoculated with TCC-4 direct-in-vat starter culture consisting of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* according to manufacturer instructions (Chr. Hansen, Bayswater, Victoria, Australia). After 40 min, diluted single-strength chymosin (1:20) was added according to the manufacturer's instructions (Chr. Hansen) and the milk coagulated in 35 min. Curd was cut into 1-cm cubes and cooked with continuous agitation at 40°C for 15 min. Whey was drained and curd was manually pressed to remove additional whey from the curd. The curd was cheddared and milled when the pH of the slabs reached to 5.3 to 5.2. The milled curd was dry-salted with 1 of 4 NaCl/KCl combinations: only NaCl (**A**; control), 3NaCl:1KCl (**B**), 1NaCl:1KCl (**C**), and 1NaCl:3KCl (**D**) at a level of 46 g/kg and the curd was allowed to mellow for 20 min. The salted curd was plasticized in 4% brine solutions (A, B, C, and D) at 80°C for 5 min and then manually kneaded. The plasticized curd was molded in circular molds 23 × 8 cm (diameter × height; ~2.5 kg per block). Cheese blocks were kept at 4°C until the temperature reached 15°C. The 2.5-kg blocks were cut into smaller blocks (500 g per block) and vacuum-packaged into barrier bags using a Multivac A300/16 machine (Multivac Sepp Haggenmuller KG, Wolfertschwenden, Germany) and then stored at 4°C for 27 d. Experimental cheeses were made in triplicate. Experimental cheeses were sampled at 0, 9, 18, and 27 d of storage. The cheese samples were shredded and then sub-sampled for the following analyses.

Gross Composition

Moisture was determined by the oven-drying method at 102°C, protein by the Kjeldahl method, fat by the Babcock method, and ash using the muffle furnace method, all according to AOAC (1995). For pH measurement, grated cheese (40 g) was macerated with 40 mL of distilled water, and the pH of the resultant slurry was measured using a calibrated digital pH meter (MeterLab, Pacific Laboratory Products, Blackburn, Victoria, Australia).

Enumeration of LAB

Counts of LAB in cheeses were enumerated according to Ayyash and Shah (2010). Grated cheese (10 g) was taken from the shredded cheese block, placed in a stomacher bag, and blended with 90 mL of sterile distilled water using a Stomacher-400 laboratory blender (Seward Medical, London, UK). Appropriate serial dilutions were made using 0.1% peptone, and LAB populations were counted using de Man, Rogosa, and Sharpe agar (Merck Pty. Ltd., Victoria, Australia). Inoculated plates in duplicate were incubated anaerobically at 37°C for 48 h using anaerobic jars. Plates having 25 to 250 colonies were counted and expressed as colony-forming units per gram of sample.

Assessment of Proteolysis

The water-soluble N (**WSN**) of the cheese samples was evaluated according to Kuchroo and Fox (1982). The nitrogen in the extract was estimated by Kjeldahl method (AOAC, 1995). Twelve percent trichloroacetic acid-soluble N (**TCA-SN**) and 5% phosphotungstic acid-soluble N (**PTA-SN**) were determined according to Ayyash and Shah (2010). The TCA-SN was estimated in 9 mL of filtrate obtained after precipitation of filtered water-soluble extract (**WSE**) of cheese with 24% TCA (Sigma-Aldrich, St. Louis, MO). The extent of secondary proteolysis (PTA-SN) was assayed similarly by using 9 mL of filtrate obtained after precipitation of filtered WSN of cheese with 10% phosphotungstic acid (Sigma-Aldrich).

Urea-PAGE

Preparation of cheese samples and urea-PAGE analysis were carried out according to Ayyash and Shah (2010) with some modifications. In brief, grated (1 g) cheese was homogenized with 10 mL of treatment buffer [6 *M* urea, 0.1 *M* β-mercaptoethanol, and 0.5% bromophenol blue (0.05% wt/vol) in 50% ethanol] for 3 min at 10,000 rpm using a tissue homogenizer (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 upper fat layer was removed and 0.5 mL of the supernatant was mixed with 3.5 mL of treatment buffer. An aliquot (1 mL) from the latest mixture was placed in a 1.5-mL Eppendorf tube and centrifuged at 3,000 × *g* for 15 min. Eleven microliters of whole casein solution (2 mg/mL; Sigma) was mixed with 40 μL of treatment buffer. From each sample and prepared whole casein, 11 μL of sample 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). All gels were run in a tank buffer (10 g of Tris and 29.9 g of glycine, wt/vol, in 2 L of distilled water) using a Bio-Rad Protean II xi cell powered by a Power Pac 300 and run for 30 min at 16 mA and then for 3 h 30 mA. The gels were fixed in destaining solution A (40% methanol, 7% acetic acid) for 30 min, and then gels were stained with 0.025% Coomassie Brilliant Blue (ICN Biochemicals Inc., Aurora, OH; 40% methanol, 7% acetic acid) for 4 h. The gels were then destained in destaining solution A for 1 h followed by destaining in destaining solution B (7% acetic acid, 5% methanol) until the background became clear. The gel images were recorded using a Fuji film intelligent dark box II with Fuji film LAS-1000 L V1.3 software (Fujifilm Co., Brookvale, NSW, Australia).

Preparation of WSE for Peptide Profile, ACE Inhibitory Activity, and Total Free Amino Acids

Grated cheese (10 g) was homogenized with 90 mL of MilliQ-water using an Ultraturrax homogenizer (Jonke & Kunkel K.G., Staufen i. Breisgau, Germany) at 20,000 rpm for 3 min to prepare another WSE. The slurry was centrifuged at 4,000 × *g* for 20 min and the fat layer was removed. A 10-mL aliquot was stored at −20°C for ACE-inhibitory activity, 10 mL was used to assess total free amino acids (**TFAA**), and then 10 mL of supernatant was freeze-dried (Dynavac FD300 freeze-dryer; Airvac Engineering Pty. Ltd., Rowville, Australia) at –20°C and –100 kPa for 72 h. The freeze-dried lyophilized samples were then stored at –20°C for analysis with reverse phase (**RP**)-HPLC as detailed below.

Peptides Profile of WSE by RP-HPLC

Peptide profile of LMMC during storage was examined by HPLC according to Ayyash and Shah (2010) and Ong and Shah (2008) with some modifications. An aliquot of freeze-dried WSE (80 mg) was mixed with 2 mL of solvent A containing 0.1% trifluoroacetic acid (Sigma Aldrich), centrifuged (3,000 × *g* for 10 min) using a bench-top centrifuge (Sorvall RT7, Newtown, CT), and filtered through a 0.45-μm filter (Millipore Corp., Bedford, MA). The RP-HPLC analysis was carried out using HPLC consisting of a Varian 9012 solvent delivery system, a Varian 9100 auto-sampler, a Varian 9050 variable wavelength UV-visible tunable absorbance detector, and a 730 data module (Varian Inc., Palo Alto, CA). A sample size of 40 μL was injected into the reverse-phase column (C18, 250 mm × 4.6 mm, 5 μm; Grace Vydac, Hesperia, CA) with a guard column (10 mm, 12 mm, Grace Vydac). Separation was conducted at room temperature (~22°C) at a flow rate of 0.75 mL/min. Eluent B was 60% acetonitrile (Merck, South Granville, New South Wales, Australia) containing 0.05% trifluoroacetic acid. A linear gradient was applied from 0 to 80% eluent B over 100 min. The detection device was an UV-visible detector set at 215 nm, and RP-HPLC chromatograms were visually analyzed.

Measurement of TFAA

Concentrations of TFAA of LMMC were measured by using the Cd-ninhydrin method according to Folkertsma and Fox (1992), and WSE prepared as previously were used in the analysis. An aliquot (100 μL) was placed in a glass tube and diluted with 1 mL of Milli-Q water (Millipore Corp.). Two milliliters of Cd-ninhydrin reagent [0.8 g of ninhydrin were dissolved in a mixture of 10 mL of glacial acetic acid (100%) and 80 mL ethanol (99.5%), followed by the addition of 1 g of $CdCl_2$ dissolved in 1 mL of Milli-Q water] was added. The mixture was heated at 84°C for 5 min and cooled to room temperature; then, absorbance at 507 nm was measured. All chemicals in this analysis were purchased from Sigma (St. Louis, MO) and analyses were carried out in duplicates.

ACE Inhibitory Activity in WSE

The ACE inhibitory activity was measured according to Wanasundara et al. (2002) using an HPLC method.

Angiotensin-converting enzyme and hippuryl-histidyl-leucine (**HHL**) were purchased from Sigma and prepared in Tris buffer (50 m*M*, pH 8.3) containing 300 m*M* NaCl. The assay consisted of 50 μL of 3.0 m*M* HHL, 50 μL of 1.25 milli-units ACE enzyme (from rabbit lung), and 50 μL of assay 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 further analysis by HPLC. The hippuric acid (**HA**) released from HHL by ACE was determined by HPLC. An external standard curve of HA was prepared to quantify the resultant HA in cheese samples. An aliquot (20 μL) of the mixture was injected onto the HPLC system consisting of a Varian 9012 solvent delivery system, a Varian 9100 autosampler, a Varian 9050 variable wavelength UV-visible tunable absorbance detector, and a 730 data module. The system was fitted with reverse-phase column (C18, 250 mm × 4.6 mm, 5 μm; Grace Vydac) with a guard column (10 mm, 12 mm, Grace Vydac). The separation was conducted at room temperature (~22°C) at a flow rate of 0.8 mL/min. The mobile phase was an isocratic system consisting of 12.5% (vol/vol) acetonitrile (Merck) in MilliQ water, and the pH was adjusted to 3.0 using glacial acetic acid. The detection device was an UV-visible detector set at 228 nm. The control reaction mixture contained 50 μL of buffer instead of the assay sample; the control was expected to liberate the maximum amount of HA from the substrate due to uninhibited ACE activity. The percentage inhibition of enzyme activity was calculated as follows:

$$\text{Inhibition (\%)} = \frac{\text{HA}\left(\text{control}\right) - \text{HA (sample)}}{\text{HA (control)}} \times 100.$$

Statistical Analysis

Two-way ANOVA was performed to investigate the significant effect of salt treatment and storage period interaction on cheeses attributes. One-way ANOVA was performed to investigate the significant difference at a level of 0.05 between experimental cheeses at the same storage time using SAS software (SAS Institute, 2008). Fisher's test was carried out to examine the differences in means between experimental cheeses at same storage period (least significant difference, LSD). The significance of storage period was analyzed at same salt treatment using LSD. Pearson correlations were calculated to investigate correlation between 4 measured minerals (Ca, P, K, and Na) and proteolysis within a salt treatment.

RESULTS AND DISCUSSION

Gross Composition

Gross composition of LMMC salted with 1 of 4 ratios of NaCl and KCl mixture stored at 4°C at 0 d is presented in Table 1. Moisture, protein, fat, and ash contents did not differ ($P > 0.05$) between experimental LMMC at 0 d of storage. This suggests that variations in the LMMC measured in this study were mainly related to salt treatment. These results are in agreement with those of Ayyash and Shah (2010), Fitzgerald and Buckley (1985), and Katsiari et al. (2000) who reported similar results in Halloumi, Cheddar, and Feta cheeses, respectively. The pH values were significantly ($P < 0.05$) higher in experimental cheeses salted with NaCl/KCl mixtures (B, C, and D) compared with the control (A). This may be attributed to higher pH of KCl (by ~0.3 pH units) compared with NaCl. This is in accordance with pH results of Ayyash and Shah (2010), who reported a slight increase in pH values of Halloumi cheese kept in NaCl/KCl mixture compared with the control.

Proteolysis and LAB Growth

The WSN, TCA-SN, and PTA-SN of experimental cheeses are presented in Table 2. Growth of LAB in experimental LMMC during storage period is shown

Table 1. Moisture, protein, fat, and ash contents and pH values of low-moisture Mozzarella cheese made with 4 different salt treatments and stored at 4°C at 0 d of storage[1]

Salt treatment[2]	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	pH
A	50.99 ± 1.59[a]	26.81 ± 0.84[a]	21.70 ± 0.99[a]	3.10 ± 0.14[a]	5.14 ± 0.02[b]
B	51.13 ± 0.60[a]	26.53 ± 1.43[a]	22.20 ± 1.19[a]	3.10 ± 0.15[a]	5.18 ± 0.04[ab]
C	50.53 ± 0.86[a]	27.77 ± 0.49[a]	21.20 ± 0.44[a]	3.03 ± 0.07[a]	5.18 ± 0.03[ab]
D	50.80 ± 0.84[a]	27.93 ± 0.13[a]	21.73 ± 0.82[a]	3.07 ± 0.16[a]	5.24 ± 0.02[a]

[a,b]Means in each column with same letter do not differ significantly ($P > 0.05$).

[1]Mean values ± SE of 3 trials.

[2]Salt treatments: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt).

in Figure 1. Concentrations of WSN and TCA-SN did not differ ($P > 0.05$) between experimental LMMC at the same storage time. However, WSN and TCA-SN increased significantly ($P < 0.05$) during storage within a salt treatment. This increase was due to the increase in proteolytic activity by coagulant enzyme residues, indigenous milk enzymes, and bacterial peptidases. These enzymes are reported to be responsible for the increase in proteolysis during cheese ripening (Fox and McSweeney, 1996; Upadhyay et al., 2004). These findings are in agreement with those of Ayyash and Shah (2010) and Katsiari et al. (2000, 2001), who reported similar results in Halloumi, Feta, and Kefalograviera cheeses during storage, respectively. A significant ($P < 0.05$) difference was observed in PTA-SN (Table 2) and LAB growth (Figure 1) between experimental cheeses at the same storage time. Analysis of variance showed that PTA-SN increased ($P < 0.05$) during storage within a salt treatment. However, LAB growth was insignificant during storage within a salt treatment (Figure 1). The observed differences in PTA-SN suggested that replacement of NaCl with KCl affected proteolytic enzymes secreted by LAB, but not logarithm growth of LAB. Ayyash and Shah (2010) reported similar observations in PTA-SN of Halloumi cheese. Armenteros et al. (2009) reported that some of the proteolytic enzymes were activated when the level of KCl was increased in a meat product, whereas other enzymes were inhibited. This supports our hypothesis that the presence of higher concentrations of KCl in cheeses stimulated LAB to produce proteolytic enzymes different from those in cheeses brined in NaCl, or activated particular enzymes and inhibited others different from those in cheeses brined in NaCl. However, more studies need to be conducted to investigate the effect of NaCl/KCl mixtures on proteolytic enzymes from LAB. In addition, the initial differences in pH may also have contributed to differences in PTA-SN (Upadhyay et al., 2004). The lower pH in treatment A (control) compared with other treatments may have been caused by increased chymosin retention in cheese and improved chymosin activity. A higher pH value in cheese is not favorable for chymosin activity (Feeney et al., 2002). Thus, the increase in chymosin activity enhanced proteolytic activity of LAB enzymes, which, in turn, increased PTA-SN in control cheese. The initial difference in pH may also be affected by metabolism of LAB, which affected the enzymes produced by LAB. Hence, this caused the significant differences in PTA-SN between the experimental cheeses (Feeney et al., 2002).

Urea-PAGE and TFAA

The proteolytic pattern of the experimental LMMC salted with 4 different NaCl/KCl mixtures are shown in Figure 2. Salt treatment had no significant effect on LMMC at the same storage time. Moreover, α- and β-caseins showed a slight hydrolysis during storage within a salt treatment. This suggests that partial substitution of NaCl with KCl had similar effects on

Table 2. Water-soluble N (WSN), 12% trichloroacetic acid-soluble N (TCA-SN), and 5% phosphotungstic acid-soluble N (PTA-SN) of low-moisture Mozzarella cheese salted with 4 different salt levels of NaCl and KCl during storage at 4°C for 27 d[1]

Storage time (d)	Salt treatment[2]	% of total N		
		WSN	TCA-SN	PTA-SN
0	A	3.79 ± 0.26[a]	2.95 ± 0.26[a]	0.73 ± 0.06[a]
	B	3.94 ± 0.16[a]	2.61 ± 0.04[a]	0.65 ± 0.04[ab]
	C	3.33 ± 0.27[a]	2.58 ± 0.13[a]	0.55 ± 0.03[ab]
	D	3.78 ± 0.18[a]	2.45 ± 0.14[a]	0.53 ± 0.03[b]
9	A	5.34 ± 0.38[a]	4.03 ± 0.25[a]	0.97 ± 0.04[a]
	B	5.60 ± 0.74[a]	3.93 ± 0.51[a]	0.78 ± 0.07[ab]
	C	4.69 ± 0.33[a]	3.46 ± 0.43[a]	0.62 ± 0.09[ab]
	D	4.53 ± 0.40[a]	4.01 ± 0.22[a]	0.40 ± 0.20[b]
18	A	5.08 ± 0.12[a]	4.69 ± 0.17[a]	1.03 ± 0.06[a]
	B	3.85 ± 0.14[b]	3.05 ± 0.18[b]	0.78 ± 0.01[ab]
	C	4.07 ± 0.28[b]	3.48 ± 0.81[ab]	0.67 ± 0.06[bc]
	D	3.97 ± 0.30[b]	4.21 ± 0.38[ab]	0.62 ± 0.03[c]
27	A	8.55 ± 0.19[a]	6.56 ± 0.42[a]	1.07 ± 0.08[a]
	B	8.42 ± 0.60[a]	7.36 ± 0.83[a]	0.98 ± 0.07[a]
	C	7.09 ± 0.49[a]	6.35 ± 0.68[a]	0.80 ± 0.08[ab]
	D	7.19 ± 0.40[a]	5.20 ± 0.85[a]	0.68 ± 0.11[b]

[a–c]Means in each column and at the same storage time with same letter do not differ significantly ($P > 0.05$).

[1]Mean values ± SE of 3 trials.

[2]Salt treatments: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt).

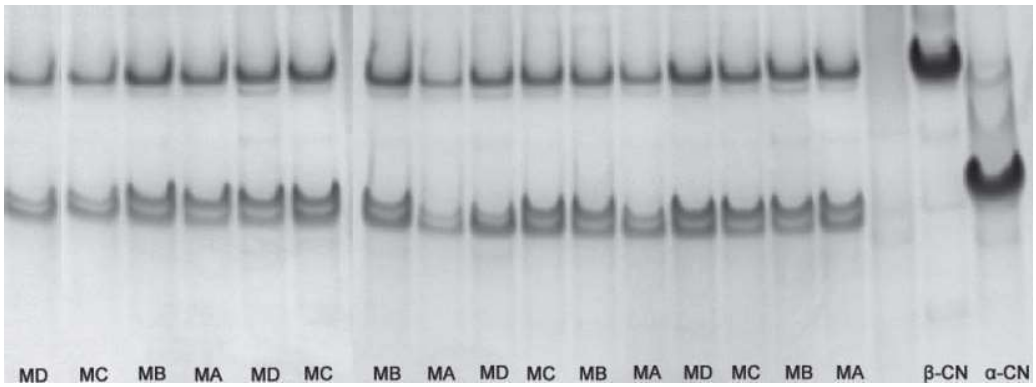

Figure 1. Urea-PAGE electropherograms of low-moisture Mozzarella cheese salted with A = only NaCl (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); and D = 1NaCl:3KCl (wt/wt) and stored at 4°C for 0, 9, 18, and 27 d.

LMMC compared with control. This finding agrees with those of Ayyash and Shah (2010) and Katsiari et al. (2000, 2001).

Concentrations of TFAA of experimental LMMC during storage are shown in Figure 3. Analysis of variance showed no significant ($P > 0.05$) difference in TFAA between experimental cheeses at the same storage time. Katsiari et al. (2000, 2001) reported similar results in Feta and Kefalograviera cheeses, respectively. Total free AA increased ($P > 0.05$) slightly during storage for all experimental cheeses. These concentrations of TFAA were lower compared with those of Katsiari et al. (2000, 2001) in Feta and Kefalograviera cheeses, respectively. This may be attributed to slower proteolytic activity in LMMC during storage regardless of the salt treatment. Cooking temperature and stretching treatment during the cheese making process may inactivate chymosin residues in LMMC. It has been reported that inactivation of coagulant residues in cheese delays the proteolytic process in cheese during storage (Fox, 1989; Fox and McSweeney, 1996; Upadhyay et al., 2004).

ACE Inhibitory Activity

The ACE inhibitory activity of LMMC salted with 4 different NaCl/KCl mixtures is shown in Figure 4; activity differed significantly ($P < 0.05$) between salt treatments at the same storage time, except at the end of storage (d 27). During storage of LMMC, ACE inhibitory activity was significantly ($P < 0.05$) higher in D cheeses compared with the other treatments. This suggests that the presence of higher concentrations of KCl increased the ACE inhibitory activity of LAB. In addition, we presumed that KCl affected LAB enzymes in a way that increased ACE activity compared with NaCl. Analysis of variance showed that the ACE inhibi-

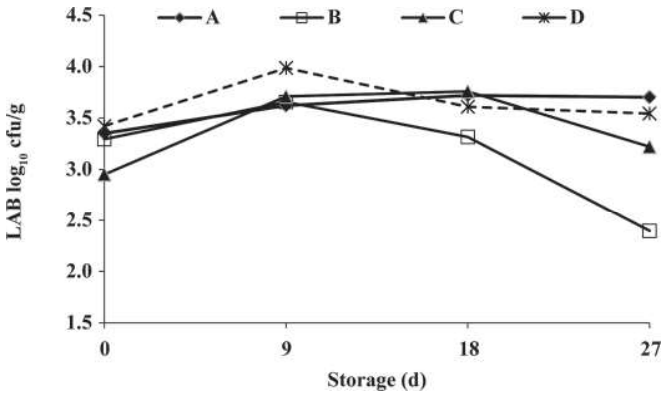

Figure 2. Lactic acid bacteria growth (LAB, cfu/g) of low-moisture Mozzarella cheese salted with 4 levels of NaCl and KCl mixture: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt), during storage at 4°C for 27 d.

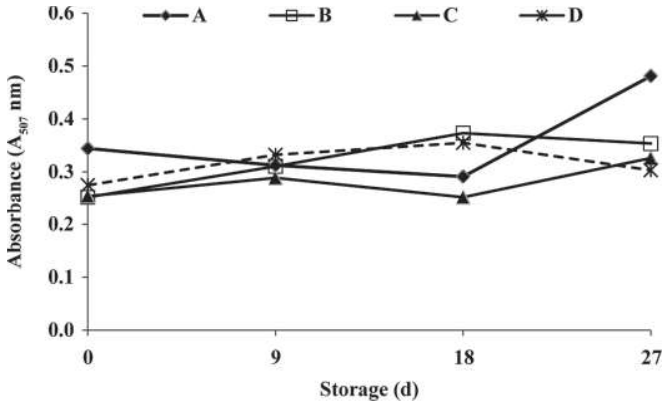

Figure 3. Total free amino acids as absorbance of water-soluble extracts of low-moisture Mozzarella cheese salted with 4 levels of NaCl and KCl mixture: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt), during storage at 4°C for 27 d.

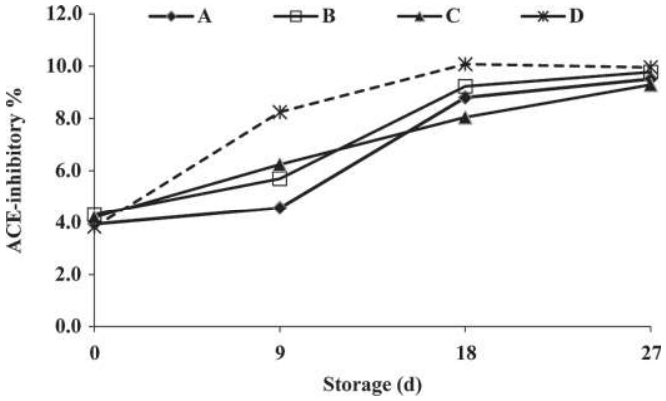

Figure 4. Angiotensin-converting enzyme (ACE) inhibitory percentage of water-soluble extracts of low-moisture Mozzarella cheese salted with 4 levels of NaCl and KCl mixture: A = NaCl only (control); B = 3NaCl:1KCl (wt/wt); C = 1NaCl:1KCl (wt/wt); D = 1NaCl:3KCl (wt/wt), during storage at 4°C for 27 d.

tory activity of LAB in LMMC increased significantly ($P < 0.05$) during storage within a salt treatment.

RP-HPLC Peptide Profile

The RP-HPLC peptide profiles of experimental LMMC during storage period are shown in Figure 5. In general, RP-HPLC chromatograms showed no significant differences in peptide profiles of experimental cheeses at the same storage time. Nonetheless, slight differences were observed at the end of storage between experimental LMMC. Peptide profiles varied slightly with LMMC ripening for all salt treatments, which may be due to slow proteolytic activity of enzymes in LMMC.

Correlations

Contents of Ca, P, K, and Na and percentage of soluble Ca are presented in the companion article (Ayyash and Shah, 2011). The Pearson correlations were calculated to investigate any correlation between Ca, P, K and Na. Our results showed negative correlations between total Ca and WSN, TCA-SN, PTA-SN, TFAA, and ACE inhibitory activity, in contrast to a positive correlation between percentage of soluble Ca and the same variables. This indicated that the conversion of insoluble Ca to soluble Ca during storage improved the proteolytic activity of LMMC in all salt treatments (data not shown). It has been reported that Ca in cheeses acts as cross-linkages for casein networks. Hence, the release of Ca from the casein network to the aqueous phase in cheeses (i.e., from insoluble Ca to soluble Ca) increased the susceptibility of caseins to hydrolysis (Feeney et al., 2002; Guinee et al., 2002).

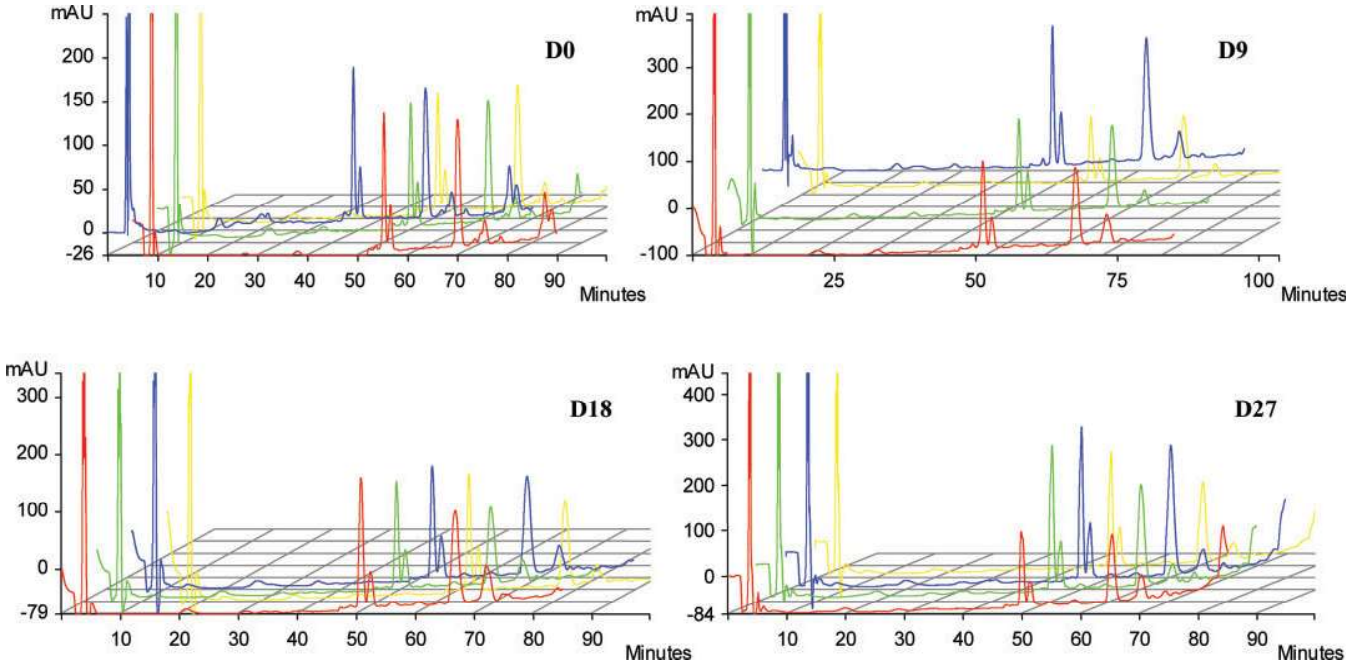

Figure 5. Reversed-phase HPLC profiles of water-soluble extracts of low-moisture Mozzarella cheese (at d 0, 9, 18, and 27) salted with A (red) = only NaCl (control); B (green) = 3NaCl:1KCl (wt/wt); C (blue) = 1NaCl:1KCl (wt/wt); D (yellow) = 1NaCl:3KCl (wt/wt) and stored at 4°C.

CONCLUSIONS

From this study, we concluded that partial substitution of NaCl with KCl did not significantly affect proteolysis of LMMC. A significant effect of partial substitution of NaCl with KCl on ACE inhibitory activity was observed; however, further investigation is needed in this area. In general, proteolysis variables and ACE inhibitory activity correlated positively with percentage of soluble Ca and negatively with Ca in LMMC within a salt treatment.

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9. Chapter 9: Effect of Partial NaCl Substitution with KCl on the texture profile, microstructure, and sensory properties of low-moisture mozzarella cheese

Introduction

Chapter nine investigates the effect of partial substitution of NaCl with KCl on the texture profile, microstructure, and sensory evaluation of LMMC and examines the relationship between soluble Ca^{2+} and texture profile as affected by NaCl substitution. A paper entitled The Effect of Partial NaCl Substitution with KCl on the texture profile, microstructure, and sensory properties of low-moisture Mozzarella cheese by Ayyash, M. M., F. Sherkat, and N. P. Shah was accepted for publication in the peer reviewed Journal of Dairy Research, 80:7-13. The microstructure images in this chapter are enlarged in Appendix C, page 172.

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

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

Declaration by [candidate name]: Mutamed Ayyash

Signature: [redacted] **Date:** 11/9/12

Paper Title:

The Effect of Partial NaCl Substitution with KCl on the Texture Profile, Microstructure, and Sensory Properties of Low-Moisture Mozzarella Cheese

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

Name	Contribution %	Nature of Contribution
Mutamed Ayyash	70	Design and perform the experiment Perform the samples analysis
		Evaluate the analytical data Perform the statistical analysis by SAS
		Prepare the major part of the manuscript
Nagendra Shah	20	Contribute to writing manuscript and submission to Journal
Frank Sherkat	10	Contribute to writing the manuscript

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
5. The original data is stored at the following location(s):

Location(s):
School of Biomedical and Health Sciences, Faculty of Health, Engineering and Science, Victoria University - Werribee campus, Victoria, Australia

and will be held for at least five years from the date indicated below:

		Date
Signature 1	[redacted]	11/9/12
Signature 2		11/09/12
Signature 3		11/09/12
Signature 4		

Ayyash, M., Sherkat, F., & Shah, N. (2013). Effect of partial NaCl substitution with KCl on the texture profile , microstructure, and sensory properties of low-moisture mozzarella cheese. Journal of Dairy Research, 80(1), 7-13. doi:10.1017/S002202991200043X

10. Chapter 10: The Effect of NaCl Substitution with KCl on Akawi Cheese: Chemical Composition, Proteolysis, ACE-inhibitory activity, Probiotic survival, Texture Profile and Sensory Properties.

Introduction

Chapter ten investigates the effect of salt substitution with KCl on chemical composition, proteolysis and ACE-inhibitory activity, probiotic survival, texture profile and sensory properties of probiotic Akawi cheese. A paper entitled The Effect of Partial NaCl Substitution with KCl on the Texture Profile, Microstructure, and Sensory Properties of Low-Moisture Mozzarella Cheese by Ayyash, M. M., F. Sherkat, and N. P. Shah was published in the peer reviewed Journal of Dairy Science 95:4747 - 4759

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

Declaration by [candidate name]: Mutamed Ayyash **Signature:** [redacted] **Date:** 11/9/12

Paper Title:

The Effect of NaCl Substitution with KCl on Akawi Cheese: Chemical Composition, Proteolysis, ACE-inhibitory activity, Probiotic survival, Texture Profile and Sensory Properties.

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

Name	Contribution %	Nature of Contribution
Mutamed Ayyash	70	Design and perform the experiment Perform the samples analysis
		Evaluate the analytical data Perform the statistical analysis by SAS
		Prepare the major part of the manuscript
Nagendra Shah	15	Contribute to writing manuscript and submission to Journal
Frank Sherkat	15	Contribute to writing the manuscript

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to a) granting bodies, b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit; and
5. The original data is stored at the following location(s):

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Signature 4		

J. Dairy Sci. 95:4747–4759
http://dx.doi.org/10.3168/jds.2011-4940

The effect of NaCl substitution with KCl on Akawi cheese: Chemical composition, proteolysis, angiotensin-converting enzyme-inhibitory activity, probiotic survival, texture profile, and sensory properties

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ABSTRACT

The effect of partial substitution of NaCl with KCl on Akawi cheese with probiotic bacteria was investigated during 30 d of storage at 4°C. Chemical composition, the survival of probiotic and lactic acid bacteria, proteolytic activity, and texture profile analysis were analyzed and sensory analysis was carried out to determine the effects of substitution. No significant differences were observed in moisture, protein, fat, and ash contents among the experimental Akawi cheeses at the same storage period. Significant differences were observed in water-soluble nitrogen and phosphotungstic-soluble nitrogen between experimental cheeses at the same of storage period. No significant difference was observed in the growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* between experimental cheeses at the same storage period. However, the growth of *Streptococcus thermophilus*, *Lactobacillus casei*, and *Lactobacillus acidophilus* was significantly affected among experimental cheeses. A significant difference was observed in soluble Ca among experimental cheeses at the same storage period. In general, no significant differences existed in hardness and adhesiveness among experimental cheeses at the same storage period. No significant differences existed in sensory attributes, including creaminess, bitterness, saltiness, sour-acid, and vinegar taste among experimental Akawi cheeses at the same storage period.
Key words: Akawi cheese, NaCl/KCl mixture, proteolysis, probiotic

INTRODUCTION

White brined cheeses produced in the Middle Eastern region are classified as rennet-coagulated cheeses with (Halloumi and Akawi cheeses) or without (Nabulsi cheese) starter culture. High salt content is a common characteristic that Middle Eastern cheeses possess (Abd El-Salam and Alichanidis, 2004). Akawi cheese is commonly consumed in Jordan, Syria, Palestine, and Lebanon (Tamime, 2006). Akawi cheese is usually consumed with breakfast and dinner. Traditionally, Akawi cheese is produced from pasteurized bovine or ovine milk (or a mixture of both). For Akawi cheese making, the milk is pasteurized (at 60°C for 30 min or 72°C for 15 s), cooled to about 35°C and then starter culture is added (1.5%). After 1 h, rennet is added to coagulate milk within an hour. The curd is cut, whey drained, and curd pieces are wrapped in cheesecloth in small portions (150 to 250 g), pressed for about 1 h, and brined in approximately 10% brine solution at 4°C. In general, Akawi cheese contains about 51.0% moisture, 21.6% fat, 22.5% protein, and 5% ash. Storage of Akawi cheese in brine solution is a critical step for maintaining quality and safety during 1 mo of storage. Because of the high salt content and health concerns, Middle Eastern people avoid consumption of Akawi cheese because of a positive correlation has been found between high sodium (Na) intake and hypertension (Kotchen, 2005), osteoporosis (Heaney, 2006), kidney stones (Massey, 2005), and cardiovascular diseases (Penner et al., 2007). The World Health Organization (Geneva, Switzerland) has recommended reducing salt in all food types to reduce health issues associated with high intake of salt (WHO, 2007). Several studies have reported that dairy products, especially cheeses, contribute to a noticeable amount of daily sodium dietary intake (Guinee, 2004a,b; Tamime, 2006). Cheese contributes to about 4% Na intake in the United Kingdom (Ash and Wilbey, 2010), 9.2% in France (Meneton et al., 2009), and 5% in Australia (NHMRC, 2003). A simple reduction in NaCl without substitution with other salts adversely affected the cheese quality (Reddy and Marth, 1991). Substitution of NaCl with KCl was successfully used to preserve cheese quality. Numerous studies have been carried out to examine the effect of NaCl substitution with KCl on Halloumi cheese (Ayyash and Shah, 2010, 2011a; Ayyash et al., 2011), Nabulsi cheese (Ayyash and

Received September 16, 2011.
Accepted June 1, 2012.
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Shah, 2011b), Feta cheese (Katsiari et al., 1997), and Kefalograviera cheese (Katsiari et al., 2001; Katsiari et al., 1998). Chemical composition, proteolysis, microbial growth and texture profile are the major attributes that have been investigated in these studies. No significant differences have been reported in most measured variables between experimental cheeses as compared with controls (Katsiari et al., 1997, 1998). However, Ayyash and Shah (2011a,b) reported significant differences in small peptides [phosphotungstic acid (**PTA**)-soluble nitrogen (**SN**) and TCA-SN contents] between experimental cheeses made using partial substitution of NaCl with KCl compared with the control. Ayyash and Shah (2011b,d) found that KCl affected the bacterial enzyme activity produced during storage, leading to qualitative or quantitative variations in enzymes produced by starter culture or nonstarter culture bacteria; this, in turn, affected cheese proteolysis. In the current study, 2 probiotic bacteria *Lactobacillus acidophilus* 2401 and *Lactobacillus casei* 290 were added to Akawi cheese to investigate for the first time the effect of salt substitution on probiotic survival. The aims of this study were to investigate the effect of salt substitution with KCl on chemical composition, proteolysis and angiotensin-converting enzyme (**ACE**)-inhibitory activity, probiotic survival, texture profile, and sensory properties of probiotic Akawi cheese.

MATERIALS AND METHODS

Bacteria Culture and Propagation

Four bacterial strains: *Streptococcus thermophilus* MS (**ST**), *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 (**LB**), *Lactobacillus acidophilus* 2401 (**LA**), and *Lactobacillus casei* 290 (**LC**) were obtained from Victoria University Culture Collection (Werribee Campus, Victoria, Australia). Cultures were stored in de Man, Rogosa, and Sharpe (**MRS**) broth (Oxoid Ltd., West Heidelberg, Victoria, Australia) with 20% glycerol at −80°C. To activate the 4 cultures, a 100-μL aliquot of each culture was individually transferred into MRS broth and incubated at 37°C for 24 h. Weekly transfer was performed to maintain the bacterial activity. Before the experiments, 2 successive culture transfers were carried out in MRS broth and a third transfer was in sterilized reconstituted skim milk (RSM 13%) and incubated at 37°C for 24 h.

Cheese Making

Full-fat (3%), homogenized, and pasteurized bovine milk was tempered at 40°C. *Streptococcus thermophilus* MS, LB, LA, and LC were added at 1.5% and mixed thoroughly for 2 min. After 45 min, the milk was coagulated using double-strength chymosin according to the manufacturer instructions (CHY-MAX; Chr. Hansen Pty Ltd., Bayswater, Victoria, Australia) for 40 min. The curd was cut into 1-cm^3 cubes using cheese knives and settled for 10 min. Whey was drained and curd cubes were transferred to cheesecloth to further drain for 25 min. Curd pieces were wrapped in cheesecloth in small portions (~150 g) and pressed for 90 min. Cheese pieces were placed in 4 brine solutions (at 10%): NaCl only (A), 3 NaCl:1 KCl (B), 1 NaCl:1 KCl (C), and 1 NaCl:3 KCl (D) and stored at 4°C for 1 mo. Samples were taken at 0, 10, 20, and 30 d of storage. The experiment was repeated in triplicate.

Chemical Composition

The chemical composition was determined according to the Association of Official Analytical Chemists methods (AOAC, 1995); moisture content was determined by the oven-drying method at 102°C, fat by the Babcock method, protein by the Kjeldahl method, and ash by the muffle furnace method. For pH measurement, 20 g of grated cheese was macerated in 20 mL of distilled water, and the pH of the resultant slurry was measured using a calibrated digital pH meter (MeterLab; Pacific Laboratory Products Australia Pty Ltd., Melbourne, Victoria, Australia).

Survival of Bacterial in Akawi Cheese

The 4 bacteria mentioned previously were enumerated according to Ong et al. (2006) and the International Organization for Standardization (ISO/IDF, 2002). Grated cheese (25 g) was blended into 225 mL of peptone water (0.1%). Serial dilutions were made and each type of bacteria was enumerated on appropriate agar. *Streptococcus thermophilus* MS were counted using M17 (Oxoid Ltd.) and aerobically incubated at 37°C for 24 h. *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 was enumerated using pH-modified (pH 4.5) MRS agar (Merck Pty Ltd., Victoria, Australia) and incubated anaerobically at 45°C for 72 h. de Man, Rogosa, and Sharpe agar supplemented with sorbitol (Merck Pty Ltd.) was used to enumerate LA. The supplemented MRS-sorbitol agar was prepared by adding 10 mL of membrane-filtered sterile 10% solutions (wt/vol) of sorbitol to 90 mL of molten MRS agar just before pouring. Plates were anaerobically incubated at 37°C for 72 h. de Man, Rogosa, and Sharpe-vancomycin (MRS-V) agar was used for the selective enumeration of LC. The MRS-V agar was prepared by adding 2 mL of 0.5 mg/mL of vancomycin (Sigma-Aldrich, St. Louis, MO) solution to 1 L

of molten MRS agar just before pouring to obtain a 1-mg/L final concentration. The plates were anaerobically incubated at 37°C for 72 h. The anaerobic condition for all bacteria was achieved using anaerobic jars and Anaerocult C (Merck Pty Ltd.) to generate an anaerobic atmosphere in anaerobic jars.

Organic Acids Analysis

Lactic, citric, and acetic acids were determined using HPLC according to Ayyash and Shah (2010), with some modifications. A 5-mL aliquot of water-soluble extract (**WSE**) was freeze dried. The freeze-dried powder was dissolved in 3 mL of 0.009 *N* sulfuric acid (Merck Pty Ltd.) and then centrifuged at 3,000 × *g* for 15 min. The supernatant was filtered using a 0.45-μm membrane filter (Millex; Millipore Corp., Bedford, MA) and 20 μL was injected into the HPLC system. The HPLC system consisted of a Varian 9012 solvent delivery system, a Varian 9100 autosampler, a Varian 9050 variable wavelength UV-visible (**UV-Vis**) tunable absorbance detector and a 730 data module (Varian Inc., Palo Alto, CA). An Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad Laboratories Inc., Richmond, CA), was used. Sulfuric acid (0.009 *N*), filtered through a 0.45-μm membrane filter (Millipore Corp.) was used as a mobile phase at a flow rate of 0.6 mL/min. The detection device was a UV-Vis detector set at 220 nm with running time of 15 min. Organic acid concentration was calculated using an external standard curve.

Assessment of Proteolysis

The WSE of the cheese samples were prepared according to Kuchroo and Fox (1982). The nitrogen in the extract was estimated by the Kjeldahl method (AOAC, 1995). Twelve percent TCA-SN and 5% PTA-SN were determined according to Ayyash and Shah (2011a). Trichloroacetic acid SN was analyzed as follows: a 5-mL sample of WSE was mixed with 24% TCA (Sigma-Aldrich) and kept overnight at room temperature. The mixture was centrifuged at 3,000 × *g* for 20 min and 9 mL of the supernatant was analyzed for SN by the Kjeldahl method (AOAC, 1995). The extent of secondary proteolysis (PTA-SN) was assayed similarly to TCA-SN using 9 mL of filtrate obtained after precipitation of filtered WSE of cheese with 10% PTA (Sigma-Aldrich).

Peptide Profile by Reverse-Phase HPLC

The peptide profile of Akawi cheese during the storage period was examined by HPLC according to Ayyash and Shah (2011a). Five milliliters of WSE was freeze dried and then mixed with 3 mL of solvent A containing 0.1% trifluoroacetic acid (TFA; Sigma-Aldrich), centrifuged (12,000 × *g* for 10 min) using a benchtop centrifuge (Sorvall RT7; Sorvall, Newtown, CT), and filtered through a 0.45-μm filter (Millipore Corp.). The reverse-phase HPLC analysis was carried out using HPLC consisting of a Varian 9012 solvent delivery system, a Varian 9100 autosampler, a Varian 9050 variable wavelength UV-Vis tunable absorbance detector, and a 730 data module. A sample size of 50 μL was injected into the C18 reverse-phase column (250-mm length × 4.6-mm diameter, 5-μm diameter of the column particles inside; Grace Vydac, Hesperia, CA) with a guard column (10 × 12 mm; Grace Vydac Separations Inc., Hesperia, CA). The separation was conducted at room temperature (~22°C) at a flow rate of 0.75 mL/min. Eluent B was 60% acetonitrile (Merck Pty Ltd.) containing 0.05% TFA. A linear gradient was applied from 0 to 80% eluent B over 100 min, followed by 10 min to equilibrate the column with 100% solvent A. The detection device was a UV-Vis detector set at 215 nm and reverse-phase HPLC chromatograms were visually analyzed.

Measurement of Total Free AA

Total free AA (**TFAA**) concentrations of water-soluble nitrogen of Akawi cheese samples were measured using the Cd-ninhydrin method according to Folkertsma and Fox (1992) and WSE, prepared as previously described, was used in analysis. An aliquot (100 μL) was placed in a glass tube and diluted with 1 mL of Milli-Q water (Millipore Corp.). Two milliliters of Cd-ninhydrin reagent [0.8 g of ninhydrin was dissolved in a mixture of 10 mL of glacial acetic acid (100%) and 80 mL of ethanol (99.5%), followed by dissolving 1 g of $CdCl_2$ in 1 mL of Milli-Q water] was added. The mixture was heated at 84°C for 5 min and then cooled at room temperature then absorbance at 507 nm was measured using a UV-VIS spectrophotometer [UV-1800; Shimadzu Scientific Instruments (Oceania) Pty Ltd., Rydalmere, New South Wales, Australia]. All chemicals were purchased from Sigma-Aldrich and analyses were carried out in duplicate.

Proteolytic Activity by o-Phthalaldehyde

Proteolytic activity of Akawi cheese samples was determined according to Church et al. (1983). Briefly, 50 μL of WSE was placed into a 1.5-mL cuvette and mixed with 1 mL of *o*-phthalaldehyde (**OPA**) reagent prepared in a 50-mL volumetric flask as follows: 25 mL of 100 m*M* disodium tetraborate (Merck Pty Ltd.), 2.5 mL of 20% (wt/wt) SDS (Merck Pty Ltd.), 40 mg of OPA dissolved in 1 mL of methanol (Merck Pty Ltd.),

and 100 μL of β-mercaptoethanol (Sigma-Aldrich). The volume was completed with Milli-Q water. The cuvette was inverted twice and the incubated for 2 min at room temperature. The absorbance was measured at 340 nm using a UV-VIS spectrophotometer [Shimadzu Scientific Instruments (Oceania) Pty Ltd.] in duplicate.

ACE-Inhibitory Activity in WSE

Angiotensin-converting enzyme-inhibitory activity was measured according to Ayyash and Shah (2011d) using HPLC. Five milliliters of WSE was freeze dried and then dissolved in 1 mL of Tris buffer (50 m*M*, pH 8.3) containing 300 m*M* NaCl. The ACE enzyme (from rabbit lung) and hippuryl-histidyl-leucine (HHL) were purchased from Sigma-Aldrich and prepared in Tris buffer. The assay consisted of 50 μL of 3.0 m*M* HHL, 50 μL of 1.25-mU/mL ACE enzyme, and 50 μL of dissolved WSE sample. The mixture was placed in a glass tube and then incubated for 30 min at 37°C in a water bath without mixing and then for an additional 30 min after mixing. Glacial acetic acid (150 μL) was added to stop ACE enzyme activity. The mixture was kept at −20°C to be analyzed using HPLC. The resulting hippuric acid from the previous reaction was determined using HPLC. An external standard curve was prepared to quantify the hippuric acid in assay samples. An aliquot (20 μL) of the mixture was injected into the HPLC system consisting of a Varian 9012 solvent delivery system, a Varian 9100 autosampler, a Varian 9050 variable wavelength UV-Vis tunable absorbance detector, and a 730 data module. The system was fitted with a reverse-phase column (Luna C18, 250-mm length × 4.6-mm diameter, 5-μm diameter of the HPLC column particles inside; Phenomenex Australia Pty Ltd., New South Wales, Australia) with a guard column (C18 4 × 3.0 mm; Phenomenex Australia Pty Ltd.). The separation was conducted at room temperature (~22°C) at a flow rate of 0.8 mL/min. The mobile phase was an isocratic system consisting of 12.5% (vol/vol) acetonitrile (Merck Pty Ltd.) in Milli-Q water, and the pH was adjusted to pH 3.0 using glacial acetic acid (Merck Pty Ltd.). The detection device was a UV-Vis detector set at 228 nm. The control reaction mixture contained 50 μL of buffer instead of the assay sample; the control was expected to liberate the maximum amount of hippuric acid from the substrate due to uninhibited ACE activity. The percent inhibition of enzyme activity was calculated as follows:

$$\text{Inhibition percentage} = \frac{\text{hippuric acid (control)} - \text{hippuric acid (sample)}}{\text{hippuric acid (control)}} \times 100.$$

Determination of Total Ca, P, Na, and K Contents by Multitype Inductively Coupled Plasma Atomic Emission Spectrometry

Calcium, K, Na, and P contents in Akawi cheese samples were determined by multitype inductively coupled plasma atomic emission (**ICPE**) spectrometry [ICPE-9000; Shimadzu Scientific Instruments (Oceania) Pty Ltd.] according to Ayyash and Shah (2011b) with some modifications. Grated cheese (5 g) was digested in a mixture of HNO_3 and $HClO_4$ (5:1; Merck Pty Ltd.) on a hot plate until the mixture was clear. The original sample was diluted 1 to 10 in Milli-Q water. The diluted samples were filtered with a 0.45-μm filter and analyzed using ICPE-9000 [Shimadzu Scientific Instruments (Oceania) Pty Ltd.]. All samples were diluted 100 times and then filtered using 0.45 μm (Millipore Corp.) before directly injecting into the ICPE-9000 spectrometer. The ICPE-9000 spectrometer consisted of an ASC-6100 autosampler, a hydride generator HVG-ICP, a hydrofluoric acid sample injection system (HFS-2), a low-temperature thermostatic chamber (NCB-1200), and a software package (ICPE-9000). Calcium, K, Na, and P concentrations were calculated in samples using an external standard curve consisting of the 4 elements, prepared at 1, 10, 20, 30, and 40 mg/kg.

Soluble Ca, P, Na, and K

The soluble Ca, expressed as percentage of total Ca in Akawi cheese, was analyzed according to Ayyash and Shah (2011c), with some modifications. Briefly, grated cheese (10 g) was taken from the shredded cheese lot and homogenized with 90 mL of Milli-Q water (Millipore Corp.) using an Ultra-Turrax homogenizer (Janke & Kunkel GmbH & Co. KG, Staufen im Breisgau, Germany) at 10,000 rpm for 3 min. The cheese slurry was centrifuged at 4,000 × *g* for 20 min and then the supernatant was filtrated using Whatman #41 filter paper (Whatman International Ltd., Maidstone, UK). The filtrate was filtered with a 0.45-μm filter (Millex; Millipore Corp.) and then soluble Ca and P were analyzed using the ICP method, which was previously mentioned. The Ca and P percentage was calculated as follows:

$$\text{Percentage of soluble Ca, P, Na, and K} = \frac{\text{soluble Ca, P, Na, and K } (\text{mg}/100\text{ g})}{\text{total Ca, P, Na, and K } (\text{mg}/100\text{ g})} \times 100.$$

Texture Profile Analysis

The texture profile (hardness, cohesiveness, adhesiveness, and gumminess) was analyzed according to Ayyash

Table 1. The description of sensory evaluation attributes[1]

Attribute	Definition	Reference
Creaminess	Flavor associated with fresh milk, creamy product, condensed milk	Ultra-high-temperature (UHT) pasteurized cream with 35% fat
Bitterness	Chemical-like, aspirin, taste sensation of caffeine	Caffeine (0.06% in water)
Saltiness	Fundamental taste sensation of which sodium chloride is typical	Sodium chloride (1% in water)
Sour-acid	Fundamental taste sensation of lactic or citric acid	Citric acid (0.08% in water) and acetic acid (0.08% in water)
Vinegar	Flavor associated with vinegar	Vinegar test from market

[1]Sensory attribute definitions and references were described according to Ong (2007) and Delahunty and Drake (2004).

et al. (2011). Cylinders of 25 × 20-mm (diameter × height) cheeses were cut from Akawi cheese blocks at the center. Specimens were kept in a refrigerator at 4°C overnight in sealed plastic containers and then texture profiles were determined. Hardness, cohesiveness, adhesiveness, and gumminess were measured using an Instron universal testing machine (model 5564; Instron Ltd., London, UK) based on the principles described by Pons and Fiszman (1996). The samples were compressed to 50% of their heights using a 100-N load cell with a flat plunger and the crosshead movement was adjusted to 50 mm/min. Two cycle compressions were carried out and the data were collected using Merlin software (Woodville, South Australia). Analyses were performed in duplicate.

Sensory Properties

Ten panelists were recruited for assessing sensory attributes. The panelists were familiar with basic sensory evaluation techniques for cheeses and were further trained for their ability to detect creaminess, bitterness, saltiness, sour-acid, and vinegar taste. Prior to sensory evaluation, they also participated in a briefing session. All panelists signed a Victoria University human subject's consent form. Sensory evaluation was conducted for Akawi cheeses at d 10, 20, and 30 of storage. Cheese samples were removed from the refrigerator, tempered at room temperature (20°C) for 1 h and cut into pieces and placed on white plates coded with random numbers. The panelists evaluated 12 cheese samples and water was provided to cheese panelists between each sample. The panelists scored the attributes using a 10-point scale as follows: creaminess: 1 = absent, 10 = extremely creamy; bitterness: 1 = absent, 10 = extremely bitter; saltiness: 1 = absent, 10 = extremely salty; sour-acid: 1 = not acidic, 10 = extremely acidic; and vinegar: 1 = not detected, 10 = high intensity. The definitions of sensory attributes and references are presented in Table 1. Panelists were trained for their ability to rank products with different concentrations of lactic acid, acetic acid, sodium chloride, and caffeine from lowest intensity to highest intensity in water and in cream cheese. Tests were repeated until the panelists were able to rank different intensities of lactic acid, acetic acid, sodium chloride, and caffeine in both water and cream cheese (Ong, 2007).

Statistical Analysis

One-way ANOVA was performed to investigate significant differences ($P < 0.05$) between experimental cheeses at the same storage period. The Fisher least significant difference (LSD) test was carried out to examine differences between means of experimental cheeses at the same storage period. The significance of storage period was examined for the same salt treatment using the LSD test.

RESULTS AND DISCUSSION

Chemical Composition

Moisture, protein, fat, and ash contents in addition to pH values of 4 experimental Akawi cheeses during 30 d of storage are presented in Table 2. In general, no significant differences ($P > 0.05$) were observed in moisture, protein, fat, and ash contents among the experimental Akawi cheeses at same storage period. These results are in accordance with those of Ayyash and Shah (2010, 2011b) who reported similar results in Halloumi and Nabulsi cheeses. As seen in Table 2, an occasional significant difference ($P < 0.05$) occurred between experimental cheeses in moisture content at d 20, protein and ash contents at d 30, and fat content at d 0 and 20. This may be attributed to differences in cheese loaves, but not due to salt treatment. Similar results have been reported by Ayyash and Shah (2011b). The pH values significantly ($P < 0.05$) differed among Akawi cheeses for the same salt treatment. After d 10 of storage, pH values of Akawi cheese in treatments C and D were higher compared with treatments A and B. This suggested that when the substitution level with KCl increased (C = 50% and D = 75%), the pH values increased. This result is in agreement with Ayyash and Shah (2011b) and Katsiari et al. (1997) who reported

Table 2. Moisture, protein, fat, and ash contents and pH values (mean values ± SE of 3 trials) of Akawi cheeses kept in 4 levels of NaCl and KCl during storage for 30 d at 4°C

Storage (d)	Salt treatment[1]	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	pH
0	A	45.5 ± 0.2^{a}	18.1 ± 0.6^{a}	20.5 ± 0.8^{b}	1.9 ± 0.1^{a}	6.60 ± 0.03^{b}
	B	46.2 ± 0.4^{a}	19.1 ± 0.4^{a}	21.1 ± 0.9^{ab}	1.9 ± 0.0^{a}	6.67 ± 0.01^{a}
	C	46.1 ± 0.5^{a}	18.6 ± 0.2^{a}	22.9 ± 0.6^{a}	1.9 ± 0.0^{a}	6.67 ± 0.01^{a}
	D	45.5 ± 0.4^{a}	18.1 ± 0.5^{a}	21.7 ± 0.2^{ab}	1.9 ± 0.0^{a}	6.68 ± 0.00^{a}
10	A	41.5 ± 1.1^{a}	15.0 ± 0.7^{a}	21.2 ± 0.4^{a}	6.1 ± 0.1^{a}	6.51 ± 0.04^{b}
	B	44.2 ± 1.2^{a}	15.9 ± 0.6^{a}	22.9 ± 0.3^{a}	6.1 ± 0.1^{a}	6.65 ± 0.02^{a}
	C	42.4 ± 0.4^{a}	15.9 ± 0.6^{a}	21.9 ± 0.8^{a}	6.0 ± 0.0^{a}	6.66 ± 0.01^{a}
	D	42.5 ± 0.5^{a}	15.8 ± 0.4^{a}	22.7 ± 7.6^{a}	6.1 ± 0.0^{a}	6.69 ± 2.23^{a}
20	A	43.6 ± 1.1^{ab}	15.3 ± 0.2^{a}	22.3 ± 0.4^{ab}	6.0 ± 0.1^{a}	6.54 ± 0.05^{ab}
	B	44.3 ± 0.4^{a}	16.3 ± 0.3^{a}	23.0 ± 0.2^{a}	6.0 ± 0.0^{a}	6.47 ± 0.01^{b}
	C	43.1 ± 0.9^{ab}	15.8 ± 0.5^{a}	21.2 ± 0.5^{b}	5.9 ± 0.1^{a}	6.51 ± 0.01^{ab}
	D	41.6 ± 0.7^{b}	16.1 ± 0.2^{a}	21.8 ± 0.2^{ab}	5.9 ± 0.1^{a}	6.59 ± 0.02^{a}
30	A	43.0 ± 1.0^{a}	17.8 ± 1.1^{a}	20.4 ± 0.6^{a}	6.0 ± 0.1^{b}	6.47 ± 0.03^{c}
	B	42.1 ± 1.1^{a}	16.7 ± 0.3^{ab}	20.8 ± 0.4^{a}	6.2 ± 0.0^{ab}	6.54 ± 0.01^{b}
	C	42.8 ± 0.6^{a}	16.2 ± 0.7^{ab}	21.3 ± 0.4^{a}	6.1 ± 0.0^{b}	6.60 ± 0.02^{a}
	D	42.1 ± 1.4^{a}	15.3 ± 0.1^{b}	19.5 ± 0.9^{a}	6.3 ± 0.1^{a}	6.64 ± 0.01^{a}

$^{a–c}$Means within a column and at the same storage time with the same superscript letter did not differ significantly ($P > 0.05$).
[1]Salt treatment: A = NaCl only (control); B = 3 NaCl:1 KCl (wt/wt); C = 1 NaCl:1 KCl (wt/wt); D = 1 NaCl:3 KCl (wt/wt).

similar results. This pH trend may be due to the higher pH of the KCl solution by about 0.3 compared with NaCl. Analysis of variance showed that moisture and protein contents significantly ($P < 0.05$) decreased and ash content increased ($P < 0.05$) during storage at the same salt treatment. This is due to migration of moisture from the cheese loaf toward the brine solution, which decreased ($P < 0.05$) the moisture content during storage. Also, the migration of salt from the brine solution toward the cheese loaf increased ($P < 0.05$) the ash content during the storage period for the same salt treatment. These results agree with those of Ayyash and Shah (2010) who reported similar results in Halloumi cheese.

Assessment of Proteolysis

Five measured indicators of proteolysis: water-soluble nitrogen, TCA-SN, PTA-SN, OPA, and TFAA are presented in Table 3. No significant differences were found in TCA-SN and TFAA between experimental cheeses at the same storage period. Starting from d 10 to the end of storage, significant ($P < 0.05$) differences were observed in water-soluble nitrogen and PTA-SN among

Table 3. Water-soluble nitrogen (WSN), TCA, phosphotungstic acid (PTA), *o*-phthalaldehyde (OPA), and total free AA (TFAA) contents (mean values ± SE of 3 trials) of Akawi cheeses kept in 4 levels of NaCl and KCl during storage for 30 d at 4°C

Storage (d)	Salt treatment[1]	WSN	TCA	PTA	OPA	TFAA
0	A	3.29 ± 0.15^{a}	1.02 ± 0.01^{a}	0.19 ± 0.01^{a}	0.51 ± 0.10^{a}	0.22 ± 0.03^{ab}
	B	3.28 ± 0.11^{a}	0.93 ± 0.05^{a}	0.18 ± 0.01^{a}	0.42 ± 0.09^{a}	0.19 ± 0.01^{ab}
	C	3.28 ± 0.09^{a}	1.00 ± 0.06^{a}	0.19 ± 0.01^{a}	0.50 ± 0.04^{a}	0.26 ± 0.01^{a}
	D	3.63 ± 0.17^{a}	1.03 ± 0.07^{a}	0.20 ± 0.01^{a}	0.46 ± 0.12^{a}	0.16 ± 0.04^{b}
10	A	5.60 ± 0.43^{ab}	1.31 ± 0.29^{a}	0.60 ± 0.04^{a}	0.25 ± 0.02^{a}	0.14 ± 0.01^{a}
	B	5.21 ± 0.27^{ab}	1.26 ± 0.17^{a}	0.42 ± 0.06^{b}	0.33 ± 0.06^{a}	0.20 ± 0.06^{a}
	C	4.59 ± 0.15^{b}	1.10 ± 0.02^{a}	0.45 ± 0.06^{ab}	0.38 ± 0.15^{a}	0.20 ± 0.03^{a}
	D	5.94 ± 0.28^{a}	1.22 ± 0.11^{a}	0.40 ± 0.01^{b}	0.60 ± 0.19^{a}	0.14 ± 0.02^{a}
20	A	5.56 ± 0.40^{a}	1.24 ± 0.07^{a}	0.69 ± 0.13^{a}	0.30 ± 0.02^{b}	0.16 ± 0.04^{a}
	B	4.66 ± 0.04^{b}	1.22 ± 0.07^{a}	0.46 ± 0.01^{b}	0.36 ± 0.05^{b}	0.11 ± 0.01^{a}
	C	4.69 ± 0.24^{b}	1.12 ± 0.08^{a}	0.39 ± 0.02^{b}	0.38 ± 0.04^{ab}	0.19 ± 0.06^{a}
	D	4.61 ± 0.04^{b}	1.24 ± 0.19^{a}	0.41 ± 0.02^{b}	0.51 ± 0.05^{a}	0.16 ± 0.06^{a}
30	A	4.77 ± 0.39^{ab}	1.53 ± 0.31^{a}	0.50 ± 0.06^{a}	0.36 ± 0.03^{b}	0.11 ± 0.01^{a}
	B	4.94 ± 0.18^{ab}	1.60 ± 0.14^{a}	0.37 ± 0.02^{b}	0.26 ± 0.04^{b}	0.09 ± 0.01^{a}
	C	4.39 ± 0.07^{b}	1.51 ± 0.09^{a}	0.42 ± 0.04^{ab}	0.30 ± 0.01^{b}	0.12 ± 0.02^{a}
	D	5.20 ± 0.12^{a}	1.66 ± 0.07^{a}	0.42 ± 0.01^{ab}	0.75 ± 0.03^{a}	0.14 ± 0.04^{a}

^{a,b}Means within a column and at the same storage time with the same superscript letter did not differ significantly ($P > 0.05$).
[1]Salt treatment: A = NaCl only (control); B = 3 NaCl:1 KCl (wt/wt); C = 1 NaCl:1 KCl (wt/wt); D = 1 NaCl:3 KCl (wt/wt).

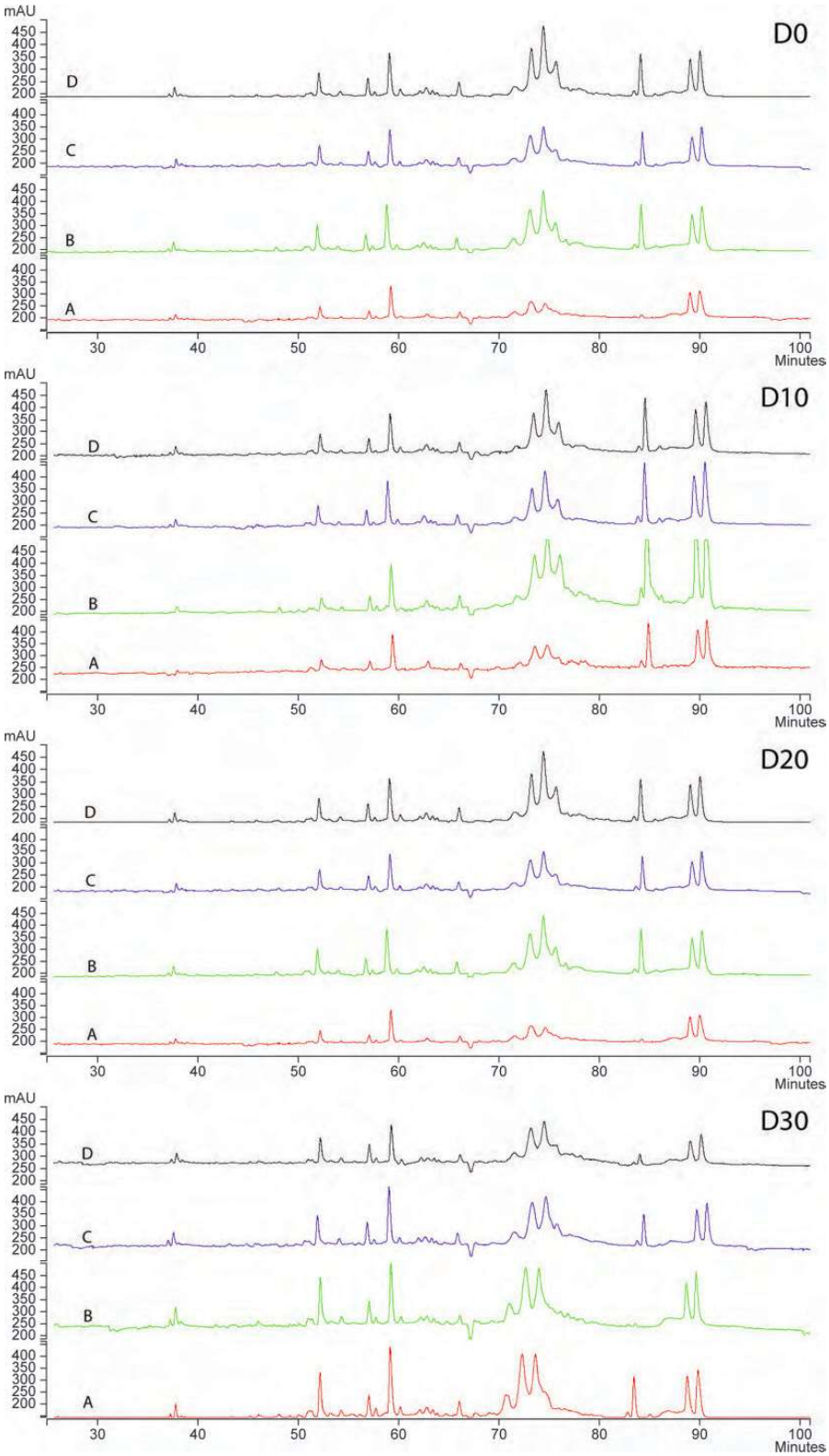

Figure 1. Reversed-phase HPLC profiles of water-soluble extract (WSE) of Akawi cheese at d 0, 10, 20, and 30 (D0, D10, D20, and D30, respectively), which was salted with A (red) = NaCl only (control); B (green) = 3 NaCl:1 KCl (wt/wt); C (blue) = 1 NaCl:1 KCl (wt/wt); or D (black) = 1 NaCl:3 KCl (wt/wt) and stored at 4°C. Color version available in the online PDF.

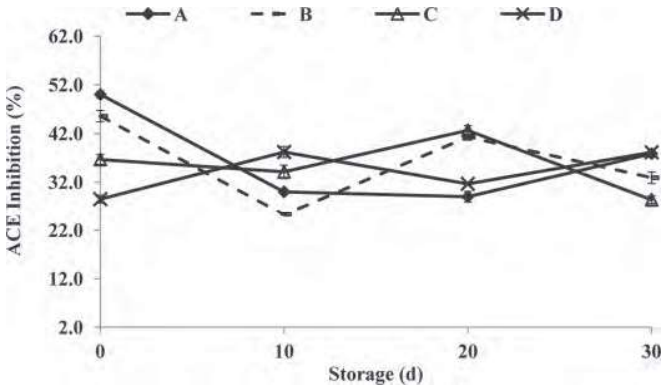

Figure 2. Angiotensin-converting enzyme (ACE)-inhibitory percentage of water-soluble extract (WSE) of Akawi cheese salted with 4 levels of an NaCl/KCl mixture, A = NaCl only (control); B = 3 NaCl:1 KCl (wt/wt); C = 1 NaCl:1 KCl (wt/wt); D = 1 NaCl:3 KCl (wt/wt), during storage at 4°C for 30 d.

experimental cheeses at the same storage period. However, OPA differed significantly ($P < 0.05$) between Akawi cheeses starting from d 20 of storage (Table 3). As seen from Table 2, PTA-SN content in Akawi cheese of treatment A (control) was higher ($P < 0.05$) compared with other treatments (B, C, and D) starting at d 10 until the end of the storage period. However, the OPA content in Akawi cheese of treatment D was higher ($P < 0.05$) compared with other treatments (A, B, and C). This trend may be attributed to the activity of proteolytic enzymes produced by lactic acid bacteria and probiotic bacteria, which, in turn, may be affected by the presence or absence of KCL. This supported the hypothesis that was proposed by Ayyash and Shah (2011b,d) and Armenteros et al. (2009) who reported that the presence of KCl inactivated or activated particular proteolytic enzymes. Water-soluble nitrogen, TCA-SN, and PTA-SN showed a significant ($P < 0.05$) increase during storage for the same salt treatment.

Peptides Profiles and ACE-Inhibitory Activity

The peptide profile chromatograms of the experimental Akawi cheeses are shown in Figure 1. The hydrophobic peptides were dominant in all peptide profiles of experimental cheeses (Figure 1). At the same storage period, the chromatograms of treatments B, C, and D were similar compared with the control (treatment A). As can be noticed, generally, the number of hydrophobic peptides in all experimental cheeses increased during the storage period (Figure 1). The ACE-inhibitory activity of Akawi cheeses during the storage period is shown in Figure 2. In general, no significant ($P > 0.05$) difference existed in ACE-inhibitory activity between experimental Akawi cheeses at the same storage period. This result is in contrast to the result of Ayyash and Shah (2011d) who reported a significant difference in ACE-inhibitory activity among experimental low-moisture Mozzarella cheeses. This variation may be attributed to the difference between cheese type and manufacturing steps. Analysis of variance showed that the ACE-inhibitory activity increased ($P > 0.05$) during the storage period for the same salt treatment.

The Survival of Bacteria

The starter culture bacterial growth (ST and LB) and that of the 2 probiotic bacteria (LC and LA) are

Table 4. *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus casei*, and *Lactobacillus acidophilus* growth (cfu/g; mean values ± SE of 3 trials) in Akawi cheeses kept in 4 levels of NaCl and KCl during storage for 30 d at 4°C

Storage (d)	Salt treatment[1]	*Strep. thermophilus*	*Lb. bulgaricus*	*Lb. casei*	*Lb. acidophilus*
0	A	7.46 ± 0.06^{a}	6.41 ± 0.01^{a}	7.64 ± 0.03^{a}	7.59 ± 0.03^{a}
	B	7.45 ± 0.08^{a}	6.46 ± 0.02^{a}	7.48 ± 0.00^{b}	7.49 ± 0.01^{a}
	C	7.57 ± 0.12^{a}	6.57 ± 0.01^{a}	7.44 ± 0.05^{b}	7.43 ± 0.04^{a}
	D	7.58 ± 0.02^{a}	6.27 ± 0.01^{a}	7.53 ± 0.06^{ab}	7.55 ± 0.04^{a}
10	A	7.69 ± 0.02^{a}	7.49 ± 0.02^{a}	7.23 ± 0.12^{b}	7.64 ± 0.04^{a}
	B	7.44 ± 0.05^{b}	7.56 ± 0.03^{a}	7.00 ± 0.15^{b}	7.38 ± 0.09^{b}
	C	7.69 ± 0.12^{a}	7.68 ± 0.05^{a}	7.65 ± 0.06^{a}	7.70 ± 0.02^{a}
	D	7.67 ± 0.02^{a}	7.68 ± 0.03^{a}	7.61 ± 0.04^{a}	7.67 ± 0.04^{a}
20	A	7.11 ± 0.10^{b}	6.20 ± 0.09^{b}	7.44 ± 0.05^{b}	7.42 ± 0.07^{c}
	B	7.40 ± 0.20^{ab}	6.64 ± 0.11^{a}	7.66 ± 0.05^{a}	7.69 ± 0.04^{a}
	C	7.41 ± 0.10^{ab}	6.75 ± 0.03^{a}	7.56 ± 0.03^{ab}	7.52 ± 0.05^{bc}
	D	7.47 ± 0.02^{a}	6.91 ± 0.13^{a}	7.61 ± 0.03^{a}	7.62 ± 0.04^{ab}
30	A	6.63 ± 0.15^{b}	6.31 ± 0.13^{a}	7.47 ± 0.02^{a}	7.13 ± 0.04^{b}
	B	7.10 ± 0.14^{a}	6.34 ± 0.08^{a}	7.47 ± 0.03^{a}	7.31 ± 0.10^{ab}
	C	7.17 ± 0.08^{a}	6.54 ± 0.02^{a}	7.44 ± 0.05^{a}	7.44 ± 0.00^{a}
	D	6.88 ± 0.08^{ab}	6.41 ± 0.02^{a}	7.19 ± 0.03^{b}	7.26 ± 0.05^{ab}

$^{a-c}$Means within a column and at the same storage time with the same superscript letter did not differ significantly ($P > 0.05$).

[1]Salt treatment: A = NaCl only (control); B = 3 NaCl:1 KCl (wt/wt); C = 1 NaCl:1 KCl (wt/wt); D = 1 NaCl:3 KCl (wt/wt).

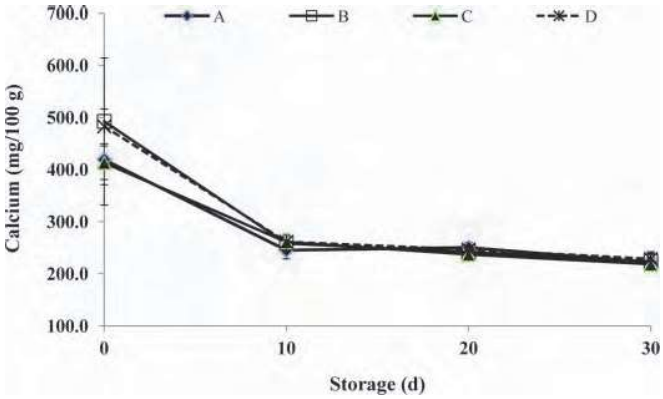

Figure 3. Calcium contents of Akawi cheeses stored in 4 levels of NaCl and KCl, A = NaCl only (control); B = 3 NaCl:1 KCl (wt/wt); C = 1 NaCl:1 KCl (wt/wt); D = 1 NaCl:3 KCl (wt/wt), during storage for 30 d at 4°C. Color version available in the online PDF.

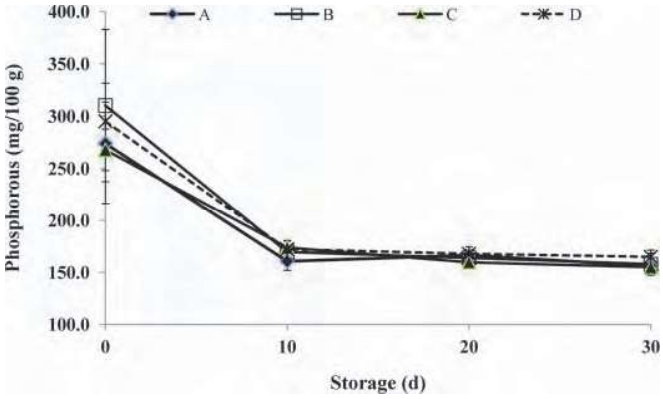

Figure 4. Phosphorous contents of Akawi cheeses stored in 4 levels of NaCl and KCl, A = NaCl only (control); B = 3 NaCl:1 KCl (wt/wt); C = 1 NaCl:1 KCl (wt/wt); D = 1 NaCl:3 KCl (wt/wt), during storage for 30 d at 4°C. Color version available in the online PDF.

presented in Table 4. No significant ($P > 0.05$) difference was observed in LB growth among experimental cheeses at the same storage period. However, the growth of ST, LC, and LA significantly ($P < 0.05$) differed among experimental cheeses. This finding is in accordance with those of Ayyash and Shah (2011d) who reported a significant difference in lactic acid bacteria growth between experimental low-molecular mass chitosans. In the current study, the differences in the bacterial growth were inconsistent during the storage period. Nonetheless, it was obvious that the substitution of NaCl with KCl affected ($P < 0.05$) probiotic growth. This may be attributed to the effect of the NaCl/KCl mixture on the bacterial enzymes activity, which, in turn, affected growth.

Organic Acids Profile

Table 5 presents citric, lactic, and acetic acid contents (mg/100 g) of Akawi cheeses with 4 different salt treatments stored for 30 d at 4°C. Significant ($P < 0.05$) differences were observed in citric, lactic, and acetic acid contents among experimental cheeses at the same storage period. These results may be attributed to the differences in bacterial growth described previously. Citric, lactic, and acetic acids were produced as a result

Table 5. Citric, lactic, and acetic acid contents (mg/100 g; mean values ± SE of 3 trials) in Akawi cheeses kept in 4 levels of NaCl and KCl during storage for 30 d at 4°C

Storage (d)	Salt treatment[1]	Citric acid	Lactic acid	Acetic acid
0	A	181.4 ± 4.9^{a}	110.9 ± 0.8^{a}	131.2 ± 2.8^{a}
	B	178.1 ± 1.3^{a}	112.1 ± 1.6^{a}	136.7 ± 2.9^{a}
	C	142.6 ± 9.2^{b}	113.3 ± 1.5^{a}	126.3 ± 3.5^{a}
	D	136.2 ± 10.8^{b}	109.7 ± 0.9^{a}	114.3 ± 3.5^{b}
10	A	157.2 ± 11.1^{a}	138.7 ± 30.1^{a}	114.6 ± 1.3^{ab}
	B	159.2 ± 4.8^{a}	108.7 ± 0.1^{a}	110.5 ± 3.2^{b}
	C	170.2 ± 6.0^{a}	109.9 ± 0.5^{a}	124.9 ± 1.9^{a}
	D	172.3 ± 2.1^{a}	110.7 ± 0.1^{a}	120.2 ± 5.4^{ab}
20	A	188.3 ± 1.8^{ab}	110.5 ± 0.4^{b}	108.8 ± 3.6^{b}
	B	204.4 ± 3.9^{a}	113.2 ± 0.2^{a}	125.2 ± 3.9^{a}
	C	190.6 ± 0.3^{ab}	111.2 ± 0.2^{b}	111.6 ± 2.5^{b}
	D	168.9 ± 12.4^{b}	109.0 ± 0.5^{c}	109.2 ± 1.8^{b}
30	A	376.8 ± 15.4^{b}	136.1 ± 3.7^{b}	282.5 ± 20.9^{b}
	B	398.1 ± 4.5^{b}	136.2 ± 0.4^{b}	259.6 ± 36.7^{b}
	C	420.8 ± 12.8^{b}	144.9 ± 3.0^{b}	325.7 ± 57.7^{ab}
	D	540.3 ± 44.3^{a}	156.5 ± 5.5^{a}	454.5 ± 38.3^{a}

[a–c]Means within a column and at the same storage time with the same superscript letter did not differ significantly ($P > 0.05$).

[1]Salt treatment: A = NaCl only (control); B = 3 NaCl:1 KCl (wt/wt); C = 1 NaCl:1 KCl (wt/wt); D = 1 NaCl:3 KCl (wt/wt).

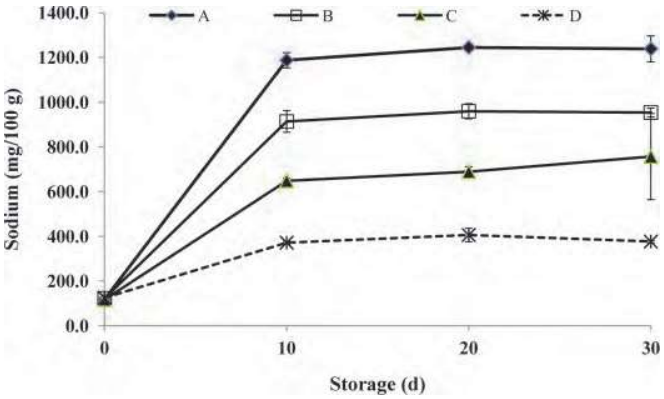

Figure 5. Sodium contents of Akawi cheeses stored in 4 levels of NaCl and KCl, A = NaCl only (control); B = 3 NaCl:1 KCl (wt/wt); C = 1 NaCl:1 KCl (wt/wt); D = 1 NaCl:3 KCl (wt/wt), during storage for 30 d at 4°C. Color version available in the online PDF.

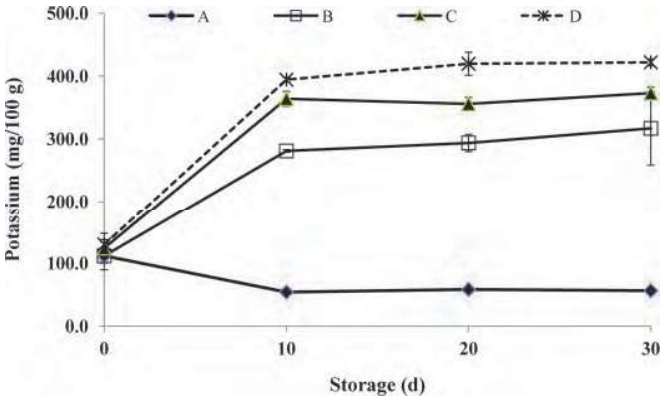

Figure 6. Potassium contents of Akawi cheeses stored in 4 levels of NaCl and KCl, A = NaCl only (control); B = 3 NaCl:1 KCl (wt/wt); C = 1 NaCl:1 KCl (wt/wt); D = 1 NaCl:3 KCl (wt/wt), during storage for 30 d at 4°C. Color version available in the online PDF.

of the bacterial metabolism in cheeses. Hence, organic acid content in Akawi cheese could be affected as a result of the salting treatment, which significantly affected the bacterial growth. In general, ANOVA showed that citric, lactic, and acetic acid contents increased ($P < 0.05$) during storage in all salt treatments (Table 5).

Total and Soluble Ca, P, Na, and K Contents

The total concentrations of Ca, P, Na, and K in experimental Akawi cheeses during the storage period are shown in Figures 3, 4, 5, and 6, respectively. Calcium and P contents in experimental cheeses decreased significantly ($P < 0.05$) during the storage period for all salt treatments. However, Ca and P contents differed insignificantly ($P > 0.05$) between Akawi cheeses at the same of storage period (Figures 3 and 4). These results agree with those of Ayyash and Shah (2010, 2011b) who reported similar results in Halloumi and Nabulsi cheeses. This reduction may be due to the migration of Ca and P toward the brine solution. Significant ($P < 0.05$) differences were found in Na and K contents in experimental cheeses at the same storage period (Figures 5 and 6). Sodium contents decreased ($P < 0.05$) in Akawi cheese along with increases in the substitution of NaCl with KCl, expectedly. The Na content gradually decreased ($P < 0.05$) in the order treatment A > B > C > D. Figures 5 and 6 show that Na and K contents

Table 6. Soluble Ca, K, Na, and P contents (mg/100 g; mean values ± SE of 3 trials) in Akawi cheeses kept in 4 levels of NaCl and KCl during storage for 30 d at 4°C

Storage (d)	Salt treatment[1]	Soluble Ca	Soluble K	Soluble Na	Soluble P
0	A	6.82 ± 0.1^{a}	15.47 ± 0.4^{ab}	9.41 ± 0.2^{bc}	5.30 ± 0.3^{ab}
	B	6.82 ± 0.1^{a}	14.40 ± 0.1^{b}	9.09 ± 0.0^{c}	4.93 ± 0.1^{b}
	C	6.86 ± 0.2^{a}	15.12 ± 0.6^{b}	9.76 ± 0.1^{ab}	5.67 ± 0.0^{a}
	D	6.81 ± 0.1^{a}	16.43 ± 0.2^{a}	9.97 ± 0.1^{a}	5.76 ± 0.1^{a}
10	A	10.91 ± 0.4^{a}	8.61 ± 0.3^{c}	597.07 ± 3.9^{a}	5.75 ± 0.1^{a}
	B	10.59 ± 0.1^{ab}	111.47 ± 1.0^{a}	439.20 ± 19.7^{b}	5.54 ± 0.2^{a}
	C	10.00 ± 0.1^{b}	109.87 ± 1.4^{a}	290.40 ± 2.1^{c}	5.77 ± 0.1^{a}
	D	9.20 ± 0.2^{c}	94.40 ± 1.2^{b}	100.53 ± 1.1^{d}	5.93 ± 0.1^{a}
20	A	8.11 ± 0.2^{ab}	9.61 ± 1.8^{d}	534.67 ± 5.6^{a}	5.76 ± 0.2^{a}
	B	7.61 ± 0.1^{ab}	87.20 ± 1.6^{b}	398.67 ± 10.5^{b}	5.75 ± 0.3^{a}
	C	6.54 ± 0.2^{b}	75.76 ± 2.1^{c}	229.33 ± 4.7^{c}	5.72 ± 0.1^{a}
	D	9.58 ± 1.7^{a}	103.81 ± 0.6^{a}	108.53 ± 3.3^{d}	6.07 ± 0.2^{a}
30	A	9.87 ± 0.0^{a}	10.72 ± 2.1^{c}	597.87 ± 17.3^{a}	5.41 ± 0.2^{a}
	B	9.36 ± 0.4^{ab}	111.47 ± 1.0^{a}	461.60 ± 6.4^{b}	5.34 ± 0.1^{a}
	C	9.15 ± 0.1^{ab}	110.67 ± 1.0^{a}	358.13 ± 56.6^{c}	5.48 ± 0.1^{a}
	D	8.83 ± 0.4^{b}	97.87 ± 0.7^{b}	113.33 ± 3.1^{d}	5.72 ± 0.2^{a}

[a–d]Means within a column and at the same storage time with the same superscript letter did not differ significantly ($P > 0.05$).

[1]Salt treatment: A = NaCl only (control); B = 3 NaCl:1 KCl (wt/wt); C = 1 NaCl:1 KCl (wt/wt); D = 1 NaCl:3 KCl (wt/wt).

Table 7. Texture profile (mean values ± SE of 3 trials) of Akawi cheeses kept in 4 levels of NaCl and KCl during storage for 30 d at 4°C

Storage (d)	Salt treatment[1]	Hardness (N)	Cohesiveness	Adhesiveness (J/m^3)	Gumminess (N)
0	A	21.07 ± 1.2^a	0.56 ± 0.1^a	0.10 ± 0.1^a	11.71 ± 1.0^a
	B	18.51 ± 0.5^a	0.56 ± 0.0^a	0.19 ± 0.1^a	10.42 ± 0.5^a
	C	18.17 ± 0.5^a	0.50 ± 0.0^a	0.19 ± 0.0^a	9.13 ± 0.3^a
	D	27.56 ± 3.9^a	0.50 ± 0.1^a	0.15 ± 0.0^a	13.74 ± 2.5^a
10	A	31.26 ± 3.9^a	0.42 ± 0.1^b	0.10 ± 0.0^b	13.45 ± 3.3^{ab}
	B	29.96 ± 2.1^a	0.52 ± 0.0^{ab}	0.21 ± 0.1^a	15.56 ± 1.2^{ab}
	C	25.89 ± 2.5^a	0.40 ± 0.0^b	0.06 ± 0.0^b	10.25 ± 0.8^b
	D	31.22 ± 3.4^a	0.63 ± 0.0^a	0.05 ± 0.0^b	19.33 ± 1.1^a
20	A	30.10 ± 0.1^a	0.61 ± 0.0^a	0.09 ± 0.0^a	18.36 ± 0.7^a
	B	27.90 ± 3.2^a	0.53 ± 0.1^a	0.23 ± 0.1^a	14.67 ± 3.3^a
	C	23.96 ± 3.1^a	0.63 ± 0.0^a	0.14 ± 0.1^a	14.82 ± 1.2^a
	D	22.51 ± 6.5^a	0.52 ± 0.1^a	0.14 ± 0.1^a	12.05 ± 4.4^a
30	A	27.62 ± 0.7^a	0.64 ± 0.0^a	0.19 ± 0.1^a	17.72 ± 0.3^a
	B	26.29 ± 2.1^a	0.57 ± 0.0^{ab}	0.19 ± 0.0^a	14.95 ± 0.6^b
	C	24.22 ± 0.2^a	0.56 ± 0.0^{ab}	0.16 ± 0.1^a	13.63 ± 0.6^b
	D	16.69 ± 0.4^b	0.46 ± 0.1^b	0.15 ± 0.1^a	7.69 ± 1.3^c

$^{a–c}$Means within a column and at the same storage time with the same superscript letter did not differ significantly ($P > 0.05$).
[1]Salt treatment: A = NaCl only (control); B = 3 NaCl:1 KCl (wt/wt); C = 1 NaCl:1 KCl (wt/wt); D = 1 NaCl:3 KCl (wt/wt).

increased ($P < 0.05$) during the storage period for the same salt treatment. These results are in agreement with those of Ayyash and Shah (2010, 2011b).

Table 6 presents the soluble Ca, P, Na, and K contents (mg/100 g) in experimental cheeses during 30 d of storage at 4°C. Soluble Na and K contents significantly differed ($P < 0.05$) between the 4 Akawi cheeses at the same storage period. In addition, the soluble Na and K content increased ($P < 0.05$) during storage for the same salt treatment. This may be attributed to the significant differences in total Na and K (Figures 5 and 6). The behavior of soluble Na and K in cheeses should be further investigated. Soluble P content insignificantly differed ($P > 0.05$) among experimental cheeses at the same storage time and at the same salt treatment (Table 6). A significant difference ($P < 0.05$) was observed in soluble Ca among experimental cheeses at the same storage period. The differences in soluble Ca between Akawi cheeses showed an inconsistent trend, which needs further investigation to understand the behavior of soluble Ca. However, the soluble Ca increased ($P < 0.05$) in Akawi cheeses during the storage period for the same salt treatment. This result agrees with findings of soluble Ca in low-moisture Mozzarella cheese salted with 4 NaCl/KCl mixtures and stored for 27 d at 4°C (Ayyash and Shah, 2011c).

Texture Profile Analysis

Hardness, cohesiveness, adhesiveness, and gumminess of experimental Akawi cheeses during 30 d of storage are presented in Table 7. In general, hardness and adhesiveness showed no significant differences ($P > 0.05$) among experimental cheeses at the same storage period.

Table 8. Sensory evaluation (mean values ± SE of 3 trials) of Akawi cheeses kept in 4 levels of NaCl and KCl during storage for 30 d at 4°C

Storage (d)	Salt treatment[1]	Creaminess	Bitterness	Saltiness	Sour-acid	Vinegar
10	A	4.1 ± 0.50^a	1.9 ± 0.46^a	5.0 ± 0.63^a	1.8 ± 0.33^a	1.8 ± 0.51^a
	B	4.4 ± 0.52^a	1.8 ± 0.33^a	4.5 ± 0.64^a	1.8 ± 0.42^a	1.8 ± 0.54^a
	C	4.2 ± 0.58^a	1.9 ± 0.46^a	4.4 ± 0.50^a	2.1 ± 0.47^a	1.7 ± 0.42^a
	D	4.6 ± 0.58^a	2.4 ± 0.52^a	4.4 ± 0.72^a	1.7 ± 0.33^a	1.9 ± 0.50^a
20	A	4.5 ± 0.65^a	2.1 ± 0.46^a	4.1 ± 0.53^a	1.9 ± 0.35^a	1.9 ± 0.53^a
	B	5.1 ± 0.60^a	2.1 ± 0.60^a	3.9 ± 0.38^a	1.9 ± 0.46^a	1.8 ± 0.51^a
	C	4.7 ± 0.60^a	2.2 ± 0.51^a	4.7 ± 0.65^a	2.5 ± 0.67^a	1.9 ± 0.46^a
	D	5.0 ± 0.63^a	2.6 ± 0.48^a	4.0 ± 0.49^a	2.1 ± 0.43^a	1.9 ± 0.41^a
30	A	5.3 ± 0.75^a	2.2 ± 0.44^a	4.7 ± 0.58^a	2.1 ± 0.45^a	1.7 ± 0.42^a
	B	5.2 ± 0.61^a	1.9 ± 0.35^a	4.1 ± 0.48^a	2.1 ± 0.41^a	2.1 ± 0.50^a
	C	4.9 ± 0.68^a	1.9 ± 0.41^a	3.9 ± 0.50^a	2.0 ± 0.42^a	2.3 ± 0.56^a
	D	4.7 ± 0.76^a	2.1 ± 0.41^a	3.7 ± 0.65^a	1.9 ± 0.38^a	2.3 ± 0.54^a

^{a}Means within a column and at the same storage time with the same superscript letter did not differ significantly ($P > 0.05$).
[1]Salt treatment: A = NaCl only (control); B = 3 NaCl:1 KCl (wt/wt); C = 1 NaCl:1 KCl (wt/wt); D = 1 NaCl:3 KCl (wt/wt).

The occasional significant difference in hardness at d 30 and in adhesiveness at d 10 may be attributed to differences between cheese loaves but was not due to salt treatment (Table 7). These results are in accordance with those of Ayyash et al. (2011) who reported similar results in Halloumi cheese. A significant difference in cohesiveness existed between experimental cheeses at d 10 and 30 of storage. A similar trend was observed in gumminess, which showed a significant difference between Akawi cheeses at d 10 and 30 of storage. We assume that these differences in cohesiveness and gumminess may be attributed to differences between cheese loaves but not due to salt treatments.

Sensory Properties

Sensory attribute scores (creaminess, bitterness, saltiness, sour-acid, and vinegar) of the 4 experimental Akawi cheeses are presented in Table 8. No significant differences ($P > 0.05$) existed in creaminess, bitterness, saltiness, sour-acid, and vinegar among experimental Akawi cheeses at the same storage period. These results are in agreement with those of Katsiari et al. (1997, 1998) who reported no significant difference in sensory attributes between cheeses made with a NaCl/KCl mixture compared with the control for Feta and Kefalograviera cheeses. This suggests that Akawi cheeses salted with the NaCl/KCl mixture had similar sensory attributes as those of the control. Treatment D numerically received lower saltiness scores compared with the other treatments. The saltiness score of treatment D decreased ($P > 0.05$) during the storage period (Table 8). Vinegar scores increased ($P > 0.05$) at the end of the storage period. This result was consistent with the increase in acetic acid content occurring during storage.

CONCLUSIONS

The effect of partial substitution of NaCl and KCl significantly affected microbial growth and proteolytic activity. The latter is due to the action of proteolytic enzymes. Salt treatment insignificantly affected the texture profile of Akawi cheese. The effect of salt treatment on chemical composition of Akawi cheese was insignificant. Further studies are needed to understand the role of K in cheese and its effect on proteolytic enzymes.

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11. Chapter 11: The impact of NaCl substitution with KCl on proteinase activities of cell-free extract and cell-free supernatant at different pH levels and salt concentrations: *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*

Introduction

Chapter eleven aims are to: 1) investigates the effect of full and partial salt substitution with KCl on the proteinase activities of cell-free and supernatant of common cheese starter cultures; *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* at different pH levels (6.0, 5.5, 5.0) and salt concentration (5% and 10%). The pH levels and salt concentrations were selected in order to cover the variation in cheese pH and brining solution used during cheese production. 2) Examines the production of ACE-inhibitory peptides and proteolytic activity of the bacterial proteinases in cell-free extract and cell-free supernatant after incubation with milk caseins (α-, β-, or κ-casein). A paper entitled the impact of NaCl substitution with KCl on proteinase activities cell-free extract and cell-free supernatant at different pH levels and salt concentrations: *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* by Ayyash, M. M., F. Sherkat, and N. P. Shah was published in Journal of Food Science 77(8):M490-M498.

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DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

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Declaration by [candidate name]: Mutamed Ayyash **Signature:** [redacted] **Date:** 11/9/12

Paper Title:

The impact of NaCl substitution with KCl on proteinase activities cell-free extract and cell-free supernatant at different pH levels and salt concentrations: Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus

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

Name	Contribution %	Nature of Contribution
Mutamed Ayyash	70	Design and perform the experiment Perform the samples analysis
		Evaluate the analytical data Perform the statistical analysis by SAS
		Prepare the major part of the manuscript
Nagendra Shah	15	Contribute to writing manuscript and submission to Journal
Frank Sherkat	15	Contribute to writing the manuscript

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
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Ayyash, M.M., Sherkat, F. and Shah, N.P. (2012), The Impact of NaCl Substitution with KCl on Proteinase Activities Cell-Free Extract and Cell-Free Supernatant at Different pH Levels and Salt Concentrations: Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus. Journal of Food Science, 77: M490-M498. doi:10.1111/j.1750-3841.2012.02802.x

12. Chapter 12: The effect of NaCl substitution with KCl on proteinase activities of cell-free extract and cell-free supernatant at different pH levels and salt concentrations: *Lactobacillus acidophilus* and *Latobacillus casei*

Introduction

Chapter twelve covers the effects of full and partial salt substitution on proteinases activities of two probiotic cultures; *Lactobacillus acidophilus* and *Lactobacillus casei* at different pH levels and salt concentrations. It also investigates the effect of salt substitution with KCl on these proteinases ability to produce ACE-inhibitory peptides from pure milk caseins individually. A paper entitled the impact of NaCl substitution with KCl on proteinase activities cell-free extract and cell-free supernatant at different pH levels and salt concentrations: *Lactobacillus acidophilus* and *Latobacillus casei* by Ayyash, M. M., F. Sherkat, and N. P. Shah was published in Journal of Dairy Science 96:40-49

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Declaration by [candidate name]: Mutamed Ayyash **Signature:** [redacted] **Date:** 11/9/12

Paper Title:

The impact of NaCl substitution with KCl on proteinase activities cell-free extract and cell-free supernatant at different pH levels and salt concentrations: Lactobacillus acidophilus and Latobacillus casei

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

Name	Contribution %	Nature of Contribution
Mutamed Ayyash	70	Design and perform the experiment Perform the samples analysis
		Evaluate the analytical data Perform the statistical analysis by SAS
		Prepare the major part of the manuscript
Nagendra Shah	15	Contribute to writing manuscript and submission to Journal
Frank Sherkat	15	Contribute to writing the manuscript

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J. Dairy Sci. 96:40–49
http://dx.doi.org/10.3168/jds.2012-5761

The effect of NaCl substitution with KCl on proteinase activities of cell-free extract and cell-free supernatant at different pH levels and salt concentrations: *Lactobacillus acidophilus* and *Lactobacillus casei*

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ABSTRACT

The aim of this study was to investigate the effect of substitution of NaCl with KCl at different pH levels and salt concentrations on proteinase activity of cell-free extract and cell-free supernatant of the probiotics *Lactobacillus acidophilus* and *Lactobacillus casei*. de Man, Rogosa, and Sharpe broth aliquots were mixed with 2 pure salts (NaCl and KCl) and 2 salt concentrations at 2 concentration levels (5 and 10%), inoculated with *Lactobacillus acidophilus* or *Lactobacillus casei*, and incubated aerobically at 37°C for 22 h. The cultures were then centrifuged at 4,000 × *g* for 30 min, and the collected cell pellets were used to prepare cell-wall proteinases and the supernatants used as a source of supernatant (extracellular) proteinases. The proteolytic activity and protein content of both portions were determined. After incubation of both portions with 3 milk caseins (α-, β-, κ-casein), the supernatants were individually subjected to analysis of angiotensin-converting enzyme (ACE)-inhibitory activity and proteolytic activity using the *o*-phthalaldehyde method. Significant differences were observed in ACE-inhibitory and proteolytic activities between salt substitution treatments of cell-free extract and cell-free supernatant from both probiotic strains at the same salt concentration and pH level.
Key words: salt substitution, *Lactobacillus acidophilus*, *Lactobacillus casei*, KCl

INTRODUCTION

High salt (sodium chloride) content in food products has emerged as a serious problem worldwide. High salt intake is associated with osteoporosis (Heaney, 2006), kidney stones (Massey, 2005), and hypertension, and the latter is directly related to cardiovascular diseases (Kotchen, 2005; Alderman, 2006). The World Health Organization recommends that food manufacturers gradually reduce salt content in their products (World Health Organization, 2007). Among dairy products, cheese contributes to about 4% of Na intake in the United Kingdom (Ash and Wilbey, 2010), 9.2% in France (Meneton et al., 2009), and 5% in Australia (National Health and Medical Research Council, 2003). A simple reduction of NaCl without substitution with other salts adversely affects cheese quality (Reddy and Marth, 1991). Substitution of NaCl with KCl has been used successfully to preserve cheese quality and safety (Reddy and Marth, 1991). Our previous studies on the effect of NaCl substitution with KCl on various cheese types, including Halloumi cheese (Ayyash and Shah, 2010, 2011a), Nabulsi cheese (Ayyash and Shah, 2011b), and low-moisture Mozzarella cheese (Ayyash and Shah, 2011c,d), showed that salt substitution with KCl affected proteinase activity of the bacterial culture, as measured by 5% phosphotungstic acid-soluble nitrogen (PTA-SN), confirming that bacterial proteinases play an important role during cheese ripening by contributing to flavor and texture of cheese (Fox, 2003; McSweeney, 2004). Information is lacking on the effects of salt substitution with KCl on bacterial proteinase activity. Therefore, growing cultures in pure broth (outside of cheese) to examine the effect of salt substitutions on their proteinase activities would provide knowledge valuable in future substitution studies. In a previous study, we evaluated the effects of salt substitution on *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*, and a substantial link between salt substitution and proteinase activities was established for these organisms (Ayyash et al., 2012). The authors concluded that salt substitution affected the proteinase activity of starter cultures at each pH level and salt concentration. The aim of the current study was to investigate the effects of full and partial salt substitution on the proteinase activities of cell-free extract and supernatant from 2 probiotic cultures—*Lactobacillus acidophilus* and *Lactobacillus casei*—at different pH levels and salt concentrations, and on production of

Received May 23, 2012.
Accepted September 8, 2012.
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angiotensin-converting enzyme (**ACE**)-inhibitory peptides after incubation with individual milk caseins at different pH levels and salt concentrations.

MATERIALS AND METHODS

Bacterial Cultivation

Lactobacillus acidophilus 2401 *and Lactobacillus casei* 290 were obtained from the Victoria University Culture Collection (Werribee, Victoria, Australia) as cultures frozen at −80°C. After thawing the frozen stock, the cultures were activated by transferring loopfuls to de Man, Rogosa, and Sharpe (**MRS**) broth (Merck Pty. Ltd., Victoria, Australia) and incubating aerobically at 37°C for 24 h. Three subsequent cultures were carried out before starting the experiment.

Experiment Design

The experimental design of this study is illustrated in Figure 1. Briefly, *L. acidophilus* and *L. casei* were cultured, separately, in pH-modified MRS broth at pH 5.0, 5.5, and 6.0 without salt addition (control). For the salt treatments, MRS was mixed with 4 salt treatments: NaCl only, 1 NaCl:1 KCl, 1 NaCl:3 KCl, and KCl only, individually at 2 salt concentrations (5 and 10%; wt/vol). Each salt treatment batch was divided into 3 equal portions to adjust the pH to 6.0, 5.5, and 5.0. The MRS samples thus prepared were sterilized and aseptically distributed into 50-mL tubes and inoculated by 1% culture, separately. The inoculated tubes were incubated aerobically at 37°C for 22 h followed by centrifugation at 4,000 × *g* for 30 min at 4°C. The clear cell-free supernatant (**CFS**) from each tube was collected and stored individually at −80°C until used for assays. The cell pellets from each tube were washed with PBS (130 m*M* sodium chloride, 10 m*M* sodium phosphate, pH 7.4), and resuspended in 3 mL of PBS containing 20% glycerol and kept at −80°C until needed for assays. To prepare cell-free extract (**CFE**), the frozen cell pellets were thawed and mixed with 300 μL of Cellytic B 10× (Sigma, St. Louis, MO) to rupture the cell walls and extract the cell contents. According to the manufacturer, Cellytic B does not affect the activity of extracted proteins or interfere in further experiments. The mixture was kept at 4°C for 10 min followed by centrifugation at 4,000 × *g*. The collected supernatant was considered as the cell-free extract containing the cell-wall proteinases. All experiments were conducted in triplicate.

Protein Content

The protein contents of CFE and CFS were determined using Bradford reagent and a 96-well plate assay protocol according to the manufacturer's instructions (Sigma-Aldrich, 2011). Briefly, 5 μL of each sample (CFE or CFS) was placed in a well of the 96-well plate (Cellstar, Greiner Bio-One, Monroe, NC) followed by

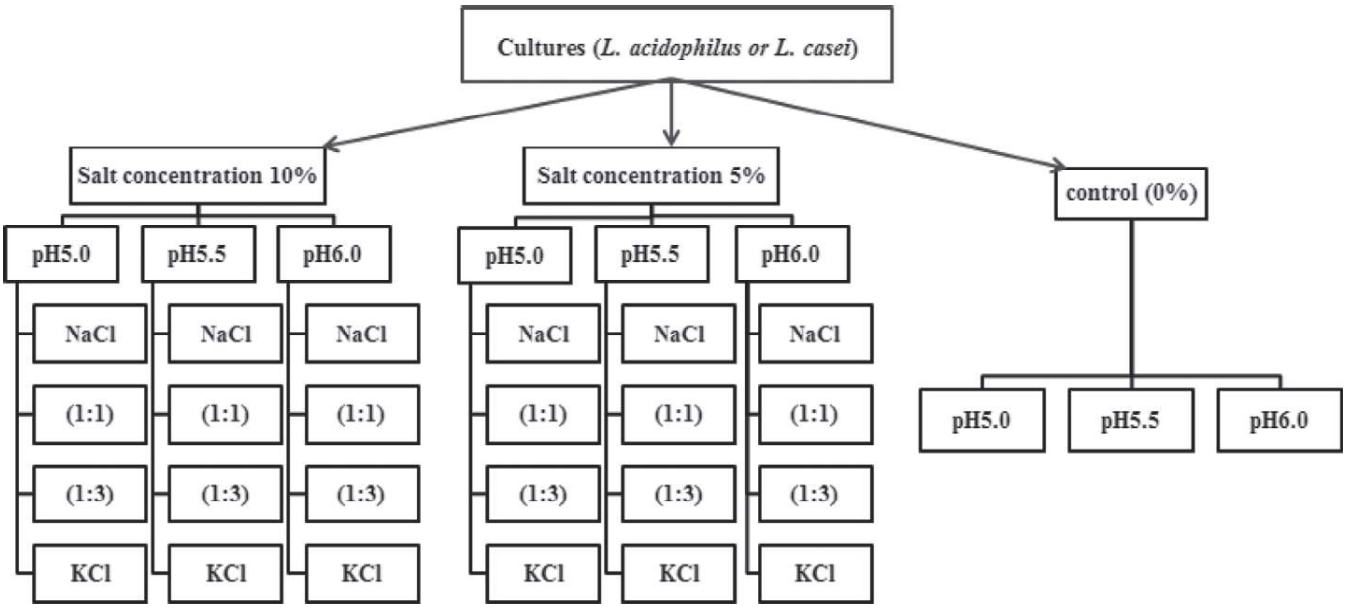

Figure 1. The experimental design of *Lactobacillus acidophilus* and *Lactobacillus casei* culture at 3 pH levels (5.0, 5.5, and 6.0), 2 salt concentrations (5 and 10%), and 4 salt treatments: Control = no salt addition; (1:1) = 1 NaCl:1 KCl; (1:3) = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

250 μL of Bradford reagent (Sigma). The 96-well plate was shaken for 30 s followed by incubation at room temperature for 10 min. Absorbance was measured at 595 nm using a microplate absorbance reader (iMark, Bio-Rad Laboratories Pty., Victoria, Australia). An external protein standard curve ranging from 0.1 to 1.4 mg/mL was prepared using a BSA standard (Sigma).

Proteinase Activity Assay

The proteinase activity of the CFE and CFS was assayed according to Shin et al. (2004) using the chromogenic substrate azocasein. One hundred microliters of specimen was mixed with 100 μL of 2% (wt/vol) azocasein solution prepared in 50 m*M* sodium phosphate buffer (pH 7.0) followed by incubation at 37°C for 16 h, as the enzyme activity was linear over a 16-h assay. Afterward, the reaction was terminated by the addition of 500 μL of 12% (wt/vol) TCA solution and mixed thoroughly. After 15 min at room temperature (21°C), the mixture was centrifuged at 12,000 × *g* for 5 min at 4°C. The supernatant (500 μL) was mixed with 500 μL of 1.0 *M* NaOH, and absorbance was measured at 440 nm against a blank without sample. One unit of proteinase activity was defined as the amount of the enzyme that resulted in an increase of 0.01 absorbance unit per hour at 440 nm.

Casein Hydrolysis

An aliquot (100 μL) of CFE or CFS was incubated with 100 μL of α-, β-, or κ-casein (Sigma; 4 mg/mL of 20 m*M* phosphate buffer, pH 6.0) individually and incubated at 37°C for 24 h. Then, the samples were immersed for 2 min into ice to minimize proteinase activity, followed by centrifugation at 12,000 × *g* for 5 min at 4°C. Two 50-μL aliquots were used for subsequent determination of proteolytic activity by *o*-phthalaldehyde (**OPA**) reagent and ACE-inhibitory activity.

OPA Assay

The OPA assay was performed according to Church et al. (1983). Briefly, 50 μL of the casein hydrolysis supernatant was placed into a 1.5-mL cuvette and mixed with 1 mL of OPA reagent [prepared in a 50-mL volumetric flask by dissolving 25 mL of 100 m*M* disodium tetraborate (Merck Pty. Ltd.), 2.5 mL of 20% (wt/wt) SDS (Merck Pty. Ltd.), 40 mg of OPA (Sigma) in 1 mL of methanol (Merck Pty. Ltd.), and 100 μL of β-mercaptoethanol (Sigma), and then making up the volume to 50 mL with Milli-Q water (Millipore, Billerica, MA)]. The cuvette was inverted twice and kept at room temperature (21°C) for 2 min before reading the absorbance at 340 nm using a UV-VIS spectrophotometer [Shimadzu Scientific Instruments (Oceania) Pty. Ltd., Rydalmere, New South Wales, Australia].

ACE-Inhibitory Activity

The ACE-inhibitory activity was measured according to Ayyash and Shah (2011d) using an HPLC method. Angiotensin-converting enzyme and hippuryl-histidyl-leucine were purchased from Sigma and prepared in Tris buffer (50 m*M*, pH 8.3) containing 300 m*M* NaCl. A mixture consisting of 50 μL of 3.0 m*M* hippuryl-histidyl-leucine, 50 μL of 1.25 mU/mL of ACE (from rabbit lung), and 50 μL of pre-hydrolyzed casein supernatant was placed in a glass tube. The mixture was incubated for 30 min at 37°C in a water bath without mixing, followed by an additional 30 min after mixing. Glacial acetic acid (150 μL) was added to stop the ACE activity. The reaction mixture was kept at −20°C for further analysis by HPLC. The hippuric acid (**HA**) resulting from the previous reaction was determined by HPLC. An external standard curve of HA was prepared to quantify the resultant HA in samples. An aliquot (20 μL) of the mixture was injected onto the HPLC system consisting of a Varian 9012 solvent delivery system, a Varian 9100 auto-sampler, a Varian 9050 variable wavelength UV-visible tunable absorbance detector, and a 730 data module (Varian Inc., Santa Clara, CA). The system was fitted with a Luna column (C18, 300 mm × 4.6 mm, 3 μm; Phenomenex Australia Pty. Ltd., New South Wales, Australia) with a guard column (C18 4 × 3.0 mm; Phenomenex). The separation was conducted at room temperature (~22°C) at a flow rate of 0.8 mL/min. The mobile phase was an isocratic system consisting of 12.5% (vol/vol) acetonitrile (Merck) in MilliQ water (Millipore), and the pH was adjusted to pH 3.0 using glacial acetic acid. The detection device was an UV-visible detector set at 228 nm. The control reaction mixture contained 50 μL of buffer solution instead of the assay sample and was expected to liberate the maximum amount of HA from the substrate due to uninhibited ACE activity. The percentage inhibition of enzyme activity was calculated as follows:

$$\text{Inhibition} = \frac{\text{HA (control)} - \text{HA (sample)}}{\text{HA (control)}} \times 100\%.$$

Statistical Analysis

One-way ANOVA was performed to investigate the effect of salt treatment on proteinase activity of CFE and CFS at the same salt concentration and pH level. Fisher's test (least significant difference) was used to examine the difference between salt treatment means at

Table 1. Protein content (mg/mL) and proteinase activity by azocasein (440 nm) of cell-free extract and cell-free supernatant of *Lactobacillus acidophilus* 2401 grown in de Man, Rogosa, and Sharpe broth for 22 h at 37°C at 3 pH levels, different salt treatments, and 5% salt concentration[1]

		Azocasein (440 nm)		Protein content (mg/mL)	
pH	Salting[2]	Cell-free extract	Cell-free supernatant	Cell-free extract	Cell-free supernatant
5.0	Control	0.11 ± 0.00[a]	0.11 ± 0.01[b]	1.11 ± 0.27[a]	0.27 ± 0.01[a]
	1:1	0.09 ± 0.00[a]	0.13 ± 0.00[a]	1.24 ± 0.37[a]	0.27 ± 0.01[a]
	3:1	0.09 ± 0.00[a]	0.13 ± 0.01[a]	1.27 ± 0.03[a]	0.26 ± 0.01[a]
	KCl	0.11 ± 0.00[a]	0.09 ± 0.00[b]	1.26 ± 0.02[a]	0.24 ± 0.01[a]
	NaCl	0.13 ± 0.04[a]	0.11 ± 0.01[b]	1.12 ± 0.07[a]	0.26 ± 0.01[a]
5.5	Control	0.09 ± 0.00[b]	0.13 ± 0.01[a]	0.72 ± 0.18[b]	0.28 ± 0.01[a]
	1:1	0.09 ± 0.00[b]	0.14 ± 0.00[a]	1.12 ± 0.29[ab]	0.27 ± 0.03[a]
	3:1	0.10 ± 0.00[b]	0.14 ± 0.00[a]	1.15 ± 0.21[ab]	0.26 ± 0.00[a]
	KCl	0.12 ± 0.00[a]	0.12 ± 0.00[b]	1.27 ± 0.02[ab]	0.29 ± 0.01[a]
	NaCl	0.09 ± 0.00[b]	0.12 ± 0.00[b]	1.32 ± 0.03[a]	0.29 ± 0.00[a]
6.0	Control	0.10 ± 0.00[ab]	0.14 ± 0.01[ab]	0.89 ± 0.28[a]	0.26 ± 0.01[a]
	1:1	0.09 ± 0.00[c]	0.16 ± 0.02[a]	1.05 ± 0.25[a]	0.26 ± 0.00[a]
	3:1	0.09 ± 0.00[b]	0.14 ± 0.00[ab]	1.10 ± 0.30[a]	0.26 ± 0.00[a]
	KCl	0.08 ± 0.00[d]	0.10 ± 0.00[c]	1.29 ± 0.02[a]	0.27 ± 0.00[a]
	NaCl	0.10 ± 0.00[a]	0.12 ± 0.01[bc]	1.09 ± 0.07[a]	0.26 ± 0.00[a]

[a–c]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

the same salt concentration and pH level. As a factorial design, 2-way and 3-way ANOVA were performed to investigate the effect of interactions of salt treatment × pH, salt treatment × salt concentration, salt concentration × pH, and salt treatment × salt concentration × pH.

RESULTS AND DISCUSSION

Azocasein and Protein Content

The effect of NaCl substitution with KCl on the proteolytic activity and protein content of CFE and CFS from *L. acidophilus* 2401 and *L. casei* 290 obtained at different pH and salt concentrations are shown in Tables 1, 2, 3, and 4.

At a salt concentration of 10% and the same pH level, proteolytic activity of *L. acidophilus* CFE and CFS differed insignificantly ($P > 0.05$) between salt treatments, except for CFS at pH 5.0 (Table 2). However, the differences were significant ($P < 0.05$) at 5% salt and the same pH level (Table 1). This implies that salt concentration affected the proteinase activity of both CFE and CFS of *L. acidophilus*. The azocasein absorbance values of CFS of *L. acidophilus* at both

Table 2. Protein content (mg/mL) and proteinase activity by azocasein (440 nm) of cell-free extract and cell-free supernatant of *Lactobacillus acidophilus* 2401 grown in de Man, Rogosa, and Sharpe broth for 22 h at 37°C at 3 pH levels, different salt treatments, and 10% salt concentration[1]

		Azocasein (440 nm)		Protein content (mg/mL)	
pH	Salting[2]	Cell-free extract	Cell-free supernatant	Cell-free extract	Cell-free supernatant
5.0	Control	0.11 ± 0.00[a]	0.11 ± 0.01[b]	1.11 ± 0.27[ab]	0.27 ± 0.01[a]
	1:1	0.08 ± 0.00[a]	0.12 ± 0.00[ab]	0.70 ± 0.38[b]	0.30 ± 0.02[a]
	3:1	0.07 ± 0.00[a]	0.12 ± 0.00[ab]	1.37 ± 0.01[a]	0.29 ± 0.01[a]
	KCl	0.09 ± 0.01[a]	0.11 ± 0.00[b]	1.37 ± 0.03[a]	0.27 ± 0.02[a]
	NaCl	0.27 ± 0.20[a]	0.14 ± 0.01[a]	1.45 ± 0.03[a]	0.29 ± 0.01[a]
5.5	Control	0.09 ± 0.00[a]	0.13 ± 0.01[a]	0.72 ± 0.18[b]	0.28 ± 0.01[a]
	1:1	0.07 ± 0.00[a]	0.12 ± 0.01[a]	1.36 ± 0.03[a]	0.28 ± 0.01[a]
	3:1	0.07 ± 0.00[a]	0.16 ± 0.01[a]	1.36 ± 0.02[a]	0.27 ± 0.01[a]
	KCl	0.10 ± 0.01[a]	0.12 ± 0.00[a]	1.36 ± 0.05[a]	0.27 ± 0.02[a]
	NaCl	0.20 ± 0.13[a]	0.20 ± 0.06[a]	1.47 ± 0.01[a]	0.28 ± 0.01[a]
6.0	Control	0.10 ± 0.00[a]	0.14 ± 0.01[a]	0.89 ± 0.28[b]	0.26 ± 0.01[b]
	1:1	0.08 ± 0.00[a]	0.15 ± 0.01[a]	1.36 ± 0.02[a]	0.27 ± 0.01[b]
	3:1	0.07 ± 0.00[a]	0.13 ± 0.01[a]	1.33 ± 0.09[a]	0.31 ± 0.01[a]
	KCl	0.09 ± 0.00[a]	0.14 ± 0.00[a]	1.38 ± 0.06[a]	0.31 ± 0.01[a]
	NaCl	0.09 ± 0.02[a]	0.16 ± 0.02[a]	1.40 ± 0.02[a]	0.27 ± 0.00[b]

[a,b]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

Table 3. Protein content (mg/mL) and proteinase activity by azocasein (440 nm) of cell-free extract and cell-free supernatant of *Lactobacillus casei* 290 growth in de Man, Rogosa, and Sharpe broth for 22 h at 37°C at 3 pH levels, different salt treatments, and 5% salt concentration[1]

		Azocasein (440 nm)		Protein content (mg/mL)	
pH	Salting[2]	Cell-free extract	Cell-free supernatant	Cell-free extract	Cell-free supernatant
5.0	Control	0.09 ± 0.00^{a}	0.15 ± 0.01^{a}	1.21 ± 0.04^{a}	0.28 ± 0.01^{a}
	1:1	0.09 ± 0.00^{a}	0.13 ± 0.00^{a}	1.20 ± 0.08^{a}	0.27 ± 0.01^{a}
	3:1	0.09 ± 0.00^{a}	0.15 ± 0.03^{a}	1.27 ± 0.03^{a}	0.26 ± 0.01^{a}
	KCl	0.09 ± 0.00^{a}	0.12 ± 0.01^{a}	0.85 ± 0.18^{b}	0.27 ± 0.00^{a}
	NaCl	0.09 ± 0.00^{a}	0.12 ± 0.01^{a}	1.24 ± 0.05^{a}	0.26 ± 0.00^{a}
5.5	Control	0.09 ± 0.00^{b}	0.18 ± 0.00^{a}	0.29 ± 0.01^{b}	0.29 ± 0.01^{a}
	1:1	0.10 ± 0.00^{ab}	0.14 ± 0.00^{b}	1.18 ± 0.09^{a}	0.24 ± 0.00^{b}
	3:1	0.10 ± 0.00^{a}	0.14 ± 0.01^{b}	1.30 ± 0.26^{a}	0.25 ± 0.01^{b}
	KCl	0.10 ± 0.01^{a}	0.13 ± 0.00^{b}	1.05 ± 0.13^{a}	0.27 ± 0.01^{a}
	NaCl	0.10 ± 0.00^{ab}	0.13 ± 0.01^{b}	1.09 ± 0.13^{a}	0.28 ± 0.01^{a}
6.0	Control	0.09 ± 0.00^{ab}	0.19 ± 0.00^{a}	1.08 ± 0.12^{a}	0.28 ± 0.01^{a}
	1:1	0.09 ± 0.00^{ab}	0.14 ± 0.00^{b}	1.06 ± 0.02^{a}	0.25 ± 0.00^{c}
	3:1	0.09 ± 0.00^{ab}	0.13 ± 0.00^{bc}	1.18 ± 0.05^{a}	0.25 ± 0.01^{c}
	KCl	0.09 ± 0.00^{b}	0.11 ± 0.01^{d}	0.99 ± 0.12^{a}	0.27 ± 0.00^{ab}
	NaCl	0.10 ± 0.00^{a}	0.12 ± 0.01^{cd}	0.96 ± 0.09^{a}	0.26 ± 0.01^{bc}

[a–d]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

salt concentrations were lower compared with those of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 (Ayyash et al., 2012).

We observed significant ($P < 0.05$) differences in protein content of *L. acidophilus* CFE and CFS between salt treatments at the same salt concentration and pH level (Tables 1 and 2), except for protein content of CFS at 5% salt (Table 1). It was clear that salt treatment and salt concentration significantly affected protein content; however, further investigation is needed to confirm these findings.

Similar trends were observed for proteolytic activity and protein content of CFE and CFS of *L. casei* (Tables 3 and 4). Significant ($P < 0.05$) differences were observed in proteolytic activity of CFE and CFS of *L. casei* between salt treatments at the same salt concentration and pH level. Thus, substitution of NaCl with KCl significantly affected the proteolytic activity of both enzymes obtained from CFE and CFS. We hypothesize that salt treatment might affect the activity of proteinases or enzyme production by *L. casei*. Armenteros et al. (2009) reported that the presence of

Table 4. Protein content (mg/mL) and proteinase activity by azocasein (440 nm) of cell-free extract and cell-free supernatant of *Lactobacillus casei* 290 growth in de Man, Rogosa, and Sharpe broth for 22 h at 37°C at 3 pH levels, different salt treatments, and 10% salt concentration[1]

		Azocasein (440 nm)		Protein content (mg/mL)	
pH	Salting[2]	Cell-free extract	Cell-free supernatant	Cell-free extract	Cell-free supernatant
5.0	Control	0.09 ± 0.00^{a}	0.15 ± 0.01^{a}	1.21 ± 0.04^{b}	0.28 ± 0.01^{a}
	1:1	0.07 ± 0.00^{b}	0.11 ± 0.00^{c}	0.31 ± 0.02^{c}	0.27 ± 0.01^{ab}
	3:1	0.08 ± 0.00^{b}	0.13 ± 0.00^{b}	1.29 ± 0.04^{b}	0.28 ± 0.01^{a}
	KCl	0.09 ± 0.00^{a}	0.12 ± 0.00^{bc}	1.41 ± 0.01^{a}	0.24 ± 0.01^{b}
	NaCl	0.07 ± 0.00^{b}	0.13 ± 0.00^{b}	1.41 ± 0.01^{a}	0.27 ± 0.01^{ab}
5.5	Control	0.09 ± 0.00^{a}	0.18 ± 0.00^{a}	0.29 ± 0.01^{b}	0.29 ± 0.01^{a}
	1:1	0.08 ± 0.00^{b}	0.13 ± 0.01^{b}	1.36 ± 0.02^{a}	0.26 ± 0.01^{ab}
	3:1	0.07 ± 0.00^{b}	0.14 ± 0.01^{b}	1.37 ± 0.02^{a}	0.27 ± 0.01^{ab}
	KCl	0.09 ± 0.00^{a}	0.12 ± 0.00^{b}	1.41 ± 0.09^{a}	0.23 ± 0.02^{b}
	NaCl	0.07 ± 0.00^{b}	0.14 ± 0.00^{b}	1.44 ± 0.01^{a}	0.28 ± 0.01^{a}
6.0	Control	0.09 ± 0.00^{a}	0.19 ± 0.00^{a}	1.08 ± 0.12^{b}	0.28 ± 0.01^{a}
	1:1	0.09 ± 0.00^{a}	0.15 ± 0.00^{c}	1.38 ± 0.02^{a}	0.25 ± 0.00^{b}
	3:1	0.08 ± 0.00^{a}	0.13 ± 0.01^{d}	1.33 ± 0.07^{a}	0.28 ± 0.01^{ab}
	KCl	0.10 ± 0.01^{a}	0.13 ± 0.00^{d}	1.38 ± 0.04^{a}	0.28 ± 0.01^{ab}
	NaCl	0.11 ± 0.04^{a}	0.18 ± 0.00^{b}	1.41 ± 0.03^{a}	0.27 ± 0.01^{ab}

[a–d]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

Table 5. *o*-Phthalaldehyde readings (340 nm) of cell-free extract and cell-free supernatant (obtained at 3 pH levels, different salt treatments, and 5% salt concentration) of *Lactobacillus acidophilus* 2401 incubated with 3 milk caseins individually for 24 h at 37°C[1]

		Cell-free extract			Cell-free supernatant		
pH	Salting[2]	α-casein	β-casein	κ-casein	α-casein	β-casein	κ-casein
5.0	Control	0.47 ± 0.07^{ab}	0.51 ± 0.02^{b}	0.51 ± 0.06^{b}	0.64 ± 0.02^{c}	0.65 ± 0.00^{b}	0.60 ± 0.02^{a}
	1:1	0.43 ± 0.06^{b}	0.50 ± 0.01^{b}	0.49 ± 0.01^{b}	0.73 ± 0.02^{a}	0.62 ± 0.00^{c}	0.66 ± 0.02^{a}
	3:1	0.42 ± 0.02^{b}	0.45 ± 0.00^{c}	0.47 ± 0.02^{b}	0.68 ± 0.02^{bc}	0.70 ± 0.01^{a}	0.68 ± 0.02^{a}
	KCl	0.59 ± 0.00^{a}	0.57 ± 0.01^{a}	0.66 ± 0.02^{a}	0.71 ± 0.00^{ab}	0.68 ± 0.00^{ab}	0.49 ± 0.21^{a}
	NaCl	0.45 ± 0.01^{b}	0.40 ± 0.01^{d}	0.47 ± 0.03^{b}	0.69 ± 0.01^{ab}	0.69 ± 0.02^{a}	0.67 ± 0.00^{a}
5.5	Control	0.45 ± 0.06^{b}	0.47 ± 0.07^{b}	0.45 ± 0.08^{b}	0.62 ± 0.01^{b}	0.68 ± 0.01^{b}	0.61 ± 0.01^{c}
	1:1	0.41 ± 0.06^{b}	0.48 ± 0.01^{b}	0.44 ± 0.01^{b}	0.75 ± 0.02^{a}	0.62 ± 0.00^{c}	0.66 ± 0.00^{b}
	3:1	0.42 ± 0.04^{b}	0.47 ± 0.01^{b}	0.43 ± 0.02^{b}	0.76 ± 0.02^{a}	0.62 ± 0.00^{c}	0.66 ± 0.01^{b}
	KCl	0.61 ± 0.00^{a}	0.58 ± 0.01^{a}	0.68 ± 0.01^{a}	0.80 ± 0.02^{a}	0.72 ± 0.01^{a}	0.75 ± 0.02^{a}
	NaCl	0.47 ± 0.01^{b}	0.44 ± 0.01^{b}	0.45 ± 0.02^{b}	0.68 ± 0.01^{b}	0.67 ± 0.01^{b}	0.67 ± 0.01^{b}
6.0	Control	0.55 ± 0.03^{a}	0.54 ± 0.00^{a}	0.64 ± 0.00^{a}	0.63 ± 0.01^{c}	0.65 ± 0.01^{a}	0.59 ± 0.00^{c}
	1:1	0.42 ± 0.06^{b}	0.48 ± 0.01^{c}	0.45 ± 0.00^{d}	0.71 ± 0.02^{b}	0.62 ± 0.00^{b}	0.65 ± 0.01^{b}
	3:1	0.42 ± 0.06^{b}	0.48 ± 0.00^{c}	0.44 ± 0.00^{d}	0.76 ± 0.01^{a}	0.62 ± 0.00^{b}	0.67 ± 0.01^{b}
	KCl	0.49 ± 0.03^{ab}	0.54 ± 0.00^{a}	0.60 ± 0.02^{b}	0.73 ± 0.02^{ab}	0.68 ± 0.01^{a}	0.73 ± 0.01^{a}
	NaCl	0.51 ± 0.01^{ab}	0.50 ± 0.01^{b}	0.50 ± 0.00^{c}	0.69 ± 0.00^{b}	0.67 ± 0.02^{a}	0.67 ± 0.01^{b}

[a–d]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

KCl activated some aminopeptidases and inactivated others in meat products.

The azocasein absorbance values of CFS were numerically higher than those of CFE at the same salt concentration, salt treatment, and pH level (Tables 3 and 4). This suggests that CFS may have a greater number of specific proteinases acting on the azocasein substrate compared with CFE.

The protein content of CFE and CFS of *L. casei* showed significant ($P < 0.05$) differences between salt treatments at the same salt concentration and pH level (Tables 3 and 4). As expected, the protein contents of CFE were higher compared with the CFS of *L. casei*. The mechanism of the effect of salt treatment on protein content needs more investigation to explore whether that effect occurred because of salt treatment or other factors.

OPA Activity of Casein Hydrolysis

The OPA absorbance values of the CFE and CFS of *L. acidophilus* with individual milk caseins at 37°C for 24 h are presented in Tables 5 and 6. We observed significant ($P < 0.05$) differences in OPA absorbance values of CFE and CFS from *L. acidophilus* between salt treatments at the same salt concentration and pH level. This suggests that substitution of NaCl with KCl significantly affected the proteinases of both cell wall and supernatant. This result is in agreement with Armenteros et al. (2009), who reported that KCl activated some aminopeptidases and inactivated others.

Tables 5 and 6 show that, at the same pH level and salt treatment, the OPA absorbance values of CFE obtained at 5% salt and incubated with milk caseins were higher ($P < 0.05$) compared with those at 10% salt. This implies that the proteinase activities (especially aminopeptidases) of *L. acidophilus* were significantly ($P < 0.05$) affected by salt concentration (5 and 10%), which in turn affected the free AA produced by these enzymes.

The OPA absorbance values of CFS were significantly ($P < 0.05$) higher with all milk caseins than those of CFE (Tables 5 and 6). This may be attributed to 2 factors: the residual AA in the supernatant after *L. acidophilus* propagation may interfere with the OPA readings, and the proteases of CFS may have greater specificity toward milk caseins compared with CFE proteinases. This could explain the higher free AA produced after incubation with CFS compared with CFE.

Similar trends were observed for OPA absorbance values of *L. casei* (Tables 7 and 8) compared with *L. acidophilus* (Tables 5 and 6). Salt treatment significantly ($P < 0.05$) affected the OPA readings of CFE and CFS at the same salt concentration and pH level (Tables 7 and 8). The control treatment (without salt) at the same pH level with all caseins had higher OPA absorbance compared with other treatments., implying that the proteolytic activities of the proteinases obtained from CFE were significantly affected by salt concentration.

The OPA absorbance values of CFS with all milk caseins were significantly ($P < 0.05$) higher than those of CFE at the same salt concentration, salt treatment, and pH level (Tables 7 and 8). Furthermore, the OPA readings of CFE obtained at 10% salt were lower ($P < 0.05$) compared with those obtained at 5% salt (Tables 7 and 8).

Table 6. *o*-Phthalaldehyde readings (340 nm) of cell-free extract and cell-free supernatant (obtained at 3 pH levels, different salt treatments, and 10% salt concentration) of *Lactobacillus acidophilus* 2401 incubated with 3 milk caseins individually for 24 h at 37°C[1]

		Cell-free extract			Cell-free supernatant		
pH	Salting[2]	α-casein	β-casein	κ-casein	α-casein	β-casein	κ-casein
5.0	Control	0.47 ± 0.07^{a}	0.51 ± 0.02^{a}	0.51 ± 0.06^{a}	0.64 ± 0.02^{b}	0.65 ± 0.00^{b}	0.60 ± 0.02^{c}
	1:1	0.31 ± 0.01^{b}	0.29 ± 0.01^{b}	0.26 ± 0.01^{b}	0.67 ± 0.01^{b}	0.65 ± 0.01^{b}	0.64 ± 0.01^{bc}
	3:1	0.29 ± 0.01^{b}	0.29 ± 0.01^{b}	0.22 ± 0.01^{b}	0.66 ± 0.01^{b}	0.64 ± 0.01^{b}	0.65 ± 0.01^{b}
	KCl	0.45 ± 0.04^{a}	0.45 ± 0.04^{a}	0.39 ± 0.07^{a}	0.82 ± 0.01^{a}	0.78 ± 0.01^{a}	0.69 ± 0.00^{a}
	NaCl	0.28 ± 0.01^{b}	0.28 ± 0.00^{b}	0.20 ± 0.00^{b}	0.66 ± 0.01^{b}	0.65 ± 0.00^{b}	0.64 ± 0.00^{b}
5.5	Control	0.45 ± 0.06^{a}	0.47 ± 0.07^{a}	0.45 ± 0.08^{a}	0.62 ± 0.01^{ab}	0.68 ± 0.01^{b}	0.61 ± 0.01^{c}
	1:1	0.32 ± 0.00^{b}	0.31 ± 0.00^{b}	0.27 ± 0.01^{b}	0.67 ± 0.01^{ab}	0.66 ± 0.02^{b}	0.64 ± 0.02^{bc}
	3:1	0.29 ± 0.01^{b}	0.28 ± 0.00^{b}	0.22 ± 0.01^{b}	0.49 ± 0.20^{b}	0.65 ± 0.01^{b}	0.65 ± 0.01^{b}
	KCl	0.52 ± 0.00^{a}	0.51 ± 0.00^{a}	0.48 ± 0.01^{a}	0.82 ± 0.01^{a}	0.78 ± 0.00^{a}	0.69 ± 0.00^{a}
	NaCl	0.28 ± 0.01^{b}	0.27 ± 0.00^{b}	0.23 ± 0.01^{b}	0.66 ± 0.01^{ab}	0.68 ± 0.01^{b}	0.63 ± 0.00^{bc}
6.0	Control	0.55 ± 0.03^{a}	0.54 ± 0.00^{a}	0.64 ± 0.00^{a}	0.63 ± 0.01^{c}	0.65 ± 0.01^{b}	0.59 ± 0.00^{d}
	1:1	0.31 ± 0.01^{b}	0.24 ± 0.01^{e}	0.29 ± 0.03^{c}	0.66 ± 0.01^{b}	0.65 ± 0.01^{b}	0.67 ± 0.02^{ab}
	3:1	0.27 ± 0.00^{b}	0.26 ± 0.00^{d}	0.20 ± 0.00^{d}	0.65 ± 0.01^{bc}	0.63 ± 0.01^{b}	0.64 ± 0.01^{bc}
	KCl	0.53 ± 0.02^{a}	0.52 ± 0.01^{b}	0.50 ± 0.01^{b}	0.83 ± 0.00^{a}	0.78 ± 0.01^{a}	0.70 ± 0.01^{a}
	NaCl	0.28 ± 0.01^{b}	0.29 ± 0.00^{c}	0.23 ± 0.01^{d}	0.68 ± 0.01^{b}	0.66 ± 0.00^{b}	0.63 ± 0.00^{c}

[a–e]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

ACE-Inhibitory Activity

The ACE-inhibitory activity of peptides released after 24-h incubation of CFE and CFS of *L. acidophilus* (obtained from different salt concentrations, pH, and salt treatments) with 3 milk caseins (α-, β-, and κ-caseins) at 37°C are presented in Tables 9 and 10.

The least significant difference test showed a significant ($P < 0.05$) difference in ACE-inhibitory activity of both CFE and CFS of *L. acidophilus* between salt treatments at the same salt concentration and pH level with all milk caseins. This suggests that substitution of NaCl with KCl may have affected the activity of proteinases of CFE and CFS, which in turn affected the ACE-inhibitory peptides quantitatively and qualitatively. Although changing the pH level significantly affected ACE-inhibitory activity at the same salt concentration and salt treatment, it was inconsistent. Changing salt concentration showed a nonsignificant ($P > 0.05$) effect on ACE-inhibitory activity of both CFE and CFS of *L. acidophilus* with all caseins at the same pH level and salt treatment (Tables 9 and 10).

Tables 9 and 10 show that ACE-inhibitory activity of CFE of *L. acidophilus* with all milk caseins were nu-

Table 7. *o*-Phthalaldehyde readings (340 nm) of cell-free extract and cell-free supernatant (obtained at 3 pH levels, different salt treatments, and 5% salt concentration) of *Lactobacillus casei* 290 incubated with 3 milk caseins individually for 24 h at 37°C[1]

		Cell-free extract			Cell-free supernatant		
pH	Salting[2]	α-casein	β-casein	κ-casein	α-casein	β-casein	κ-casein
5.0	Control	0.54 ± 0.05^{a}	0.51 ± 0.05^{a}	0.55 ± 0.05^{a}	0.70 ± 0.01^{a}	0.64 ± 0.02^{a}	0.63 ± 0.00^{bc}
	1:1	0.46 ± 0.01^{a}	0.46 ± 0.03^{ab}	0.37 ± 0.03^{b}	0.65 ± 0.00^{b}	0.62 ± 0.00^{b}	0.62 ± 0.00^{c}
	3:1	0.49 ± 0.02^{a}	0.45 ± 0.01^{ab}	0.41 ± 0.00^{b}	0.65 ± 0.01^{b}	0.65 ± 0.01^{a}	0.63 ± 0.01^{bc}
	KCl	0.49 ± 0.00^{a}	0.47 ± 0.02^{ab}	0.44 ± 0.01^{b}	0.72 ± 0.01^{a}	0.66 ± 0.06^{a}	0.72 ± 0.01^{a}
	NaCl	0.49 ± 0.02^{a}	0.43 ± 0.01^{b}	0.44 ± 0.01^{b}	0.66 ± 0.02^{b}	0.67 ± 0.01^{a}	0.64 ± 0.01^{b}
5.5	Control	0.46 ± 0.03^{b}	0.48 ± 0.02^{b}	0.41 ± 0.03^{b}	0.68 ± 0.01^{b}	0.63 ± 0.01^{b}	0.66 ± 0.01^{b}
	1:1	0.50 ± 0.01^{ab}	0.48 ± 0.02^{b}	0.41 ± 0.03^{b}	0.67 ± 0.02^{b}	0.62 ± 0.00^{c}	0.62 ± 0.00^{b}
	3:1	0.50 ± 0.01^{ab}	0.49 ± 0.00^{ab}	0.42 ± 0.01^{b}	0.70 ± 0.02^{b}	0.62 ± 0.00^{c}	0.62 ± 0.01^{b}
	KCl	0.55 ± 0.01^{a}	0.52 ± 0.00^{a}	0.51 ± 0.01^{a}	0.76 ± 0.02^{a}	0.72 ± 0.01^{a}	0.72 ± 0.02^{a}
	NaCl	0.52 ± 0.03^{a}	0.49 ± 0.01^{b}	0.46 ± 0.02^{ab}	0.66 ± 0.01^{b}	0.64 ± 0.01^{b}	0.62 ± 0.01^{b}
6.0	Control	0.44 ± 0.06^{c}	0.42 ± 0.01^{b}	0.37 ± 0.06^{b}	0.69 ± 0.00^{b}	0.61 ± 0.01^{d}	0.65 ± 0.00^{ab}
	1:1	0.46 ± 0.01^{bc}	0.46 ± 0.02^{ab}	0.39 ± 0.05^{ab}	0.69 ± 0.02^{b}	0.62 ± 0.00^{c}	0.63 ± 0.00^{b}
	3:1	0.50 ± 0.01^{abc}	0.49 ± 0.00^{ab}	0.39 ± 0.03^{ab}	0.67 ± 0.01^{bc}	0.62 ± 0.00^{c}	0.62 ± 0.01^{b}
	KCl	0.54 ± 0.02^{ab}	0.50 ± 0.02^{ab}	0.49 ± 0.01^{a}	0.73 ± 0.01^{a}	0.68 ± 0.00^{a}	0.69 ± 0.03^{a}
	NaCl	0.55 ± 0.00^{a}	0.52 ± 0.03^{a}	0.50 ± 0.00^{a}	0.64 ± 0.01^{c}	0.66 ± 0.01^{b}	0.63 ± 0.01^{b}

[a–d]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

Table 8. *o*-Phthalaldehyde readings (340 nm) of cell-free extract and cell-free supernatant (obtained at 3 pH levels, different salt treatments, and 10% salt concentration) of *Lactobacillus casei* 290 incubated with 3 milk caseins individually for 24 h at 37°C[1]

		Cell-free extract			Cell-free supernatant		
pH	Salting[2]	α-casein	β-casein	κ-casein	α-casein	β-casein	κ-casein
5.0	Control	0.54 ± 0.05^{a}	0.51 ± 0.04^{a}	0.55 ± 0.05^{a}	0.70 ± 0.01^{b}	0.64 ± 0.02^{b}	0.63 ± 0.00^{b}
	1:1	0.34 ± 0.03^{b}	0.28 ± 0.01^{b}	0.25 ± 0.01^{c}	0.64 ± 0.02^{c}	0.63 ± 0.00^{b}	0.60 ± 0.01^{c}
	3:1	0.29 ± 0.00^{b}	0.26 ± 0.00^{b}	0.20 ± 0.01^{c}	0.63 ± 0.00^{c}	0.63 ± 0.01^{b}	0.60 ± 0.02^{c}
	KCl	0.50 ± 0.00^{a}	0.46 ± 0.01^{a}	0.42 ± 0.01^{b}	0.82 ± 0.01^{a}	0.78 ± 0.00^{a}	0.68 ± 0.01^{a}
	NaCl	0.27 ± 0.00^{b}	0.26 ± 0.00^{b}	0.23 ± 0.03^{c}	0.66 ± 0.00^{c}	0.65 ± 0.02^{b}	0.62 ± 0.01^{bc}
5.5	Control	0.46 ± 0.03^{a}	0.48 ± 0.01^{a}	0.41 ± 0.03^{a}	0.68 ± 0.01^{ab}	0.63 ± 0.01^{b}	0.66 ± 0.01^{ab}
	1:1	0.34 ± 0.00^{b}	0.31 ± 0.00^{b}	0.27 ± 0.00^{b}	0.64 ± 0.01^{ab}	0.63 ± 0.00^{b}	0.59 ± 0.01^{d}
	3:1	0.29 ± 0.01^{c}	0.27 ± 0.01^{c}	0.22 ± 0.01^{bc}	0.47 ± 0.19^{b}	0.63 ± 0.00^{b}	0.63 ± 0.00^{c}
	KCl	0.50 ± 0.02^{a}	0.47 ± 0.02^{a}	0.41 ± 0.02^{a}	0.82 ± 0.01^{a}	0.77 ± 0.01^{a}	0.68 ± 0.01^{a}
	NaCl	0.29 ± 0.01^{c}	0.27 ± 0.00^{c}	0.21 ± 0.02^{c}	0.66 ± 0.01^{ab}	0.64 ± 0.01^{b}	0.63 ± 0.00^{bc}
6.0	Control	0.44 ± 0.06^{a}	0.42 ± 0.06^{a}	0.37 ± 0.06^{a}	0.69 ± 0.00^{b}	0.61 ± 0.01^{b}	0.65 ± 0.00^{b}
	1:1	0.32 ± 0.01^{b}	0.29 ± 0.01^{b}	0.27 ± 0.01^{b}	0.64 ± 0.00^{c}	0.63 ± 0.01^{b}	0.64 ± 0.00^{bc}
	3:1	0.32 ± 0.01^{b}	0.27 ± 0.01^{b}	0.21 ± 0.00^{b}	0.63 ± 0.01^{c}	0.63 ± 0.00^{b}	0.60 ± 0.01^{d}
	KCl	0.51 ± 0.02^{a}	0.48 ± 0.02^{a}	0.44 ± 0.02^{a}	0.81 ± 0.00^{a}	0.76 ± 0.00^{a}	0.68 ± 0.01^{a}
	NaCl	0.28 ± 0.00^{b}	0.27 ± 0.00^{b}	0.24 ± 0.01^{b}	0.65 ± 0.01^{c}	0.62 ± 0.01^{b}	0.62 ± 0.00^{c}

[a–d]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

merically higher ($P > 0.05$) compared with CFS at the same salt concentration, pH level, and salt treatment. This may suggest that the proteolytic action of the cell-wall proteinases releases extra ACE-inhibitory peptides compared with that of CFS. Further investigation is required to explore cell-wall proteinases and their effect on production of ACE-inhibitory peptides.

Tables 11 and 12 illustrate the ACE-inhibitory activity of CFE and CFS of *L. casei* (obtained at different salt concentrations, pH values, and salt treatments) incubated with milk caseins for 24 h at 37°C. The ACE-inhibitory activity of CFE with the 3 caseins was higher ($P < 0.05$) compared with CFS at the same salt concentration, pH level, and salt treatment (Tables 11 and 12). This may be due to the higher activity of cell-wall proteinases compared with those of CFS, or may indicate that CFE proteinases had more specificity toward milk caseins compared with those obtained from CFS.

We observed significant ($P < 0.05$) differences in ACE-inhibitory activities of CFE and CFS of *L. casei* between salt treatments at the same salt concentra-

Table 9. Angiotensin-converting enzyme (ACE)-inhibitory activity of cell-free extract and cell-free supernatant (obtained at 3 pH levels, different salt treatments, and 5% salt concentration) of *Lactobacillus acidophilus* 2401 incubated with 3 milk caseins individually for 24 h at 37°C[1]

		Cell-free extract			Cell-free supernatant		
pH	Salting[2]	α-casein	β-casein	κ-casein	α-casein	β-casein	κ-casein
5.0	Control	56.47 ± 0.86^{d}	58.71 ± 0.37^{b}	60.95 ± 0.22^{a}	45.46 ± 0.47^{b}	47.78 ± 0.26^{ab}	48.74 ± 0.36^{ab}
	1:1	74.97 ± 1.10^{a}	63.04 ± 0.56^{a}	51.11 ± 0.96^{c}	42.99 ± 0.93^{c}	49.19 ± 0.22^{a}	48.78 ± 0.35^{ab}
	3:1	68.79 ± 0.54^{b}	56.69 ± 1.71^{bc}	49.17 ± 0.78^{c}	45.94 ± 0.44^{b}	45.41 ± 0.32^{b}	46.28 ± 0.28^{c}
	KCl	60.60 ± 0.89^{c}	57.13 ± 0.48^{bc}	53.63 ± 0.93^{b}	48.31 ± 0.35^{a}	49.29 ± 0.91^{a}	50.02 ± 0.60^{a}
	NaCl	60.16 ± 0.68^{c}	56.00 ± 0.23^{c}	50.36 ± 0.73^{c}	44.96 ± 0.45^{b}	46.54 ± 1.49^{b}	47.44 ± 1.15^{bc}
5.5	Control	55.05 ± 0.37^{d}	56.67 ± 0.76^{c}	58.29 ± 1.62^{a}	43.85 ± 0.31^{b}	48.77 ± 0.36^{ab}	47.56 ± 0.20^{ab}
	1:1	78.56 ± 0.52^{a}	65.36 ± 0.16^{a}	52.16 ± 0.76^{b}	46.43 ± 0.86^{a}	49.78 ± 0.14^{a}	50.35 ± 0.16^{a}
	3:1	72.66 ± 0.55^{b}	61.20 ± 0.24^{b}	49.75 ± 0.20^{b}	40.45 ± 1.31^{c}	45.57 ± 0.10^{c}	45.91 ± 1.80^{b}
	KCl	62.05 ± 1.13^{c}	56.60 ± 0.50^{c}	52.13 ± 0.58^{b}	47.38 ± 0.48^{a}	47.61 ± 1.03^{b}	48.66 ± 0.76^{ab}
	NaCl	61.13 ± 0.59^{c}	55.85 ± 0.39^{c}	52.57 ± 2.10^{b}	47.17 ± 0.07^{a}	47.59 ± 0.60^{b}	48.79 ± 0.30^{a}
6.0	Control	54.61 ± 1.61^{c}	72.05 ± 2.13^{a}	52.22 ± 0.82^{ab}	42.80 ± 0.83^{b}	46.97 ± 0.15^{b}	47.96 ± 0.26^{c}
	1:1	57.26 ± 1.28^{c}	55.64 ± 0.85^{c}	54.01 ± 0.81^{a}	46.85 ± 0.43^{a}	49.47 ± 0.30^{a}	49.08 ± 0.16^{ab}
	3:1	75.60 ± 0.33^{a}	63.81 ± 0.83^{b}	52.03 ± 1.53^{ab}	41.22 ± 0.24^{c}	48.00 ± 0.36^{ab}	47.53 ± 0.34^{c}
	KCl	66.24 ± 1.58^{b}	54.84 ± 1.84^{c}	50.37 ± 0.96^{b}	46.82 ± 0.32^{a}	47.00 ± 0.64^{b}	48.41 ± 0.40^{bc}
	NaCl	62.20 ± 1.33^{b}	56.48 ± 0.39^{c}	51.45 ± 0.27^{ab}	47.05 ± 0.26^{a}	48.41 ± 1.14^{ab}	49.54 ± 0.30^{a}

[a–d]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

Table 10. Angiotensin-converting enzyme (ACE)-inhibitory activity of cell-free extract and cell-free supernatant (obtained at 3 pH levels, different salt treatments, and 10% salt concentration) of *Lactobacillus acidophilus* 2401 incubated with 3 milk caseins individually for 24 h at 37°C[1]

		Cell-free extract			Cell-free supernatant		
pH	Salting[2]	α-casein	β-casein	κ-casein	α-casein	β-casein	κ-casein
5.0	Control	56.47 ± 0.86^{d}	58.71 ± 0.37^{a}	60.95 ± 0.22^{ab}	45.46 ± 0.47^{ab}	47.78 ± 0.26^{a}	48.74 ± 0.36^{a}
	1:1	77.05 ± 0.63^{c}	53.59 ± 0.23^{bc}	55.94 ± 3.64^{bc}	48.35 ± 0.94^{a}	38.22 ± 4.94^{a}	48.42 ± 0.39^{a}
	3:1	91.21 ± 0.34^{b}	55.85 ± 0.96^{b}	63.26 ± 0.27^{a}	47.55 ± 0.64^{a}	40.59 ± 4.21^{a}	49.70 ± 0.85^{a}
	KCl	93.20 ± 0.10^{a}	51.11 ± 0.88^{c}	60.69 ± 0.46^{ab}	41.15 ± 3.07^{b}	39.20 ± 4.99^{a}	46.53 ± 0.45^{b}
	NaCl	54.81 ± 0.32^{e}	54.00 ± 1.21^{b}	54.81 ± 0.32^{c}	49.21 ± 0.18^{a}	46.69 ± 0.54^{a}	49.21 ± 0.18^{a}
5.5	Control	55.05 ± 0.37^{c}	56.67 ± 0.76^{a}	58.29 ± 1.62^{bc}	43.85 ± 0.31^{c}	48.77 ± 0.36^{a}	47.56 ± 0.20^{ab}
	1:1	53.40 ± 0.32^{c}	51.24 ± 1.05^{c}	57.38 ± 2.87^{c}	46.86 ± 0.64^{b}	34.70 ± 3.64^{b}	45.81 ± 1.45^{b}
	3:1	85.29 ± 2.12^{b}	56.66 ± 0.61^{a}	63.43 ± 0.48^{a}	48.52 ± 0.52^{a}	38.97 ± 4.65^{ab}	49.57 ± 0.54^{a}
	KCl	92.58 ± 0.04^{a}	53.69 ± 0.24^{b}	62.17 ± 0.38^{ab}	47.43 ± 0.38^{ab}	40.04 ± 3.86^{ab}	48.68 ± 0.34^{a}
	NaCl	55.16 ± 0.48^{c}	55.16 ± 0.48^{ab}	55.16 ± 0.48^{c}	47.81 ± 0.51^{ab}	46.00 ± 0.51^{a}	47.81 ± 0.51^{ab}
6.0	Control	54.61 ± 1.61^{d}	72.05 ± 2.13^{a}	52.22 ± 0.82^{c}	42.80 ± 0.83^{b}	46.97 ± 0.15^{a}	47.96 ± 0.26^{a}
	1:1	56.76 ± 0.49^{d}	54.32 ± 0.25^{b}	49.32 ± 0.63^{d}	44.15 ± 0.11^{b}	46.11 ± 1.33^{a}	46.48 ± 0.27^{a}
	3:1	80.92 ± 0.26^{b}	54.73 ± 0.36^{b}	63.45 ± 0.36^{b}	48.19 ± 0.34^{a}	38.74 ± 4.95^{a}	49.23 ± 0.22^{a}
	KCl	91.90 ± 0.23^{a}	55.01 ± 0.05^{b}	62.16 ± 0.10^{b}	47.72 ± 0.21^{a}	40.74 ± 4.26^{a}	49.19 ± 0.26^{a}
	NaCl	70.01 ± 0.46^{c}	70.03 ± 0.48^{a}	70.03 ± 0.48^{a}	48.22 ± 1.90^{a}	44.51 ± 1.03^{a}	48.22 ± 1.90^{a}

[a–e]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

tion and pH level (Tables 11 and 12). These differences were found to be inconsistent when pH level changed, suggesting that pH level affected the proteinase activities of CFE and CFS significantly. In conclusion, substitution of NaCl with KCl was found to significantly affect the proteinase activities of starter culture strains *L. acidophilus* and *L. casei*. Further investigation is required to explore the effect of KCl on proteinase activity and production during fermentation or product storage. The salt treatment effect was highly dependent on pH.

CONCLUSIONS

Complete or partial replacement of NaCl with KCl significantly affected the proteinase activities of CFE and CFS from probiotic strains *L. acidophilus* and *L. casei*. The proteolytic activity following incubation with individual milk caseins showed that the presence of KCl in the bacterial culture medium had a significant effect on the proteinases. Salt treatment also significantly affected ACE-inhibitory activity. The effect of KCl on the bacterial proteinases depended on pH, casein type,

Table 11. Angiotensin-converting enzyme (ACE)-inhibitory activity of cell-free extract and cell-free supernatant (obtained at 3 pH levels, different salt treatments, and 5% salt concentration) of *Lactobacillus casei* 290 incubated with 3 milk caseins individually for 24 h at 37°C[1]

		Cell-free extract			Cell-free supernatant		
pH	Salting[2]	α-casein	β-casein	κ-casein	α-casein	β-casein	κ-casein
5.0	Control	56.05 ± 1.29^{d}	57.12 ± 0.79^{ab}	58.19 ± 2.01^{a}	44.56 ± 0.67^{a}	46.65 ± 0.53^{a}	46.85 ± 1.26^{a}
	1:1	75.11 ± 0.58^{a}	62.25 ± 0.82^{a}	49.39 ± 1.47^{bc}	43.51 ± 0.07^{a}	45.22 ± 0.61^{a}	46.34 ± 1.34^{a}
	3:1	65.41 ± 1.77^{b}	50.98 ± 6.83^{b}	46.15 ± 0.98^{c}	43.28 ± 1.31^{a}	47.65 ± 1.81^{a}	43.28 ± 1.20^{a}
	KCl	60.43 ± 0.41^{c}	54.51 ± 2.08^{ab}	51.50 ± 1.45^{b}	45.55 ± 0.55^{a}	47.53 ± 0.79^{a}	46.24 ± 0.74^{a}
	NaCl	59.58 ± 0.58^{c}	51.03 ± 1.75^{b}	48.90 ± 2.07^{bc}	43.55 ± 0.83^{a}	40.49 ± 1.98^{b}	46.33 ± 1.05^{a}
5.5	Control	53.42 ± 2.55^{c}	56.38 ± 1.66^{b}	59.34 ± 0.89^{a}	42.98 ± 0.84^{ab}	44.58 ± 1.22^{bc}	46.13 ± 0.36^{a}
	1:1	77.61 ± 0.38^{a}	63.98 ± 1.09^{a}	50.35 ± 2.25^{b}	41.24 ± 2.15^{ab}	46.59 ± 0.97^{ab}	46.75 ± 0.64^{a}
	3:1	73.74 ± 0.94^{a}	61.36 ± 0.50^{a}	48.99 ± 1.07^{b}	41.05 ± 0.44^{b}	42.15 ± 0.38^{c}	40.78 ± 0.03^{b}
	KCl	60.10 ± 0.92^{b}	53.10 ± 1.90^{b}	48.83 ± 0.90^{b}	44.66 ± 1.09^{ab}	48.54 ± 0.85^{a}	44.65 ± 1.02^{a}
	NaCl	60.59 ± 0.45^{b}	52.82 ± 0.56^{b}	49.27 ± 1.63^{b}	45.01 ± 0.91^{a}	43.69 ± 1.35^{bc}	46.08 ± 1.39^{a}
6.0	Control	51.36 ± 3.14^{d}	56.87 ± 6.95^{a}	53.59 ± 2.53^{a}	40.13 ± 0.90^{b}	44.84 ± 0.91^{a}	44.89 ± 0.83^{ab}
	1:1	64.71 ± 4.40^{bc}	57.42 ± 0.90^{a}	50.13 ± 2.62^{ab}	44.85 ± 1.53^{a}	47.65 ± 0.78^{a}	46.63 ± 0.81^{a}
	3:1	73.31 ± 0.43^{a}	61.91 ± 1.26^{a}	50.50 ± 2.51^{ab}	40.18 ± 0.96^{b}	44.52 ± 1.01^{a}	43.26 ± 1.41^{b}
	KCl	71.73 ± 1.54^{ab}	51.77 ± 2.89^{a}	46.46 ± 0.90^{b}	43.95 ± 1.13^{a}	47.66 ± 1.84^{a}	44.83 ± 1.00^{ab}
	NaCl	61.88 ± 0.60^{c}	54.70 ± 2.20^{a}	49.00 ± 1.93^{ab}	45.92 ± 0.70^{a}	44.84 ± 1.96^{a}	45.86 ± 0.96^{ab}

[a–d]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

Table 12. Angiotensin-converting enzyme (ACE)-inhibitory activity of cell-free extract and cell-free supernatant (obtained at 3 pH levels, different salt treatments, and 10% salt concentration) of *Lactobacillus casei* 290 incubated with 3 milk caseins individually for 24 h at 37°C[1]

		Cell-free extract			Cell-free supernatant		
pH	Salting[2]	α-casein	β-casein	κ-casein	α-casein	β-casein	κ-casein
5.0	Control	56.05 ± 1.29^{c}	57.12 ± 0.79^{a}	58.19 ± 2.01^{abc}	44.56 ± 0.67^{cd}	46.65 ± 0.53^{a}	46.85 ± 1.26^{b}
	1:1	78.39 ± 0.06^{b}	52.78 ± 0.17^{bc}	56.45 ± 3.94^{bc}	45.74 ± 0.59^{c}	18.73 ± 2.04^{b}	49.08 ± 1.02^{b}
	3:1	91.12 ± 0.07^{a}	54.85 ± 1.55^{ab}	63.44 ± 0.38^{a}	47.28 ± 0.13^{b}	22.38 ± 1.90^{b}	52.18 ± 0.41^{a}
	KCl	92.91 ± 0.02^{a}	48.37 ± 1.29^{d}	60.69 ± 0.57^{ab}	44.17 ± 0.44^{d}	19.11 ± 3.92^{b}	49.05 ± 0.76^{b}
	NaCl	54.07 ± 0.26^{d}	50.30 ± 0.65^{cd}	54.07 ± 0.26^{c}	49.09 ± 0.07^{a}	43.59 ± 0.25^{a}	49.09 ± 0.07^{b}
5.5	Control	53.42 ± 2.55^{c}	56.38 ± 1.66^{a}	59.34 ± 0.89^{bc}	42.98 ± 0.84^{b}	44.58 ± 1.22^{a}	46.13 ± 0.36^{bc}
	1:1	66.52 ± 3.64^{b}	51.97 ± 2.09^{ab}	57.44 ± 1.90^{cd}	43.35 ± 0.88^{b}	17.26 ± 2.26^{b}	46.02 ± 0.82^{bc}
	3:1	89.98 ± 0.05^{a}	52.76 ± 1.12^{a}	62.68 ± 0.47^{a}	46.98 ± 0.71^{a}	18.78 ± 1.71^{b}	50.68 ± 1.10^{a}
	KCl	92.98 ± 0.09^{a}	47.73 ± 1.41^{b}	61.76 ± 0.62^{ab}	45.85 ± 0.69^{ab}	20.63 ± 2.67^{b}	48.84 ± 0.49^{ab}
	NaCl	54.56 ± 0.42^{c}	54.56 ± 0.42^{a}	54.55 ± 0.41^{d}	45.56 ± 1.43^{ab}	43.80 ± 0.64^{a}	45.56 ± 1.43^{c}
6.0	Control	51.36 ± 3.14^{c}	56.87 ± 6.95^{a}	53.59 ± 2.53^{b}	40.13 ± 0.90^{c}	44.84 ± 0.91^{a}	44.89 ± 0.83^{c}
	1:1	55.39 ± 0.42^{c}	51.26 ± 1.32^{a}	47.31 ± 0.10^{c}	43.13 ± 0.59^{b}	43.16 ± 0.63^{a}	45.24 ± 0.37^{bc}
	3:1	80.76 ± 0.45^{b}	53.40 ± 0.55^{a}	63.38 ± 0.75^{a}	46.98 ± 0.34^{a}	17.99 ± 2.37^{b}	50.14 ± 0.47^{a}
	KCl	92.08 ± 0.12^{a}	55.18 ± 0.86^{a}	62.47 ± 0.46^{a}	47.24 ± 0.48^{a}	20.22 ± 4.09^{b}	47.53 ± 1.13^{b}
	NaCl	54.08 ± 0.57^{c}	54.08 ± 0.57^{a}	54.08 ± 0.57^{b}	44.24 ± 0.72^{b}	41.24 ± 0.97^{a}	44.24 ± 0.72^{c}

[a–d]Means in each column and within a pH level with the same letter do not differ significantly ($P > 0.05$).

[1]Values are means ± standard error of 3 replicates.

[2]Control = no salt addition; 1:1 = 1 NaCl:1 KCl; 1:3 = 1 NaCl:3 KCl; KCl = KCl only; NaCl = NaCl only.

and the concentration of each salt. Further investigation is needed to study the effect of KCl on bacterial proteinases in food (especially cheese).

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13. Chapter 13: Conclusions, further research and recommendations

13.1. Conclusions

This study shows that KCl can be used successfully to partially substitute NaCl during cheese production and ripening. Chemical composition (moisture, protein, fat, ash, and pH), texture profile (hardness, adhesiveness, cohesiveness, and gumminess), microstructure and sensory properties of partially substituted cheeses were similar compared with the control.

Moisture content of cheese is affected by several factors during production and ripening. Salt (NaCl) addition is a major factor that affects the moisture content in cheese. The chemical composition differed insignificantly between experimental cheeses. This implies that the differences in molecular weight between KCl molecules (74.55 g/mol) and NaCl (58.44 g/mol) did not significantly affect the chemical composition of cheeses. Similarly, texture profile of experimental cheeses partially salted by NaCl/KCl mixture showed no significant difference compared with control. Chemical composition especially moisture content is highly related to texture profile of cheese. This study showed that KCl molecules may diffuse and interact with cheese components in the same way as the NaCl molecules do.

Sensory attributes of experimental cheeses salted by different NaCl/KCl mixtures were similar compared with control. Among the experimental cheeses salted with NaCl:KCl mixtures, the mixtures 3NaCl:1KCl and 1NaCl:1KCl had similar scores compared with control. This suggests that 50% partial substitution of NaCl with KCl could be the maximum salt replacement could be performed in cheese production. The presence of NaCl in the salting or brining might maintains the salty taste and masks the bitterness taste derived from KCl.

This study shows that replacement of NaCl with KCl affected insignificantly the primary proteolysis stage in cheeses which water soluble nitrogen (WSN) is an indicator to estimate this stage. Primary proteolysis occurred as a result of rennet action remains in cheese curd and/or indigenous milk enzymes. Hence, salt substitution with KCl did not significantly affect the rennet and indigenous milk enzymes activities. Replacement of NaCl with KCl insignificantly affected the intermediate stages of proteolysis which 12% TCA-SN is used as indicator to evaluate this stage.

The advanced stage of proteolysis was significantly affected by salt substitution with KCl. In this stage, the bacterial enzymes hydrolyse the large peptides to tripeptides, dipeptides and free amino acids. This implies that replacement of NaCl with KCl significantly affected the activity of proteolytic enzymes secreted by starter cultures.

In conclusion, this study shows that KCl could be successfully used to partially substitute salt during cheese production. Overall characteristics of cheeses salted with NaCl/KCl mixtures were similar with control cheese (only NaCl). However, proteolysis of these cheeses salted with NaCl/KCl mixtures differed than control. KCl did not maintain the cheese proteolysis as it was with NaCl. The presence of KCl affected the proteolytic agents in cheese. The 1NaCl:1KCl mixture was the maximum salt substitution could be used in cheese production with satisfactory characteristics. This project shows that the effect of salt substitution is dependent in cheese type. Also, it shows that salt substitution affected the proteolytic activities of the starter culture and probiotic proteinases. However, the mechanism of this effect is still unclear.

13.2. Further research and recommendations

Further investigations are required to explore the impact of salt substitution on proteolytic agents in cheese (rennet, indigenous milk enzymes and bacterial enzymes). Model cheeses could be used to examine the effect of salt substitution on the activities of chymosin and indigenous milk enzymes remain in cheese. The influence of salt replacement on the activity of starter culture proteinases should be examined intensely. The effect of salt replacement on these enzymes should be tested individually in artificial media with pure milk caseins as substrate. In the artificial media, we able to control the other factors effect which could interfere with salt replacement treatment. These proteinases must be extracted and purified and then test their proteolytic activity in presence of KCl. Worthwhile information will be accomplished if the future studies investigate the effect of salt replacement on caseins hydration, individually, which in turn would affect proteolysis level in cheese. Model cheese could be used to investigate the effect of salt replacement on caseins hydration. The experiment conditions (moisture content, protein, pH …etc.) are much controllable in cheese model and would be first step before investigating the real cheeses.

Lipolysis in cheeses of this study did not play a significant role could affect the overall quality of cheeses. However, the effect of salt substitution on lipolytic enzymes in cheeses needs to be investigated deeply. The lipolytic enzymes in cheese should be extracted and then test the effect of salt replacement with KCl on their activities individually.

One of the major challenges associates with salt replacement is the sensory properties of cheeses. The impact of salt replacement with KCl on sensory properties of cheeses during storage should be intensely investigated. The studies should focus on how to

mask bitterness of KCl in cheese in order to increase the replacement ratio of NaCl with KCl.

A valuable knowledge will be achieved when expand the applications of salt replacement on other cheeses types especially the high salt content due to the individuality of each cheese characteristics and manufacturing. A great knowledge would be gained if this salt replacement technique is applied on mould-ripened cheeses. Hence, the effect of salt substitution mould ripening will be investigated.

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15. Appendices

Appendix A: Sensory evaluation of Halloumi and Nabulsi cheeses

This supplementary section provides results of sensory properties of Halloumi and Nabulsi cheeses brined in four different salt treatments (A = NaCl only (control); B = 3NaCl:1KCl (w/w); C = 1NaCl:1KCl (w/w); D = 1NaCl:3KCl (w/w)). Due to the delay in Victoria University's Human Ethics Committee approval, the sensory results of Halloumi and Nabulsi cheeses were not publish together with previous results.

Ten panellists were recruited for assessing sensory attributes and all signed a Victoria University Human Subject's Consent Form. The panellists were familiar with basic sensory evaluation techniques for cheeses and were further trained for their ability to detect creaminess, bitterness, saltiness, sour-acid, and vinegar taste and to rank products with different concentration of lactic acid, acetic acid, sodium chloride and caffeine from lowest intensity to highest intensity in water and in cream cheese. Tests were repeated until the panellists were able to rank different intensity of lactic acid, acetic acid, sodium chloride and caffeine in both water and cream cheese. The panellist questionnaire and scoring form is presented in the Appendix B. Prior to sensory evaluation, they also participated in briefing sessions. Sensory evaluation was conducted for Halloumi and Nabulsi cheeses at different storage period using ranking sensory test. Cheese samples were removed from the refrigerator tempered at room temperature (20°C) for 1 h and cut into pieces (~ 2 cm^3) and placed on white plates coded with random numbers. The panellists evaluated 12 cheese samples over three sessions (4 samples per session) and water was provided to cheese panellists between each sample. The panellists were asked to score the following attributes: creaminess, bitterness, saltiness, sour- acid, and vinegar tests from 1 (not detected) to 10 (extreme). The definitions of sensory attributes and references are presented Table 4.

Table 4: The description of sensory evaluation attributes

Attribute	Definition	References
Creaminess	Flavour associated with fresh milk, creamy product, condensed milk.	UHT cream with 35% fat
Bitterness	Chemical-like, aspirin, taste sensation of caffeine	0.06% Caffeine
Saltiness	Fundamental taste sensation of which sodium chloride is typical	1.0% NaCl
Sour-acid	Fundamental taste sensation of citric acid	0.08% Citric acid
Vinegar	Flavour associated with vinegar	Vinegar (~ 4%) from market

Table 4 defines the five sensory attributes in order for panelists to have better understanding of these attributes. UHT milk from market was mixed with cream to increase fat content to 35%. This 35% fat UHT milk was used as reference to creaminess taste. Caffeine, citric acid, and sodium chloride were separately dissolved in drinkable water by 0.06%, 0.08%, and 1%, respectively, to be used as reference to bitterness, sour-acid, and saltiness respectively. White vinegar (~ 4%) was purchased from local market and used as reference to vinegar taste. One-way ANOVA was performed to investigate significant difference at $P < 0.05$ between experimental cheeses at the same storage period. Fisher`s test (least significant difference, LSD) was carried out to examine differences between means of experimental cheeses at the same storage period (least significant difference, LSD).

a) Sensory evaluation of Halloumi cheese

The effect of salt replacement with KCl on sensory properties of Halloumi cheese during storage is presented in Table 5. Analysis of variance showed that salt replacement with KCl had no significant effect (P > 0.05) on the five sensory attributes.

Table 5: Sensory evaluation of Halloumi cheeses kept in 18% brines at 4 levels of NaCl and KCl during storage for 56 days at 4ºC

Storage (day)	Salting[1]	Creaminess	Bitterness	Saltiness	Sour-acid	Vinegar
14	A	3.90±0.78[a]	2.60±0.62[a]	8.45±0.54[a]	2.85±0.58[a]	2.40±0.58[a]
	B	4.25±0.89[a]	2.90±0.64[a]	8.75±0.47[a]	2.85±0.54[a]	3.00±0.65[a]
	C	3.70±0.80[a]	2.90±0.53[a]	8.35±0.51[a]	3.00±0.60[a]	2.80±0.83[a]
	D	4.60±0.92[a]	3.15±0.47[a]	7.80±0.53[a]	3.15±0.60[a]	2.60±0.72[a]
28	A	4.15±0.68[a]	2.50±0.34[a]	7.50±0.78[a]	3.65±1.15[a]	2.30±0.58[a]
	B	4.40±0.73[a]	2.90±0.41[a]	7.70±0.76[a]	2.65±0.53[a]	2.60±0.54[a]
	C	4.40±0.75[a]	3.05±0.37[a]	7.75±0.67[a]	2.00±0.33[a]	2.10±0.48[a]
	D	4.35±0.80[a]	2.80±0.44[a]	7.60±0.75[a]	2.30±0.37[a]	2.00±0.37[a]
56	A	5.40±0.73[a]	3.30±0.63[a]	7.40±0.70[a]	2.65±0.48[a]	2.60±0.76[a]
	B	5.00±0.67[a]	3.10±0.57[a]	7.25±0.63[a]	2.25±0.31[a]	2.35±0.41[a]
	C	4.15±0.89[a]	3.25±0.63[a]	7.60±0.56[a]	2.30±0.45[a]	2.75±0.66[a]
	D	4.60±0.97[a]	3.90±0.71[a]	7.90±0.62[a]	3.05±0.50[a]	3.20±0.74[a]

[1]Salt treatment: A = NaCl only (control); B = 3NaCl:1KCl (w/w); C = 1NaCl:1KCl (w/w); D = 1NaCl:3KCl (w/w)

[a – a] Means in each column and at the same storage time with same letter did not differ significantly (P > 0.05).

At the same storage time, no significant differences (P > 0.05) in creaminess, bitterness, saltiness, sour-acid, and vinegar were observed among experimental Halloumi cheeses (Tables 5). These results are in agreement with those of Katsiari et al. (1997; 1998) who reported no significant difference in sensory attributes between cheeses made with NaCl/KCl mixture compared with the control Feta and Kefalograviera cheeses. Also, these results are in accordance with results of low-moisture Mozzarella and Akawi cheeses (chapters 9 and 10). This suggests that cheeses salted with NaCl/KCl mixture had similar sensory attributes with the control. However, during storage at the same salting treatment bitterness scores increased (P > 0.05) in experimental cheeses (Table 5). Saltiness decreased (P > 0.05) after day 14 of storage in all experimental Halloumi

cheeses. The increase in bitterness may be attributed to the increase in salt replacement with KCl.

b) Sensory evaluation of Nabulsi cheese

Sensory evaluation results of Nabulsi cheeses brined in 18% brines at 4 levels of NaCl and KCl during storage for 5 months at room temperature are presented in Table 6.

Table 6: Sensory evaluation of Nabulsi cheeses kept in 18% brines at 4 levels of NaCl and KCl during storage for 5 months at room temperature

Storage (month)	Salting[1]	Creaminess	Bitterness	Saltiness	Sour-acid	Vinegar
1	A	4.20±0.77[a]	2.40±0.45[a]	5.90±0.80[a]	2.20±0.61[a]	1.90±0.38[a]
	B	4.05±0.68[a]	2.50±0.40[a]	5.15±0.77[a]	2.15±0.47[a]	2.00±0.49[a]
	C	3.90±0.55[a]	2.60±0.54[a]	5.60±0.56[a]	2.85±0.65[a]	2.20±0.59[a]
	D	3.85±0.73[a]	3.90±0.74[a]	6.50±0.73[a]	2.55±0.46[a]	2.20±0.39[a]
3	A	3.90±0.46[a]	3.90±0.81[a]	6.65±0.68[a]	2.85±0.56[a]	2.40±0.54[a]
	B	4.25±0.49[a]	3.30±0.63[a]	6.70±0.73[a]	2.75±0.45[a]	2.30±0.50[a]
	C	4.50±0.69[a]	3.30±0.67[a]	6.60±0.76[a]	2.40±0.48[a]	2.15±0.38[a]
	D	3.85±0.61[a]	3.10±0.69[a]	5.70±0.63[a]	2.60±0.48[a]	2.45±0.51[a]
5	A	3.70±0.56[a]	2.85±0.58[a]	5.95±0.58[a]	2.60±0.40[a]	2.15±0.39[a]
	B	3.40±0.60[a]	3.05±0.65[a]	6.00±0.60[a]	2.50±0.40[a]	2.40±0.45[a]
	C	3.20±0.65[a]	3.00±0.56[a]	5.85±0.65[a]	2.60±0.48[a]	2.70±0.68[a]
	D	3.70±0.62[a]	3.50±0.64[a]	6.30±0.54[a]	2.45±0.44[a]	2.80±0.83[a]

[1]Salting: A = NaCl only (control); B = 3NaCl:1KCl (w/w); C = 1NaCl:1KCl (w/w); D = 1NaCl:3KCl (w/w)

[a – a] Means in each column and at the same storage with same letter did not differ significantly (P > 0.05).

Creaminess, bitterness, saltiness, sour-acid, and vinegar showed no significant differences (P > 0.05) between experimental Nabulsi cheeses at the same storage period. These results agree with Halloumi cheese sensory results (Table 6) and LMMC (chapter 10). This suggests that cheeses salted with NaCl/KCl mixtures had similar sensory attributes with the control (only NaCl). The presence of NaCl during brining might mask the bitterness of KCl (Guinee, 2004b).

Appendix B: **Panellist questionnaire**

Panellist questionnaire and scoring form

Name:

Age:

Gender:

Description of attributes

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.
Saltiness: salty taste

Attributes grading

Creamy / milky 1 = not creamy 10 = extremely creamy
Sour- acid 1 = not acidic 10 = extremely acidic
Vinegary 1 = not detected 10 = high intensity
Bitterness 1 = not bitter 10 = extremely bitter
Saltiness 1 = absent 10 = extremely salty

Attributes	H1†				H2†				H3†			
	1	2	3	4	5	6	7	8	9	10	11	12
Creamy												
Bitterness												
Saltiness												
Sour-acid												
Vinegary												
Attributes	N1‡				N2‡				N3‡			
	13	14	15	16	17	18	19	20	21	22	23	24
Creamy												
Bitterness												
Saltiness												
Sour-acid												
Vinegary												

† H1, H2, and H3 = Halloumi cheese at day 0, 14 and 28 of ripening, respectively.
‡ N1, N1, and N3 = Nabulsi cheese at month 0, 3 and 5 of ripening, respectively

Appendix C: ESEM images

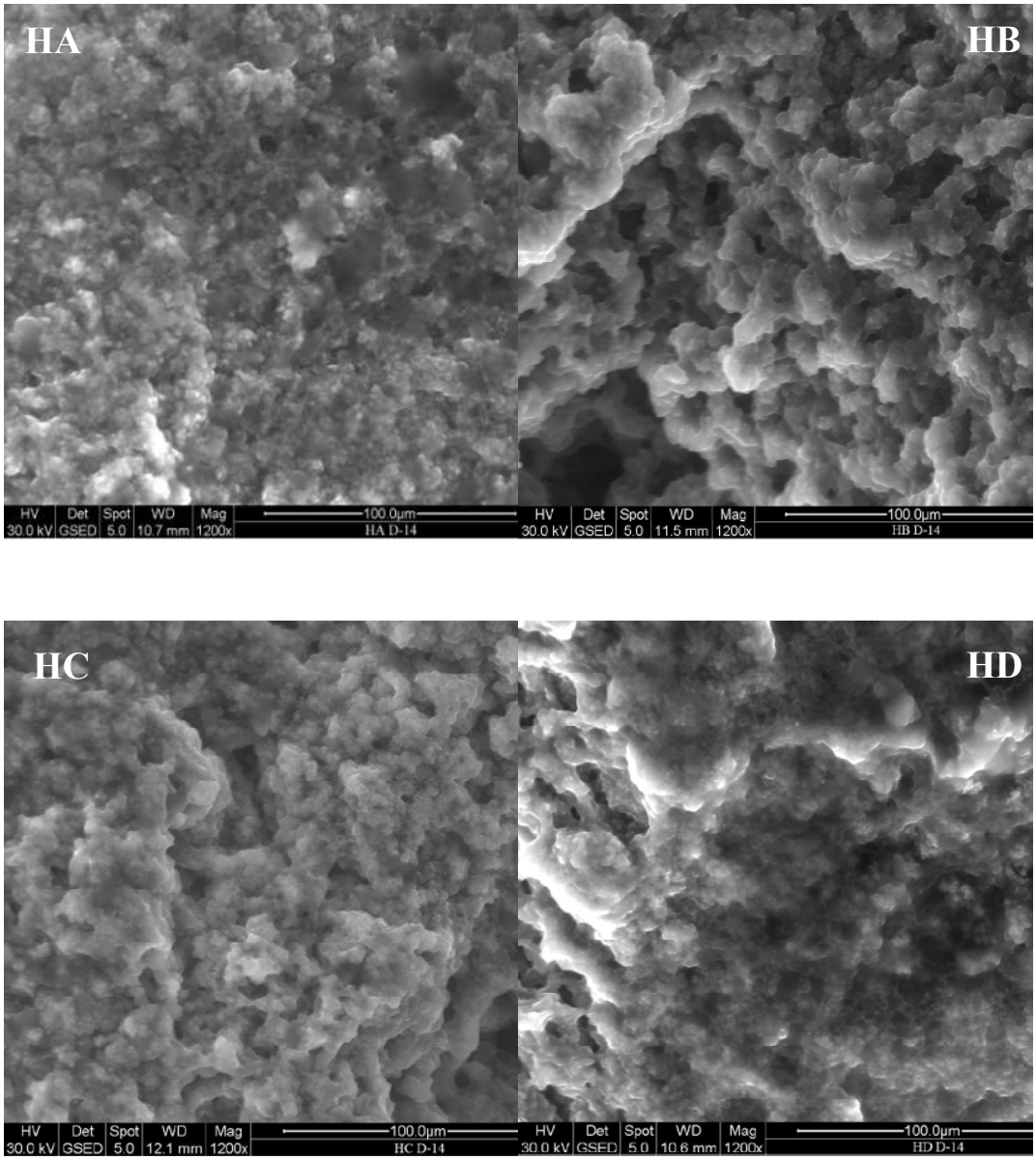

Figure 6: Environmental scanning electron micrograph (ESEM) of Halloumi cheese made with HA = only NaCl (control); HB = salt with 3NaCl:1KCl (w/w); HC = salt with 1NaCl:1KCl (w/w); HD = salt with 1NaCl:3KCl (w/w), at 14 day of storage.

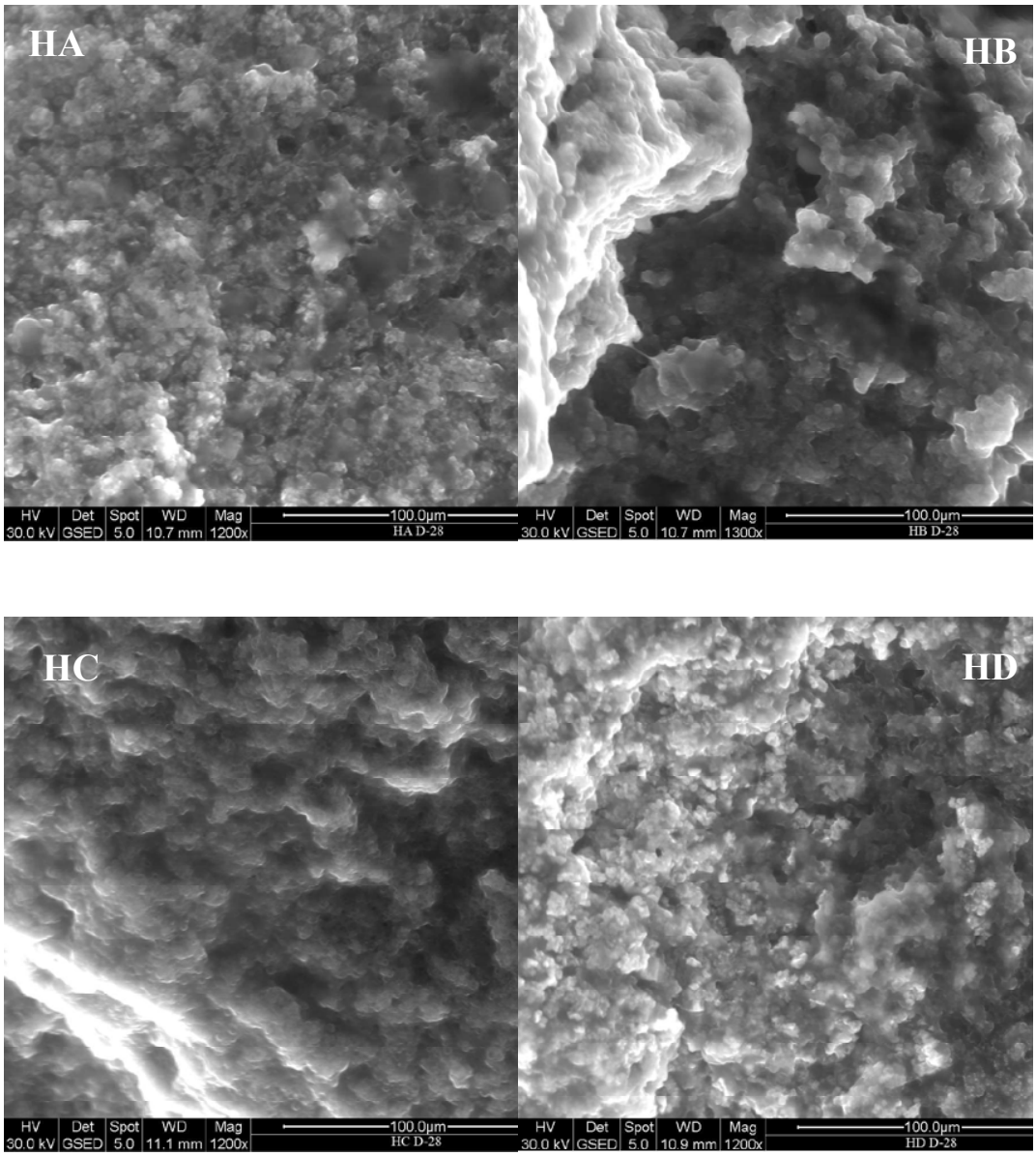

Figure 7: Environmental scanning electron micrograph (ESEM) of Halloumi cheese made with HA = only NaCl (control); HB = salt with 3NaCl:1KCl (w/w); HC = salt with 1NaCl:1KCl (w/w); HD = salt with 1NaCl:3KCl (w/w), at 28 day of storage.

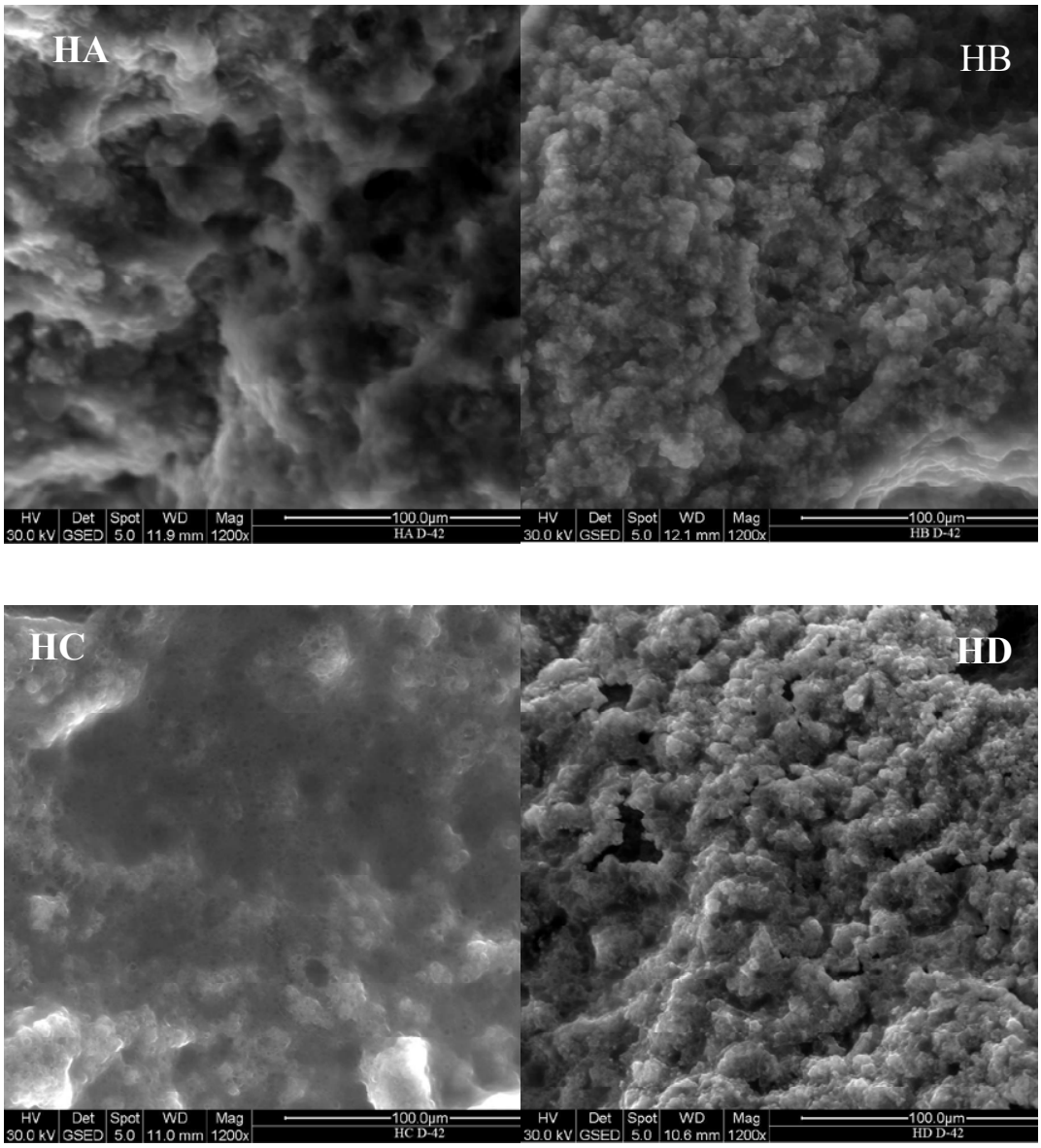

Figure 8: Environmental scanning electron micrograph (ESEM) of Halloumi cheese made with HA = only NaCl (control); HB = salt with 3NaCl:1KCl (w/w); HC = salt with 1NaCl:1KCl (w/w); HD = salt with 1NaCl:3KCl (w/w), at 42 day of storage.

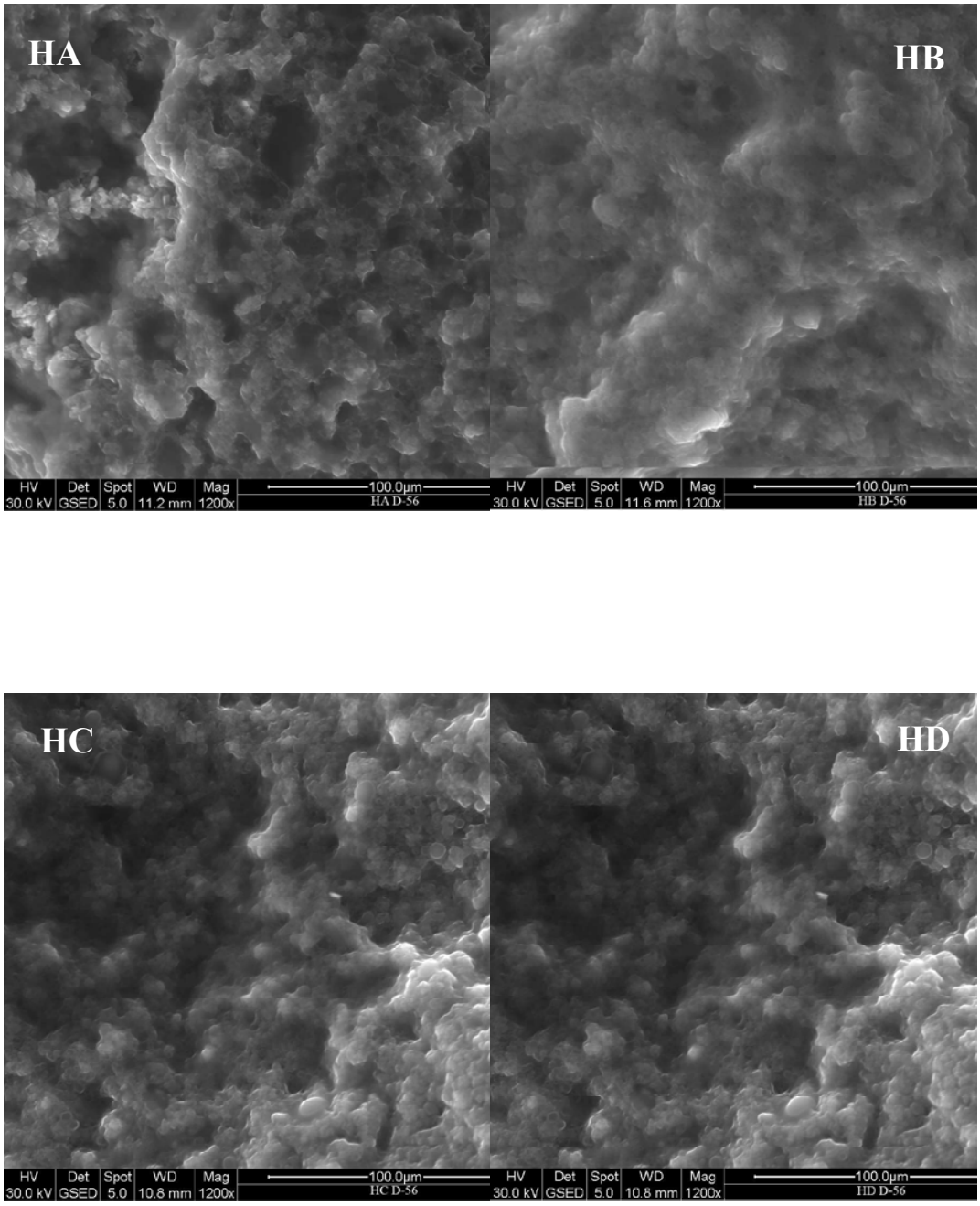

Figure 9: Environmental scanning electron micrograph (ESEM) of Halloumi cheese made with HA = only NaCl (control); HB = salt with 3NaCl:1KCl (w/w); HC = salt with 1NaCl:1KCl (w/w); HD = salt with 1NaCl:3KCl (w/w), at 56 day of storage.

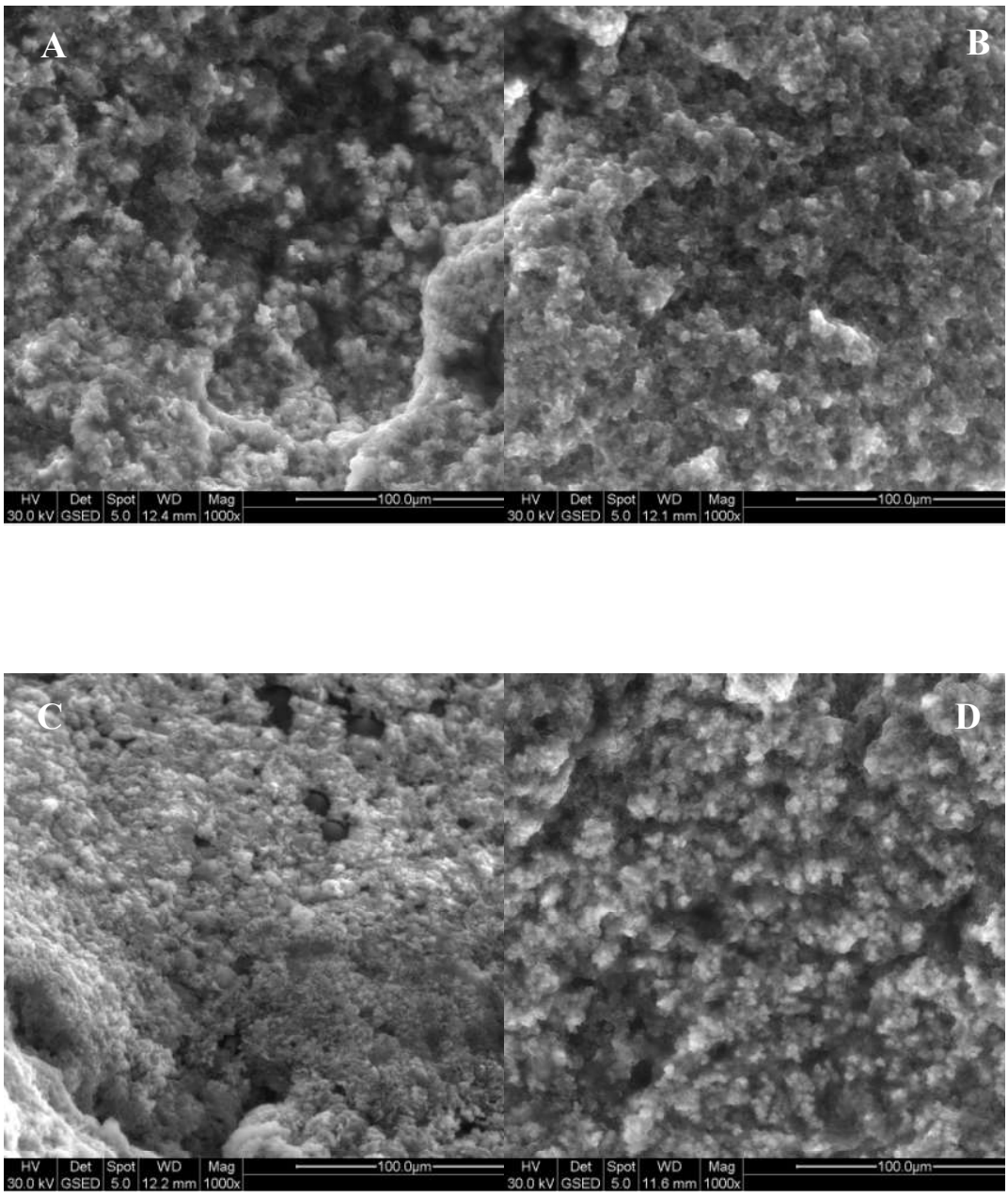

Figure 10: Environmental scanning electron micrograph (ESEM) of Nabulsi cheeses kept with 4 levels of NaCl and KCl; A = NaCl only (control); B = 3NaCl : 1KCl (w/w); C = 1NaCl : 1KCl (w/w); D = 1NaCl : 3KCl (w/w), during storage for 0 month at room temperature.

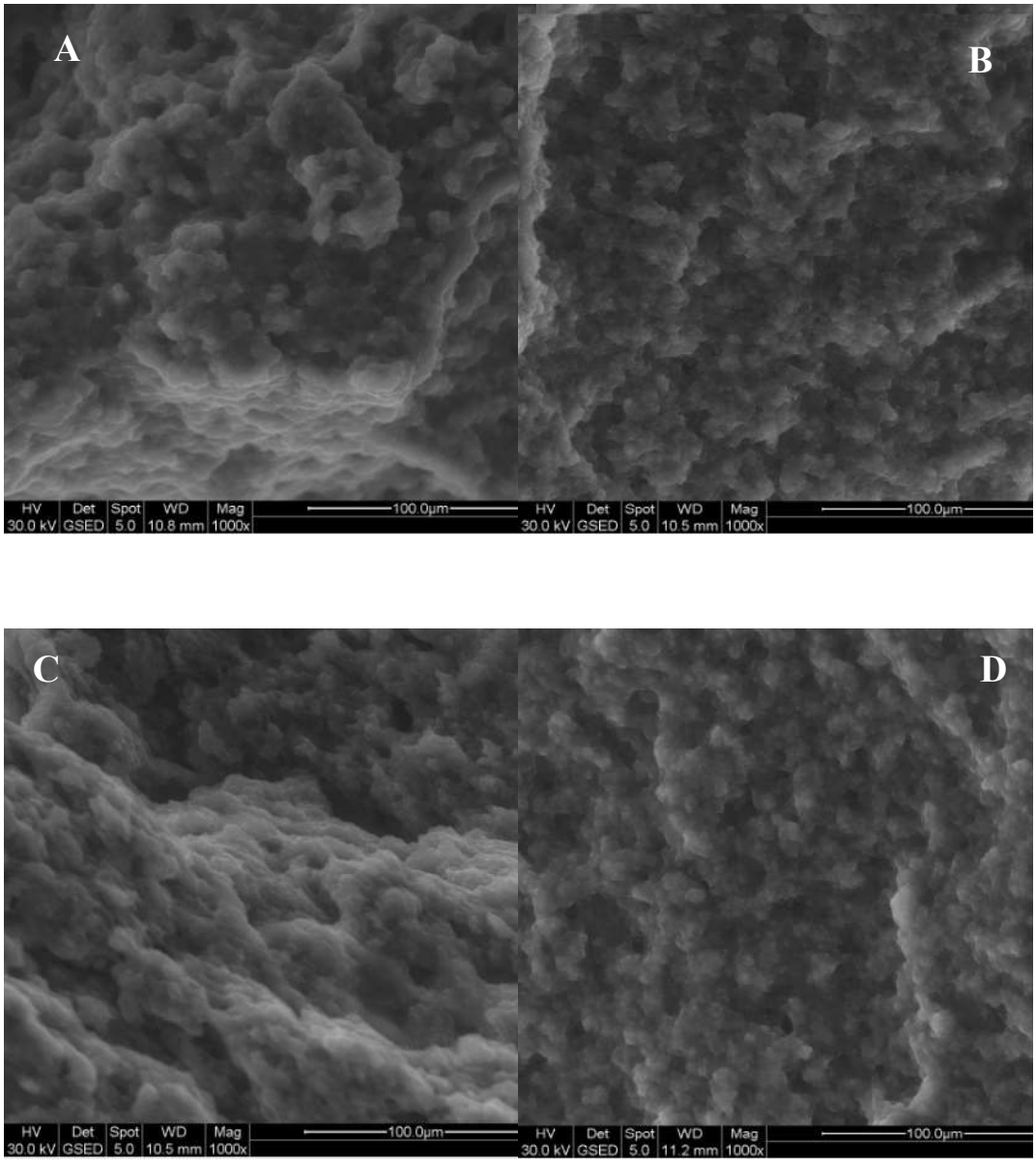

Figure 11: Environmental scanning electron micrograph (ESEM) of Nabulsi cheeses kept with 4 levels of NaCl and KCl; A = NaCl only (control); B = 3NaCl : 1KCl (w/w); C = 1NaCl : 1KCl (w/w); D = 1NaCl : 3KCl (w/w), during storage for 5 months at room temperature.

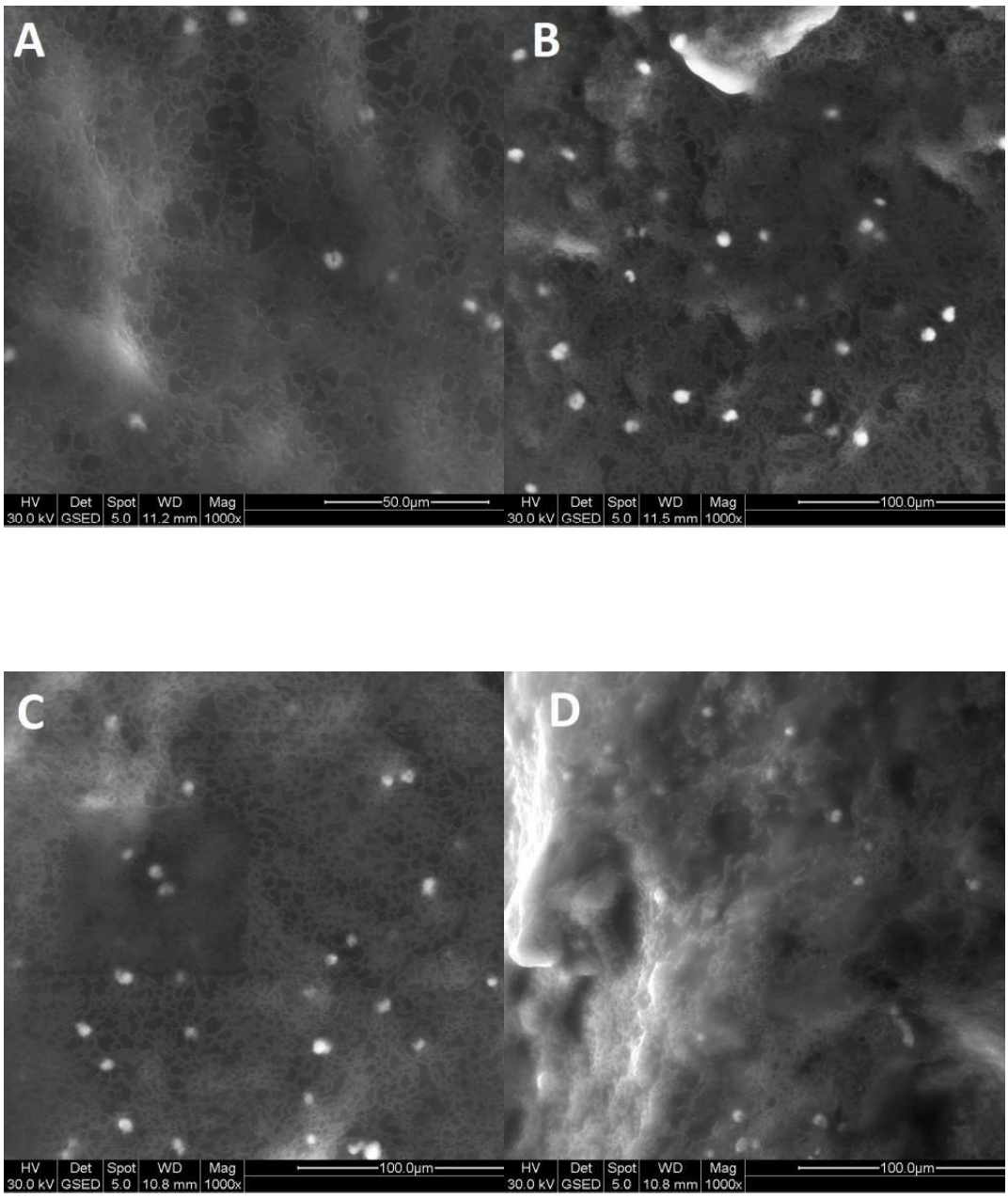

Figure 12: The ESEM images of 4 experimental LMMC samples; A = NaCl only (control); B = 3NaCl:1KCl (w/w); C = 1NaCl:1KCl (w/w); D = 1NaCl:3KCl (w/w) at day 27 of storage.