IMPACT OF PRE-ACIDIFICATION, FAT REPLACERS AND EXOPOLYSACCHARIDE PRODUCING STARTER CULTURES ON FUNCTIONALITY OF LOW FAT MOZZARELLA CHEESE

A thesis submitted for the degree of Doctor of Philosophy

By

Bogdan Zisu

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School of Molecular Sciences

Victoria University, Werribee Campus, VIC, Australia

DEDICATED TO

THE ZISU FAMILY

I.   Abstract

Eighty strains of cocci and twenty of lactobacilli were screened for potential exopolysaccharide (EPS) production.   Eleven cocci strains were identified as capsular EPS producing *Streptococcus thermophilus* (strains 801, 285, 371, 760, 1275, 751, 753, 1439, 287, 288 and 486) and two bacilli strains with the ability to synthesise capsular EPS were identified as *Lactobacillus delbrueckii* ssp. *bulgaricus* (strains 756 and 840).   Strain 820 was also a capsular EPS producer, however, it was identified as *Lactococcus lactis* ssp. *lactis*.   *S. thermophilus* 1275 was identified as a mixed capsular/ropy EPS producer.   Genetic differentiation of *S. thermophilus* strains by pulsed field gel electrophoresis (PFGE) identified two genetically similar groups.   The first group included strains 287, 288, 371 and 801 and the second group consisted of strains 751 and 753.   All other strains were genetically independent.   Seven different fragment patterns were identified from the eleven strains of *S. thermophilus*.   EPS production required the cultivation of bacterial strains in a complex medium (milk medium).   Freeze drying had no apparent impact on capsular EPS synthesis.   Viscosity of milk was greatest when fermented with the ropy producing *S. thermophilus* 1275, however, capsular EPS had no effect on this parameter.   EPS production in milk was also greatest for *S. thermophilus* 1275 at 360 mg/L after 24 h of incubation at 37°C.   *S. thermophilus* 753, 1439, 285 produced 90, 99 and 84 mg/L of EPS after 24 h of fermentation, respectively.   *S. thermophilus* 287, 288 and 486 synthesised a more modest 11, 18 and 19 mg/L of EPS after 24 h.   *S. thermophilus* strains 1275 and 285 were selected for cheese making trials.

S. thermophilus 1275 produced the greatest amount of EPS during screening. Accordingly, the effects of pH, temperature, supplementation with whey protein concentrate (WPC) and non-EPS culture on the EPS production by *S. thermophilus* 1275 were studied.   The organism was grown in 10% reconstituted skim milk (RSM) in a Biostat® B fermenter for 24 h at various pH (4.5, 5.5 and 6.5), and temperatures (30, 37, 40 and 42°C), and supplementation with

WPC 392, and non-EPS producing *S. thermophilus* 1303 and the amount of EPS produced was determined. Bacterial counts were enumerated and the concentrations of lactic acid, lactose, glucose and galactose were also determined. A maximum of 406 mg/L of EPS was produced in RSM at 37°C after 24 h of fermentation at pH 4.08 when the pH was not controlled. A pH of 5.5 and temperature of 40°C were found to be optimal for EPS production by *S. thermophilus* 1275, yielding 458 mg/L. The EPS production increased when RSM was supplemented with WPC 392. At optimum pH and at 37°C with WPC supplementation, the level of EPS increased to 1029 mg/L. Co-culturing *S. thermophilus* 1275 with non-EPS *S. thermophilus* 1303 increased EPS production at 37°C and pH 5.5 to 832 mg/L. High temperature (42°C) reduced the amount of EPS production, and EPS production ceased at pH 4.5 when maintained constantly at this pH. The level of lactose utilization and lactic acid production depended on growth conditions of the organism. No glucose was detected, while galactose was found to accumulate in the medium.

The microbial EPS producing strains of *S. thermophilus* 285 (producing capsular EPS) and 1275 (producing capsular and ropy EPS, respectively) were used to make low fat Mozzarella cheeses containing 6% fat. The effects of EPS on moisture retention and textural and functional characteristics were investigated over 28 d of storage at 4°C. Texture profile analysis (TPA), meltability, stretchability, and pizza bake performance were analysed. Control cheeses made with non-EPS strains (*S. thermophilus* 1303) had the lowest moisture content of 53.77%. Control cheeses exhibited poor meltability and stretchability and greater hardness, springiness and chewiness. Capsular- and ropy- EPS increased moisture retention in cheeses to 57.17% and 58.39%, respectively, and EPS was isolated from curd at 31.09 mg/g and 51.65 mg/g, respectively. Cheeses made with EPS were softer, showed lower degree of springiness and chewiness and exhibited better stretch and melt as compared to control cheeses, although melt of capsular EPS cheeses was not significant ($P > 0.05$). Adhesiveness and cohesiveness were greater in EPS cheeses compared to control cheeses. Adhesiveness was similar between the two types of EPS cheeses, but cohesiveness was greater in ropy EPS type cheeses. Ropy type

cheeses were, however, coated in an undesired layer of slime. Pizza bake performance was poor for all cheeses although those made with EPS showed greater Hunter L-values and lower a-values to signify a reduction in scorching.

In an attempt to improve the quality of low fat Mozzarella cheeses containing 6% fat by reducing hardness and increasing meltability and stretchability, they were made by pre-acidification of cheese milk with citric acid and fermentation with capsular or ropy microbial exopolysaccharide producing cultures. Moisture retention in cheeses and their textural and functional characteristics were investigated. The changes in TPA, cheese meltability, stretchability, and pizza bake performance were analysed over 90 d of storage at 4°C. Control cheeses made with non-EPS *S. thermophilus* 1303 without pre-acidification and those made from pre-acidified milk without EPS starters had the lowest moisture content at 54.84% and 55.28%, respectively. Meltability, stretchability and hardness of control cheeses were inferior during the initial stages of storage, but those properties improved significantly with time. Pizza bake performance, however, remained poor. Pre-acidification led to some improvement in hardness and meltability of low fat Mozzarella cheeses. Capsular- and ropy- EPS were quantified at 30.42 mg/g and 30.55 mg/g of cheese, respectively, and increased moisture retention in pre-acidified cheese to 56.67% and 56.21%, respectively. These cheeses were softer and exhibited lower springiness and chewiness. Adhesion was low in all cheeses in general. This characteristic was negligible during the initial 28 d of storage followed by a slight increase over time. A greater meltability was observed initially but became similar to control cheeses after 90 d of storage. Ropy type cheeses made with pre-acidified milk were coated in an undesired layer of slime. Stretch distance was similar in all cheeses, however, those made from pre-acidified milk exhibited a dense and fibrous characteristic. When baked after 45 d of storage, EPS cheeses showed a substantial improvement in cheese meltability and flow and there was less scorching.

Due to the limited improvement in cheeses resulting from pre-acidification with citric acid alone and citric acid with the addition of EPS producing cultures, low fat Mozzarella

cheeses containing 6% fat were made by pre-acidification of milk, pre-acidification combined with capsular EPS producing *S. thermophilus* 285, used independently or as a co-culture with non-EPS *S. thermophilus* 1303, and pre-acidification combined with WPC and EPS. The parameters of co-culturing and supplementation with WPC were conceived from the fermentation experiments conducted earlier where they were found to stimulate EPS synthesis. It was anticipated that this may occur in a cheese system, and the impact of these treatments on moisture retention, texture and functionality of cheeses were evaluated. The changes in TPA, cheese meltability, stretchability, and on pizza bake performance were investigated over 45 d of storage at 4°C. Pre-acidified cheeses without EPS (control) had the lowest moisture content (53.75%). These cheeses were hardest and exhibited greatest springiness and chewiness. Melt and stretch properties of these cheeses improved most during the first 28 d of storage. The moisture content in cheeses increased to 55.08%, 54.79% and 55.82% with EPS starter (containing 41.18 mg/g of EPS), co-culturing (containing 28.61 mg/g of EPS), and WPC (containing 44.23 mg/g of EPS), respectively. The production of EPS was not increased with co-culturing or supplementation with WPC in a cheese system. EPS improved texture and functionality of low fat cheeses made with pre-acidified milk in general. EPS reduced hardness, springiness and chewiness and exhibited an increase in cohesiveness and meltability. Although stretch distance was similar in all cheeses, those containing EPS were softer than the control. Co-cultured cheeses exhibited the greatest meltability. Cheeses containing WPC were softest in general, however, hardness remained unchanged over 45 d. There was least improvement in meltability of cheeses made with WPC over time. Incorporation of WPC did not produce good pizza bake characteristic, however, there was an improvement in this property between d 7 and 45. Coating of the cheese shreds with oil was necessary for adequate browning, meltability and flow characteristics during pizza baking in all cheese types.

We proceeded to examine ways to improve the quality of the product by using carbohydrates of non-microbial origins. The influence of capsular EPS, combined with pre-

acidification and use of two fat replacers, OptaMax® (FR1) and Versagel® (FR2) on the textural and functional characteristics of Mozzarella cheeses containing 6% fat was studied. Changes in TPA, cheese meltability, stretchability, yield point, microstructure, proteolysis and pizza bake performance were investigated over 60 d of storage. Control cheeses made with capsular EPS producing *S. thermophilus* 285 had the lowest moisture (52.84%) and yield while hardness and melt properties of these cheeses improved with storage. Stretch properties improved in the initial 28 d of storage before stabilising. Addition of FR1 and pre-acidification of milk increased the moisture content in cheeses to 56.21% and 60.25%, respectively and cheeses made with FR2 and pre-acidification had 57.89% moisture. All cheeses were softer than the control. Cheeses made with FR1 exhibited the greatest stretchability and meltability, while those made with FR2 showed poor stretch and melt results. Each cheese showed a unique microstructure with varying porosity prior to, and after the hydration of the protein matrix. The amount of α- and β-casein proteolysis was greater in cheeses made with pre-acidified milk and those having a higher moisture content. Pizza bake performance was improved with FR1, however, FR2 cheeses had the least favourable bake characteristics. Coating cheese shreds with oil was necessary for adequate browning, meltability and flow during pizza baking.

The most successful variables of cheese made at pilot scale were selected and low fat Mozzarella cheeses containing 6% fat were made with pre-acidification of cheese milk with citric acid and use of capsular EPS producing *S. thermophilus* 285 at semi commercial quantities. Control cheeses were made with non-EPS starter bacteria and without pre-acidification. EPS cheeses were made with or without acid treatment and by co-culturing with a non-EPS strain and pre-acidification. Our aim was to determine whether moisture retention, texture and functionality observed in cheeses made on a pilot scale (25 L batch size) could be replicated in a semi-automated system (using 200 L batches). For the scale-up trials, cheeses were made with freshly pasteurised milk and mechanical agitation was applied. Stretching, however, was carried out manually. The composition of cheeses was determined. Texture and functionality of

cheeses was monitored over 90 d of storage at 4°C. Pizza bake was also carried out at 28, 45, 60 and 90 d. EPS cheeses showed lower moisture retention to those made at pilot scale. The moisture content of control cheeses was 50.07%. Cheeses made with EPS alone (containing 79.29 mg/g of EPS) had a significantly higher moisture content of 51.37% ($P < 0.05$). EPS containing cheeses made by pre-acidification (containing 28.69 mg/g of EPS) and when co-cultured (containing 16.19 mg/g of EPS), had moisture contents of 50.08% and 50.78%, respectively. The changes in the procedure when manufacturing low fat Mozzarella cheeses in a semi-automated setup at scale-up did not alter the general texture and functionality of cheeses compared to those made manually at a pilot scale. Meltability and texture improved with time and EPS pre-acidified cheeses had higher meltability. Hardness, springiness and chewiness were in general lower in EPS containing cheeses. Stretch distance was similar between each type of cheese. Pizza bake was best in cheeses made with EPS, however, a surface coating with oil was necessary for adequate shred fusion and browning.

II. Certificate

Dr. Nagendra P. Shah (M. Sc., Ph. D)

Professor – Food Technology

School of Molecular Sciences,

Victoria University,

Werribee Campus, Victoria, Australia

CERTIFICATE

This is to certify that the thesis entitled "IMPACT OF PRE-ACIDIFICATION, FAT REPLACERS AND EXOPOLYSACCHARIDE PRODUCING STARTER CULTURES ON FUNCTIONALITY OF LOW FAT MOZZARELLA CHEESE" submitted by Bogdan Zisu in partial fulfillment of the requirements 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.

Werribee, Australia

Date:

(Prof. N. P. Shah)

Thesis Supervisor

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Werribee, Australia

Date: x   BOGDAN ZISU

IV.   List of Publications

Journal Publications

Amatayakul, T., Zisu, B., Sherkat, F., and Shah, N.P. 2005. Physical characteristics of set yoghurts as affected by co-culturing with non-EPS- and EPS starter cultures and supplementation with WPC. Aust. J. Dairy Technol. (Accepted for publication).

Zisu, B. and Shah, N.P. 2003. Effects of pH, temperature, supplementation with WPC, and adjunct cultures on the production of exopolysaccharides by *Streptococcus thermophilus* 1275. J. Dairy Sci. 86:3405-3415.

Zisu, B. and Shah, N.P. 2004. Role of microbial exopolysaccharides on moisture retention and texture and functionality of low fat mozzarella cheeses. Aust. J. Dairy Technol. (Under review).

Zisu, B. and Shah, N.P. 2004. Texture and functionality of low fat Mozzarella cheeses as influenced by pre-acidification with citric acid and use of capsular and ropy EPS starter. Int. Dairy J. (Under review).

Zisu, B. and Shah, N.P. 2005. Low-fat Mozzarella as influenced by microbial exopolysaccharides, pre-acidification and whey protein concentrate. J. Dairy Sci. (In press).

Zisu, B. and Shah, N.P. 2005. Textural and functional changes of low fat Mozzarella cheeses in relation to proteolysis and microstructure as influenced by the use of fat replacers, pre-acidification and EPS starter. Int. Dairy J. (In press).

Chaired Poster Presentations

Amatayakul, T., Zisu, B., Sherkat, F., and Shah, N.P. 2003. Effects of co-culturing EPS and non-EPS starter cultures and supplementation with WPC on syneresis, textural and rheological properties of set yoghurt. American Dairy Science Association Annual Meeting, Phoenix, AZ, June 22-26, 2003. (J. Dairy Sci. 86 (Supp. 1):362).

Zisu, B., Bhaskaracharya, R.K., and Shah, N.P. 2004. Incorporation of polysaccharides of microbial and non-microbial origin in cheese system. International Dairy Federation Symposium on Cheese, Prague, Czech Republic, March 21-25, 2004.

Zisu, B., Harvey, G., and Shah, N.P. 2003. EPS and lactic acid production by *Streptococcus thermophilus* 1275: Influence of pH, nutrients, temperature and adjunct cultures. American

Dairy Science Association Annual Meeting, Phoenix, AZ, June 22-26, 2003. (J. Dairy Sci. 86 (Supp. 1):360).

Zisu, B. and Shah, N.P. 2002. Effects of exopolysaccharide producing *Streptococcus thermophilus* strains on functionality of low fat Mozzarella cheeses. Institute of Food Technologists Annual Meeting, Anaheim, CA, June 15-19, 2002. (Abstract No. 15B-3).

Zisu, B. and Shah, N.P. 2004. Functionality of low fat Mozzarella cheese as affected by pre-acidification and microbial EPS. 37th Australian Institute of Food Science and Technology Annual Convention, Brisbane, Australia, July 25-28, 2004. (Abstract No. P34).

Chaired Oral Presentations

Bhaskaracharya, R., Zisu, B., and Shah, N.P. 2004. Carbohydrate-based fat replacers and functionality of low fat mozzarella cheese. International Dairy Federation Symposium on Cheese, Prague, Czech Republic, March 21-25, 2004.

Zisu, B. and Shah, N.P. 2003. Influence of pH, nutrients, temperature and adjunct cultures on the production of exopolysaccharides by *Streptococcus thermophilus* 1275. Institute of Food Technologists Annual Meeting, Chicago, IL, July 12-16, 2003 (Abstract No. 41-3, page 95).

Zisu, B. and Shah, N.P. 2004. Influence of microbial EPS, pre-acidification and use of fat replacers on texture and functionality of low fat Mozzarella cheeses. 37th Australian Institute of Food Science and Technology Annual Convention, Brisbane, Australia, July 25-28, 2004.

Zisu, B. and Shah, N.P. 2004. Yield and functionality of low fat Mozzarella made by pre-acidification and use of carbohydrate-based fat replacers and microbial EPS. 37th Australian Institute of Food Science and Technology Annual Convention, Brisbane, Australia, July 25-28 July, 2004. (Abstract No. A39).

Awards Received

Manfred Kroger Graduate Paper Competition in Dairy Foods (2nd Place) - Institute of Food Technologists Annual Meeting, Chicago, IL, July 12-16, 2003.

Malcom Bird Commemorative Award / Graduate Paper Competition (Runner-up) - 37th Australian Institute of Food Science and Technology Annual Convention, Brisbane, Australia, July 25-28, 2004.

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VIII.   List of Abbreviations

Abbreviation key:

AA = amino acid (s)

AAS = atomic absorption spectroscopy

ADC = Australian Dairy Corporation

AOAC = Association of Official Analytical Chemists

ANOVA = one-way analysis of variance

ASCRC = Australian starter culture research centre

CFU = colony forming units

Cps = centipoise

CSLM = confocal scanning laser microscopy

DNA = deoxyribonucleic acid

EPS = microbial exopolysaccharides

FDM = fat in dry matter

HPLC = high performance liquid chromatography

HTST = high temperature short time

Hunter a-value (a value) = measurement of redness

Hunter b-value (b value) = measurement of yellowness

Hunter L-value (L value) = measurement of whiteness

LAB = lactic acid bacteria

M17 agar = agar for enumeration of *Streptococcus thermophilus*

M:P = moisture to protein ratio

MNFS = moisture in non-fat substance

MRS agar = de Man-Rogosa Sharpe - agar for enumeration of *Lactobacillus delbrueckii* ssp. *bulgaricus*

PFGE = pulsed field gel electrophoresis

RSM = reconstituted skim milk

SDS-PAGE = sodium dodecyl sulphate – poly acrylamide gel electrophoresis

SE = standard error

S/M = salt in moisture

SMP = skim milk powder

TBE = tris/boric acid/EDTA buffer

TE = tris/EDTA buffer

TPA = texture profile analysis

UTM = Instron universal testing machine

UV/VIS = ultra violet and visible range of the light spectrum

WG = wheat germ

WPC = whey protein concentrate

Chemicals:

Ca = calcium

CaCl$_2$ = calcium chloride

EDTA = ethylene diamine tetra acetic acid

H$_2$O = water

HCl = hydrochloric acid

LMT = low melting temperature agarose

NaCl = sodium chloride

NaOH = sodium hydroxide

SDS = sodium dodecyl sulphate

***Sma* I** = DNA restriction endonuclease buffer

TCA = tri chloroacetic acid

TEMED = N,N,N',N' – tetra methyl ethylene diamine

Tris = tris (hydroxymethyl) aminomethane

Tri-Cl = tris (hydroxymethyl) aminomethane -chloride

Units of measure:

μ = micro

μg = microgram

μL = microlitre

^{o}C = degree Celsius

cm = centimeter

d = day (s)

g = gram

h = hour (s)

kg = kilogram

L = litre

M = molar concentration

mA = milliampre

mg = milligram

min = minute (s)

mL = millilitre

mm = millimeter

mM = millimolar

ppm = parts per million

rpm = revolutions per minute

s = second (s)

U = units

V = volts

vol/vol = volume by volume

wt/vol = weight by volume

wt/wt = weight by weight

CHAPTER 1.0

Introduction to Thesis

Research showing a correlation between fat intake and coronary heart disease has put a great deal of emphasis on reducing dietary fat intake. Consumer awareness of dietary fat intake has seen a dominant trend in the sales and production of reduced fat dairy products (Fife *et al.*, 1996; Tunick *et al.*, 1995). Sales of reduced fat dairy foods including cheeses have reported a gradual increase in recent years.

Full fat Mozzarella cheeses are described as a 'semi-hard' cheese, with a firm body. These cheeses exhibit good melt, flow and stretch properties with an absence of surface scorching when heated. The textural and functional properties of Mozzarella cheeses are of primary importance as these cheeses are often used as a pizza ingredient. These characteristics are mostly attributed to the fat content of the cheese which acts as a lubricant and provides a physical barrier to the evaporation of surface moisture (Rudan and Barbano, 1998b). Reducing the fat content to make low fat Mozzarella cheeses creates an excessively firm and rubbery body due to a reduction in the moisture to protein ratio and the development of a compact protein matrix (McMahon and Oberg, 1998a). The meltability and stretchability of reduced fat Mozzarella are also hindered. Such cheeses lack complete shred fusion and show excessive amounts of surface scorching when baked (Fife *et al.*, 1996). Low fat Mozzarella cheeses could be baked to exhibit similar melting and browning characteristics to those of the full fat variety by manipulation of their surface behaviour. This was achieved by coating cheese shreds with a hydrophobic material prior to baking to prevent surface dehydration and subsequent skin formation (Rudan and Barbano, 1998b). The fresh and slightly acidic flavour of full fat Mozzarella is also reduced with the removal of fat, however, the flavour is of secondary importance to texture and functionality.

The calcium content is also important for texture and functionality of Mozzarella cheeses. Cheese firmness is associated with its calcium content; firmness increases as calcium content increases and low fat Mozzarella cheeses tend to have higher calcium levels (Geurts *et al.*, 1972; Metzger *et al.* 2000b). The adversities associated with reduced fat Mozzarella cheeses and their

higher calcium composition have led cheese industries to look for ways and means of manufacturing low fat cheeses of acceptable quality.

Full fat Mozzarella cheeses contain 24 to 27% fat. Low fat Mozzarella cheeses containing 18% fat have been manufactured successfully but any further reduction in the fat content creates a product of unacceptable textural and functional properties. Several studies have examined the effects of using carbohydrate- and protein-based fat replacers to make low fat Mozzarella cheeses with some success (Rudan *et al.*, 1998). Some researchers have used the approach of increasing the moisture to protein ratio by incorporating polysaccharides of microbial origin (Perry *et al.*, 1997). Other researchers have removed some of the calcium by pre-acidification of cheese milk with an acid to improve functionality of low fat cheeses (Metzger *et al.*, 2000b).

This study broadly aimed at developing low fat Mozzarella cheeses containing 6% fat with improved textural and functional characteristics. The specific aims of this study were to:

1. identify, screen and select strains of EPS producing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* to be used as starter cultures for the production of low fat Mozzarella cheeses,

2. examine the EPS production of a selected EPS producing strains and identify conditions such as, pH, temperature, co-culturing, fortification with WPC, influencing the EPS production so the processing conditions used to make low fat Mozzarella cheeses can be manipulated accordingly,

3. examine the physical, compositional, textural and functional characteristics of low fat Mozzarella cheeses made with EPS producing starter cultures,

4. examine the physical, compositional, textural and functional characteristics of low fat Mozzarella cheeses made by combining the use of EPS producing starter cultures and pre-acidification of cheese milk with citric acid to pH 6.1,

5. study the physical, compositional, textural and functional characteristics of low fat Mozzarella cheeses (including coating the surface of cheeses with a hydrophobic coating of

oil) made by combining the use of EPS producing starter cultures, pre-acidification of cheese milk with citric acid to pH 6.1 and co-culturing and supplementation with WPC (as identified in step 2),

6. investigate the effects of incorporating fat replacers in low fat Mozzarella cheeses made by combining EPS producing starter cultures and pre-acidification of cheese milk with citric acid to pH 6.1 on the physical, compositional, textural and functional characteristics, and

7. select the most successful variables from the preliminary trials and pilot scale trials to develop low fat Mozzarella cheeses at a semi commercial scale.

Chapter 2 of the thesis deals with the literature review to highlight the principles involved in the development of Mozzarella cheeses and the relevant issues affecting this field of research, specifically the problems associated with the development of low fat Mozzarella. Chapter 3 deals specifically with screening of potential EPS producing starter cultures, while Chapter 4 examines the factors affecting EPS production of a selected strain of EPS producing bacterium. Chapter 5 contains results of cheeses made with EPS producing starter cultures and Chapter 6 discusses the combined effects of pre-acidifying cheese milk and using EPS producing starters. Chapter 7 focuses on the production of low fat Mozzarella cheeses made with EPS cultures and pre-acidification by examining the effects of co-culturing starter bacteria and supplementation with WPC (based on the findings of Chapter 4). The combined effects of using fat replacers with EPS cultures and pre-acidification on texture and functionality of low fat cheeses are discussed in Chapter 8. Chapter 9 focuses on the development of low fat cheeses at a semi-commercial scale based on the findings of Chapters 5 to 8. Overall conclusions have been given in Chapter 10, and the future directions of research are discussed in Chapter 11. References have been listed in Chapter 12.

CHAPTER 2.0

Literature Review

2.1 Mozzarella Cheese

Mozzarella cheese is a member of the pasta filata (kneaded/stretched curd) cheese family that originated in Italy. This group of cheeses falls into two sub-groups, the kneaded cheese, and the plasticised and formed cheese (including Mozzarella) (Figure 2.1). It is made from whole or partly skimmed cow's milk, buffalo milk and milk powder. Mozzarella cheeses undergo a specialised cooking and stretching treatment of fresh curd in hot water, allowing the development of a fibrous protein structure with high melting and stretching properties. Mozzarella is a fresh, unripened cheese often consumed within weeks of manufacture. A typical full fat Mozzarella cheese offers a slightly acidic and salty flavour, has an acceptably firm body, and is slightly yellow in colour depending on the source of the milk.

Enzyme coagulated curd
↓
Acidified plastic mass
↓
Cutting into small pieces
↓
Reduction to a plastic mass in hot water

KNEADED CHEESE   **PLASTICISED CHEESE**

Plastic curds
↓
Kneaded and piled in layers
↓
Formed into moulds/shaped

Plastic curds formed into bands and cord strips

Bands rolled into spiral shape   Rounded into smaller shapes   Large pieces cut, kneaded and layered into large shapes

Loaf shape   Decorated shapes pressed in moulds

Kashkaval   *Ostiepok*   *Parencia*   ***Mozzarella***   *Provolone*
                                                                     Caciocavallo

Figure 2.1. Variety of the pasta filata group of cheeses.

In 2002/03, 42% of manufacturing milk in Australia was used in processing cheese. In the same year, over 368,000 tonnes of cheese was produced and more than 208,000 tonnes was exported. Australian cheese exports sales have increased from $250 million (Aus.) in 1990/91 to approximately $800 million in 2002/03 (Figure 2.2).
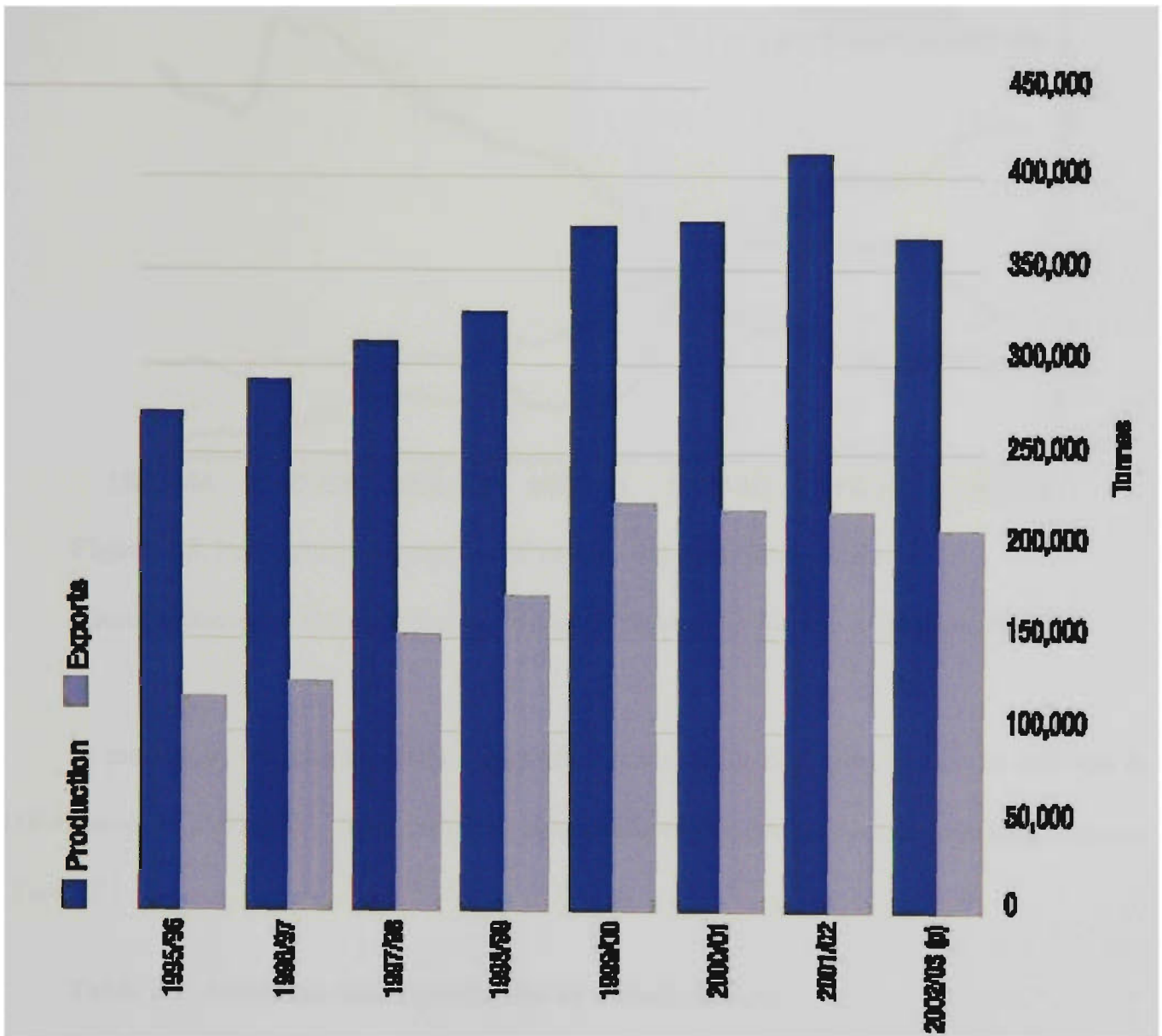
Figure 2.2. Australian cheese production and exports (tonnes).

Source: Dairy Australia (2003).

7

Per capita consumption of cheese has risen from 10.7 kg in 1997/98 to 12.0 kg in 2002/03. The trends in the consumption of various dairy products in Australia are shown in Figure 2.3.
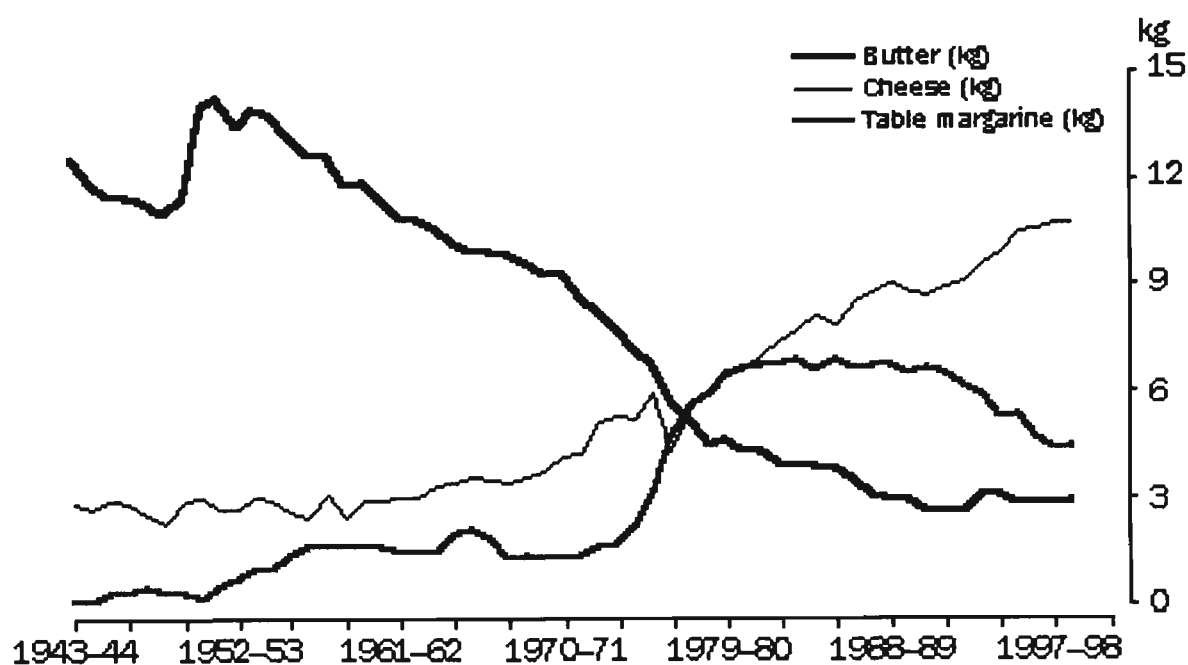
Figure 2.3. Per capita consumption of various dairy products, Australia.

Source: Australian Dairy Corporation and the Australian Bureau of Statistics (2001).

In particular, Mozzarella cheese production has risen from 27,043 tonnes in 1997/98 to 50,064 tonnes in 2002/2003. The growth in Mozzarella cheese production in Australia is shown in Table 2.1.

Table 2.1. Australian cheese production by variety (tonnes).

Semi-hard cheese	1997/98	1998/99	1999/00	2000/01	2001/02	2002/03
Mozzarella	27,043	34,752	43,007	55,743	57,829	50,064
Pizza	11,319	9,925	7,277	5,976	7,070	8,612
Other stretch curd and shredding	6,807	7,769	9,346	5,665	5,099	4,479
Edam	1,588	2,168	2,228	1,034	241	638
Gouda	8,779	14,732	14,381	10,944	17,260	12,529
Other eye type cheese (3)	2,124	3,391	3,914	2,159	1,531	1,475
Other semi-hard cheese (4)	2,945	1,986	1,847	2,604	4,657	6,026
Total semi-hard cheese	**60,606**	**74,722**	**82,000**	**84,124**	**93,688**	**83,824**

Source: Dairy Australia (2003).

This trend has also been observed in the United States, the leading manufacturer of Mozzarella cheese. Due to the specialised functional characteristics that distinguish the pasta filata type cheeses, the increase in the manufacture of Mozzarella is partly attributed to its increased use as a pizza ingredient. Stemming from its popularity in the pizza industry, it is predicted that the growth in Mozzarella production will continue to develop to resemble the US market, where 70% of the Mozzarella cheese produced constitutes for this specific industry (Perry *et al.*, 1998; Rowney *et al.*, 1999). This increased demand has put competitive pressure on cheese makers to produce high quality cheeses more efficiently at lower costs through higher yields and reduced aging.

Past research has shown a correlation between coronary heart disease and dietary fat intake. Given the much publicised potential adverse health implications of consuming dietary fat, consumers are becoming more concerned about their consumption of fat in cheese and other dairy products. As a result of consumer awareness of consumption of nutritional food and increased willingness to follow dietary guidelines, dietary trends have focused on reducing the total fat intake, particularly that of saturated fats (Fife *et al.*, 1996; Tunick *et al.*, 1995; Tunick *et al.*, 1993b). The sales of low fat cheeses other than Mozzarella cheese are steadily increasing. Low fat Mozzarella cheeses therefore have a potential of finding an important market among light dairy products. The development of low fat Mozzarella cheeses has, however, been limited due to the adverse effects imparted in the functionality of such cheeses by the removal of fat.

2.1.1 Functional properties of melted Mozzarella cheeses

The functional characteristics attributed to melted full fat Mozzarella cheese are largely responsible for consumer perception. Melt, stretch, free-oil release and browning are among the most important attributes of a good quality traditional Mozzarella. Accordingly, a multitude of methods have been developed for assessing the functional properties of Mozzarella cheese.

The ability of Mozzarella cheese to stretch and melt has made it the cheese of choice for pizza toppings. Many complex rheological assessment techniques of specific parameters and many subjective methods have been used to determine the functionality of Mozzarella cheese and only those of importance to our work have been described.

2.1.2 Meltability

Heated Mozzarella cheeses begin to melt when the structure is no longer able to support its weight and begins to deform and flow under gravity (Rudan and Barbano, 1998b). At this stage Mozzarella cheese should melt uniformly and exhibit complete shred fusion so that individual cheese shreds coalesce into a homogenous mass. Traditionally, the meltability of cheese has been assessed by the Arnott (Arnott *et al.*, 1957) and Schreiber (Kosikowski, 1977) test. A modified Schreiber test has since been developed by Muthukumarappan *et al.* (1999). Microwave assessment of the meltability of cheeses has also been employed, however, comparisons of the traditional methods and the microwave methods showed no correlation (Park *et al.*, 1984). The results were influenced by the type of heating used (*ie.* convection or forced-draft), and the heating temperature and time applied (Park *et al.*, 1984). More recently, the meltability of Mozzarella cheeses has also been measured by the time taken for cheese to form a molten mass and the resulting area size in an exposed environment (Joshi *et al.*, 2003) or by the distance the cheese flows down a glass tube after heating (Olson and Price, 1958; Poduval and Mistry, 1999). Metzger and Barbano (1999) have also described the measurement of post-melt chewiness of low fat Mozzarella cheeses.

2.1.3 Stretchability

The ability of melted Mozzarella cheese to form fibrous strands that elongate under tension without breaking describes the cheese's stretchability. This parameter was originally assessed by the 'fork' test, where the cheese was cooked on a pizza base and subjectively

assessed by lifting the melted cheese with an implement such as a fork. As can be imagined, the reliability of this test was poor, as it was difficult to maintain uniformity in sampling and recording of measurements. Helical viscometry measurements are often used to measure the resistance exerted by the melted cheese to give a measure of stretchability when the measuring spindle is withdrawn in a helical path (Kindstedt *et al.*, 1989a; Kindstedt *et al.*, 1989b). More recently, a texture analyser was used to lift the melted Mozzarella vertically and, in addition to resistance measurements, the stretch distance at the point of breakage was recorded (Bhaskaracharya and Shah, 2002). In the same year, Fife *et al.* (2002) also measured the stretchability of melted cheese with a texture analyser in a similar manner as Bhaskaracharya and Shah (2002). The apparent viscosity of cheeses was tested using a helical viscometer and the fork test results were compared after pizza baking.

2.1.4 Browning and free oil release

When baking Mozzarella cheeses, browning occurs due to Maillard reaction, primarily as a result of the reaction between lactose and its constituents and the amino moiety of milk proteins. The reaction can be facilitated by increased proteolysis to release more available amino groups. Alternatively, starter cultures that have the ability to ferment galactose may be used to reduce browning. At the same time, fat in the liquid form separates from the melted cheese, often at the surface. An excessive oil release as well as insufficient release of oil (see section 2.4) cause problems when baking and are serious defects of Mozzarella cheeses. Browning has been assessed by cooking the cheese and determining the colour subjectively (Rudan and Barbano, 1998a; Rudan and Barbano, 1998b), or objectively by using a colour meter (Fife *et al.*, 1996; McMahon *et al.*, 1993; Metzger *et al.*, 2000a). The methods used for cooking the cheese include baking (may be on a pizza base topped with tomato paste as melting is affected by the diffusion of ions and other compounds between the cheese and tomato sauce to closely simulate

a pizza bake) and boiling tubes packed with cheese in a water bath for a sufficient time.

2.1.5 Textural properties of unmelted Mozzarella cheese

Complex methods have been used to determine the textural parameters of Mozzarella cheese to understand their behaviour. Rheological properties of specific parameters of Mozzarella cheese have been measured to develop an understanding of the possible influences on the curd texture (Yun *et al.*, 1994b). An Instron Universal Testing Machine (UTM) has been used to assess the textural characteristics of unmelted Mozzarella cheeses to serve as a bridge between the instrumental and sensory evaluation of texture (Bhaskaracharya and Shah, 1999; Metzger *et al.*, 2001; Shakeel-Ur-Rehman *et al.*, 2003; Tunick *et al.*, 1995; Yun *et al.*, 1998). The UTM is used to simulate the masticating action of the human mouth by a two-cycle compression of the food sample with a vertical reciprocating movement at a constant speed. These compressions generate a typical texture profile analysis (TPA) curve (Figure 2.4). A compression of intact samples subjected to the forced applied by the UTM has allowed the cheese hardness, cohesiveness, springiness, adhesiveness, fracturability and chewiness to be evaluated from the TPA curve (Figure 2.4). Pons and Fiszman (1996) have provided a detailed and comprehensive evaluation of texture and the parameters involved when using the UTM.
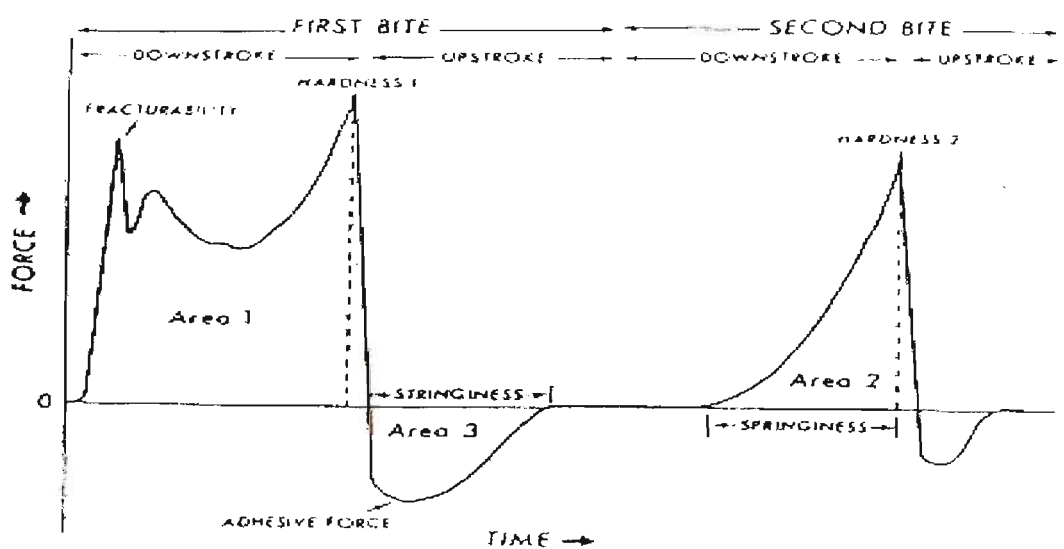
Figure 2.4. A generalised TPA curve from the UTM (Source: Pons and Fiszman, 1996).

The textural properties of Mozzarella cheeses have also been correlated to their microstructure. Several forms of microscopy have been used to examine the microstructure of cheeses at high magnifications, however, these methods alter the actual state of the product during sample preparation. More recently, confocal scanning laser microscopy (CSLM) has been used to study the microstructure of Mozzarella cheeses to monitor the changes in the protein matrix that occur with time. The advantage of this form of microscopy is that samples remain relatively unaffected during preparation, not requiring freezing, dehydration or the removal of fat and can be viewed close to their natural form. Individual components such as protein, fat and EPS may be visualised and comparisons of their distribution can be made. Microscopic observations are, however, limited by the resolution that can be achieved and therefore images can only be viewed at relatively low magnifications. Confocal microscopy has been used to study the microstructure of dairy products by several authors (Auty *et al.*, 2001; Hassan *et al.*, 2002b; Hassan *et al.*, 1995).

2.2 Manufacture of Mozzarella Cheeses

Mozzarella cheeses are produced from whole or partly skimmed milk to which starter culture or small amounts of organic acids are added, followed by a milk coagulator, rennet extract. The process for the manufacture of Mozzarella cheese broadly involves achieving a smooth texture by stretching and moulding the curd in hot water at pH 5.2. The process can be divided into the following steps (Figure 2.5):
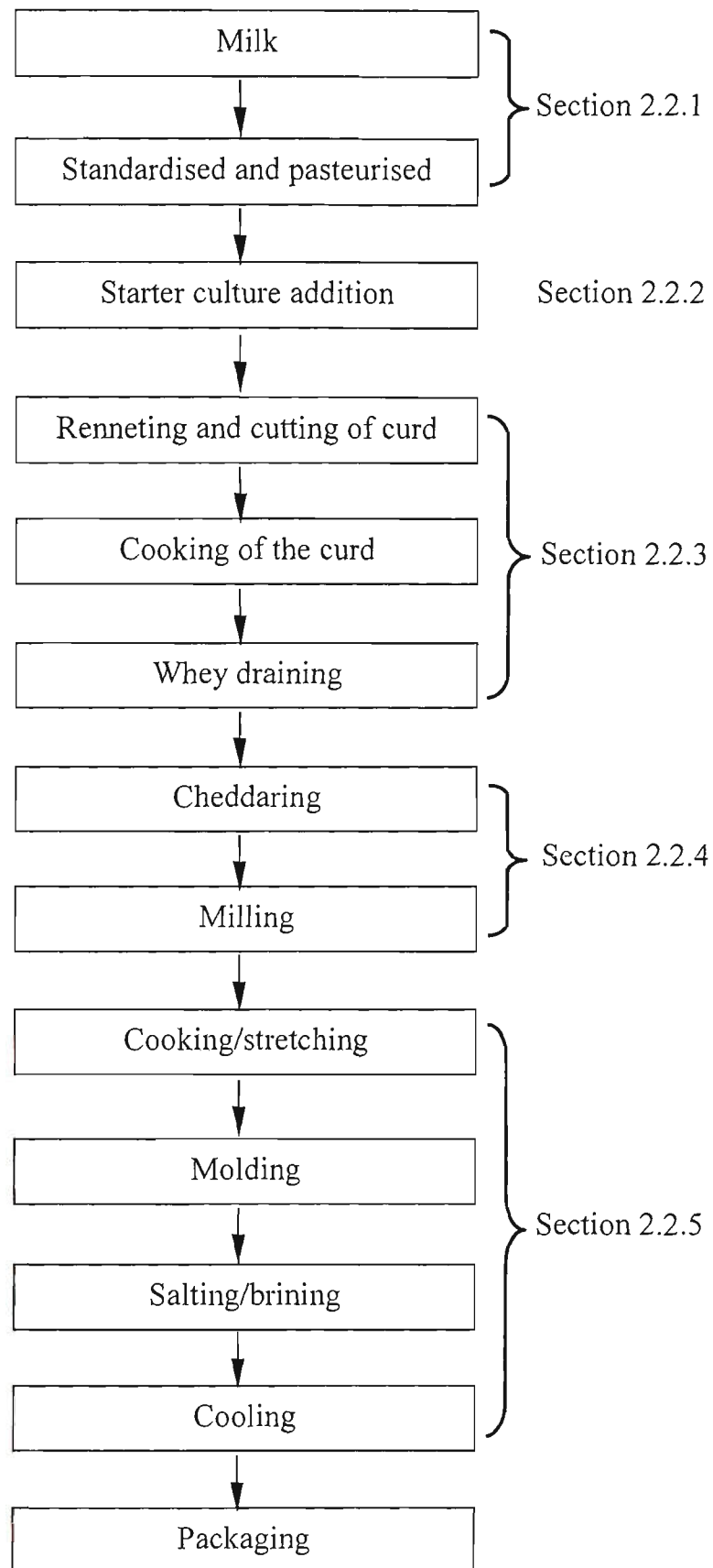
Figure 2.5. Steps involved in the manufacture of Mozzarella cheese.

2.2.1 Preparation of cheese milk

Cheese milk is often standardised, homogenised and pasteurised. Standardisation of the milk achieves uniformity in cheeses, _eg._ fat content to compensate for the variations in the milk composition brought about by seasonal changes or the breed of animal and to be able to use recombined milk. Standardisation allows for production of cheeses with a maximum yield and better quality. The process also allows milk components to be used economically.

The homogenisation process is used to reduce the size of the fat globules and to distribute the fat evenly in the milk to reduce fat separation during storage and to give the milk a whiter colour. The milk proteins also divide into a fine network, preventing them from easily fusing together in the curd structure to allow more moisture retention.

The heat treatment of milk by pasteurisation is used to standardise the bacteriological quality by destroying spoilage microorganisms and enzymes, to facilitate the removal of carbon dioxide and to remove off flavours. Pasteurisation time and temperature combinations are strictly controlled to prevent under- and over- pasteurisation. Under pasteurisation of milk allows the unwanted survival of spoilage microorganisms that may cause uncontrollable fermentation problems during cheese making. An excessive heat treatment of milk may potentially denature the whey proteins and hinder the complex formation with κ-caseins to inhibit rennet coagulation and results in excessive losses through whey (Fennema, 1996). A high temperature, short time (HTST) treatment of 72°C with a holding time of 15 sec is commonly used to achieve the desired results.

2.2.2 Selection of starter cultures

Lactic acid bacteria (LAB) often used for the manufacture of Mozzarella cheese include thermophilic strains of _Streptococcus thermophilus, Lactobacillus delbrueckii_ ssp. _bulgaricus_ and/or _Lactobacillus delbrueckii_ ssp. _helveticus_, and starter culture combinations are used. Strains of _S. thermophilus_ and _L. delbrueckii_ ssp. _bulgaricus_ hydrolyse lactose to its monomers,

glucose and galactose by β–galactosidase activity. The glucose moiety is metabolised through the glycolytic pathway, however, these strains are not equipped to metabolise galactose, which is excreted. Residual galactose may increase non-enzymic Maillard browning when the cheese is baked on a pizza by reacting with the amino acid fractions of the milk proteins. *L. delbrueckii* ssp. *helveticus* strains do metabolise galactose via glucose-6-phosphate using the Leloir pathway (Walstra *et al.*, 1999) and Maillard browning may be reduced. The simultaneous determination of lactose, glucose and galactose as well as the lactic acid produced by LAB can be achieved by high performance liquid chromatography (HPLC). Recent technological advances in HPLC columns and equipment have greatly improved the analysis capabilities in foods to prevent the co-elution of similar compounds such as lactose, glucose and galactose. Hydrolysis of lactose in cultured dairy products can be measured rapidly and reliably by HPLC and is a technique preferred over a variety of other methods previously employed including paper chromatography, thin layer chromatography, automated liquid chromatography, gas liquid chromatography or ion exchange chromatography (Laye *et al.*, 1993; Richmond *et al.*, 1982, 1987).

Cheese milk does not contain sufficient amino acids to support extensive growth of LAB, therefore, the proteolytic ability to breakdown proteins to oligopeptides, smaller peptides and amino acids is necessary. *S. thermophilus* shows less proteolytic activity than *L. delbrueckii* ssp. *bulgaricus* (Shihata and Shah, 2000). Furthermore, the extent of proteolysis exhibited between different strains of *L delbrueckii* ssp. *bulgaricus* was shown to vary widely, as did their effect on the functionality of Mozzarella cheese, with *L. delbrueckii* ssp. *helveticus* having a wider spectrum of proteolysis than *L. delbrueckii* ssp. *bulgaricus* (Rowney *et al.*, 1999). Therefore, the specific mixture of starter cultures used in cheesemaking may effectively influence the ultimate functionality of Mozzarella cheeses.

With the increased production of Mozzarella cheeses and the extensive use of starter bacteria comes the increased risk of a possible phage attack. *S. thermophilus* in particular has a relatively small genome (O'Sullivan and Fitzgerald, 1998). Analysis of the genetic makeup of

LAB by pulsed field gel electrophoresis (PFGE) has the potential to differentiate between strains to identify the different genomes within a species. This allows selection of starter bacteria with unique genetic makeup to use in the event of a strain specific phage attack. PFGE makes it possible to analyse large fragments of DNA and strains can be compared according to the restriction patterns of their chromosomes. These restriction patterns have been shown to be strain specific (Tanskanen _et al._, 1990). Desai _et al._ (2002) used PFGE to differentiate 22 strains of lactobacilli.

2.2.3 Development of curd structure during manufacture

During cheese making, starter cultures metabolise lactose and acidify the milk. The decrease in pH causes solubilisation of micellular calcium phosphate. The stable α_{s1}-caseins along with bound β–casein network and the calcium linked to κ–casein micelles through phosphate bonding undergo transformational changes and the casein bound calcium is expelled $(Ca_9(PO_4)_6 \rightarrow 9Ca^{2+} + 6PO_4^{3-})$ (Fox and McSweeney, 2003). There is an increase in the ratio of soluble-to-micellar calcium. As the pH continues to drop, disaggregation of β–casein occurs and with an increase in H^+ ions the α_{s1}-caseins and κ–caseins acquire opposite charges and aggregate, causing a shrinkage of the network. The β– and κ–caseins are re-incorporated into the casein network and there is an increase in hydration and bonding between caseins. This acts as a cementing agent between the casein micelles/sub-micelles. At pH values in the region of 6.0 to 5.2, solubilisation of micellar calcium phosphate is dominant as decreasing pH results in an increase in the hydration of para-casein.

Following the addition of starter cultures to cheese milk during the manufacture of Mozzarella cheese, the enzyme rennet is added to coagulate milk. Rennet is an extract from the stomach of a calf and contains the clotting factor, chymosin. Chymosin loses its activity below 20°C, has an optimum working temperature between 30 to 48°C and works best in acidic milk. After a short lag period, the milk coagulates to form a coagulum. The cheese milk physically

changes from a dispersed colloidal system of casein micelles to a weak three-dimensional gel network entrapping fat globules and starter culture cells. Structurally, this weakly formed gel network comprises of clusters and chains of individual micelles that are in surface contact (McMahon *et al.*, 1993). During cheese making, all subsequent changes to milk proteins come about as a result of the change in free energy of the system as the macropeptide portion of κ–casein is removed by renneting (Coultate, 1996). The hydrolytic activity of chymosin on casein micelle is best divided into three phases. The primary phase is an enzymic step which leads to activation of the milk clotting particles, the casein micelles. The enzyme itself has a negative site, which attaches to the positive charged site between the amino acid (AA) residues 105 and 106. There is destabilisation of κ–casein by hydrolysis of the susceptible AA bond at the 105-106 (phenylalanine-methionine) linkage to yield para-κ–casein (AA's 1 to 105; insoluble), glycomacropeptide (AA's 106 to 148; soluble) and terminal peptide (AA's 149 to 169; soluble) (Coultate, 1996). The glycomacropeptide and terminal peptide fractions are soluble and are lost in whey after clotting (Coultate, 1996). The para-κ–casein is insoluble, highly hydrophobic and in the presence of divalent ions (mainly Ca^{2+} and Mg^{2+}, phosphates and citrates) aggregates to form a coagulum. Some of the enzyme is adsorbed onto para-casein, and at lower pH the affinity of the enzyme increases leading to increases in the reaction rate.

The secondary phase is the clotting phase during which the enzyme modified casein micelles begin to aggregate with each other. During this time, the milk must remain stationary and the casein micelles are allowed to coalesce. The stabilisation effect of κ–casein over casein michelles is lost, hence by partial hydrolysis to form a gel structure enclosing other components of milk. The factors controlling this phase are the quantity of rennet (0.006% to 0.03% increases the curd tension and further increases in rennet are irrelevant), temperature (up to 40°C increases the curd tension and then reduces it) and lowering pH to 5.8 increases curd tension but beyond pH 5.8 reduces the tension. The composition of milk including casein to fat ratio, total fat content, total calcium content also influence the properties of curd. High fat in milk decreases

firmness of the curd, while an increase in calcium makes the curd firmer. The amount of rennet inhibiting agents such as whey proteins present in milk may also decrease the curd tension (Fox and McSweeney, 2003). The curd particles that have an internal structure consisting of an open matrix made up of protein strands. Between these protein strands, fat globules, bacterial cells and whey (including its water constituent) become trapped and fill up all open spaces. The secondary phase begins before the primary phase is completed.

The tertiary phase involves changes in the properties and structure of the coagulum once it has formed, including the preliminary proteolysis of α-caseins and β–casein by the residual rennin (approximately 6% of the rennin remains adsorbed in the casein) during the maturation period and contributes to the flavour and texture development (Scott, 1986).

When the coagulum achieves a desired firmness, often based on a setting time of approximately 20 to 30 min and subjective assessment by a knife test (an incision is made on a slant in the set curd and the knife is tilted upwards to reveal a firm curd with clear whey separation), it is cut into cubes. The curd formed is cut with cheese knives with a grid of desired size and allowed to matt. The coagulum which is a gel formed by cross linking casein particles and consists of chains of casein particles (approximately 10 particles long), is porous through which moisture (whey) leaks out with increasing acidification. The rate of moisture expulsion from the cubes and the amount of mineral retention depend on the surface area of the curd size available for syneresis (_i.e._ the expulsion of the moisture and firming of the curd). Heating the curd (to ~40°C) in the whey encourages syneresis. Scalding causes shrinkage of the protein matrix (syneresis) and more whey is expelled. Higher temperatures cause faster bacterial metabolism, which produces more lactic acid, causing a further drop in the pH. This leads to more shrinkage and the dissolution of some of the divalent ions (including calcium) which permeate in whey. Depending on the pH of the milk gel, the strength of the cross-linking is determined. An increase in acidification increases the reactive sites of the casein particles and reduces the bond strength. During syneresis the casein particles, which have several reactive

sites, tend to form new bonds after local bonds with other caseins are broken. When the weaker bonds are broken there is an increase in pore size, which allows more syneresis. Generally, caseins when bonded are unable to move, are held tightly and retained in the gel network. The breaking and forming of new bonds continues until the caseins are compacted and strongly bonded (Walstra *et al.*, 1999). The amount of syneresis occurring leads to a concentration of milk components (including fat globules) in the curd cubes and determines the moisture content in the finished cheese. When the curd is firm and the desired pH of the whey (~6.1) is reached, usually 25 to 30 min after the curd is cut, the whey is drained in order to allow the texturising of the curd. The whey contains expressible serum and some serum/whey proteins.

2.2.4 Cheddaring and milling of the curd

After complete whey removal from the cheese vats, the curd is cheddared. This involves packing the curd into layers on each side of the vat to matt the curd together over time and to continue whey expulsion from curd particles to control the ultimate moisture content in the final cheese. Cheddaring of the blocks also involves systematic turning (every 10 to 15 min) and piling to maintain an uniform temperature distribution throughout. The external pressure from re-piling of the blocks increased deformation forces within the curd to continue breaking some bonding between casein particles to aid syneresis and increase the compactness of the casein particles as new bonds are formed as explained earlier. An exposure of the drained curd to warm temperatures permits a mild acid ripening to pH 5.2 by the LAB, upon which it is heated in hot salted water, stretched and moulded.

Before stretching, the cheddared curd appears mellow and has a rubbery texture. It is milled into smaller pieces using a cheese mill or a similar device to promote further whey removal and to increase the surface area so that salts can be applied more evenly when the curd is dry salted. Salt acts as a preservative by inhibiting non-starter organisms from growing and a flavour enhancer and helps reduce the metabolic activity of the starter cultures. The salt further

reduces the moisture content of traditional full fat Mozzarella cheese and shrinks the curd. Salting improves emulsification of fat because of calcium exchange in protein, reducing the chances of oiling off, and is independent of the moisture content of the product. Dry salting of the curd before stretching has been shown to be most effective (Oberg *et al.*, 1993). In a separate study (McMahon and Oberg, 1998b), fat-free Mozzarella cheeses in which the curd had been salted had higher moisture levels than those that were not salted. This was believed to be a result of the salt inducing hydration of proteins on the surface of the curd particles causing the whey within the curd to be trapped and remain in the final product. When salt concentration was > 0.85%, no expressible serum was obtained. It was believed that the salt had lowered the tendency for the proteins to aggregate such that no regions of free serum remained within the protein matrix, and the moisture in the cheese was entrapped. In unsalted cheese, approximately 7% of the moisture in the cheese was expressible.

2.2.5 Stretching and development of Mozzarella structure by the pasta filata process

The principle of the 'pasta filata' nature of Mozzarella cheese is achieving a smooth texture and grain in cheese through stretching of the curd particles in hot salted water ($\sim65^{\circ}$C to raise the curd temperature to $\sim55^{\circ}$C) until the cheese appears as a homogenous mass. In contrast to increased hydration of para-casein between pH 6.0 to 5.2, charge neutralisation dominates in the pH range of 5.2 to 4.6, and leads to a decrease in para-casein hydration. At this pH, the concentration of calcium in the curd and the ratio of soluble-to-colloidal calcium permits adequate hydration of the para-casein for proper plasticisation. The characteristics of Mozzarella cheese come about due to the action of lactic acid on dicalcium paracaseinate, which is converted to monocalcium paracasinate and form para-casein fibers at a pH of approximately 5.2 (Guinee *et al.*, 2002). The relatively low curd pH and the high temperature are conducive to limited aggregation of the casein and the formation of para-casein fibres of relatively high tensile strength. The latter provides the strings and sheen of the cheese (Kosikowski and Mistry, 1997).

At this stage, the three-dimensional network of the protein matrix is changed, and the proteins become aligned and elongated into fibres separated by channels containing closely packed fat globules, bacterial cells and whey serum (McMahon *et al.*, 1993). Heating and stretching of the curd particles into a homogenous mass allows the development of the pasta filata nature of Mozzarella cheese, which provides the desired levels of stretch and stringiness when the cheeses are cooked on pizzas. The temperature and time of stretching will impact the survival of starter bacteria and coagulant, and affect the rate and extent of proteolysis in stored cheeses. High stretch temperatures have also been shown to increase losses of fat globules. Micro-structural examination shows that the protein alignment changes during processing (Oberg *et al.*, 1993). When the stretched curd was still warm, the protein alignment had a continuous appearance that was interconnected, smooth walled fibres separated by channels which contained molten fat globules, serum, bacteria and water soluble constituents. Upon cooling and brining, the protein matrix around the serum channels had developed a rough texture. These serum channel walls showed indentations from previous contact with fat globules and starter bacteria.

Protein is a major constituent of Mozzarella cheese and gives body to the product. The protein gives the desired firmness, stretch and meltability to cheeses. However, cheese firmness is affected by the relative amounts of water, protein and fat. The dominant factor in determining the firmness of the cheese is the amount of moisture, governing the ratio of moisture to protein.

2.2.6 Physico-chemical and proteolytic changes in Mozzarella cheeses during storage

The unique functional properties of Mozzarella cheese first developed during manufacturing when the curd structure is established. The second, inter-dependent phase occurs during storage when the curd structure alters and influences functionality.

In Mozzarella cheeses, the principal biochemical change is due to proteolysis. Although Mozzarella is a fresh cheese and there is limited proteolysis as compared to ripened cheese varieties, it is sufficient to affect the functional and rheological characteristics of cheeses. The

changes that occur during ripening in regards to the protein degradation of Mozzarella cheese are believed to occur as a result of proteolytic activity that breaks down the casein matrix. Primary proteolysis is believed to occur due to the action of residual coagulant which breaks down casein into peptides. Peptides are further degraded into smaller peptides and amino acids during secondary proteolysis caused by the release of enzymes of the starter culture used in the cheese manufacture as well as endogenous milk proteases (plasmin). As a combined result of proteolysis and degradation of the milk protein matrix, the meltability of Mozzarella cheese has been shown to improve (Fife *et al.*, 1996). Tunick *et al.* (1995) reported that proteolysis and rheology of cheese are affected more by the fat content and storage rather than by fat globule size or homogenisation of milk. In addition to improving cheese meltability, the increase in proteolysis decreased hardness, springiness, and chewiness. Various methods of assessing the compositional and proteolytic effects in Mozzarella cheese have been reported including electrophoresis examination of selected milk protein fractions over time by SDS-PAGE (Fife *et al.*, 1996).

At a micro-structural level, the appearance of the serum channels continued to change. In the first days of storage following manufacture, there is redistribution of protein and water with the intact fat globules. The protein matrix has clear, round indentations of fat and a fresh cheese shows approximately 30% expressible serum. A honeycomb-like structure develops by day 14 of storage and expressible serum is reduced to zero (Kindstedt and Guo, 1997). With storage time, the protein matrix swells and extended into the serum channels and connected channel walls and partially occupies the space previously occupied by the serum and fat globules. Eventually the interstitial space between fat globules is almost entirely filled by the protein matrix. These changes occur as a result of protein and water redistribution from within the serum channels to hydrate the protein matrix.

Many factors influence the textural and functional properties of Mozzarella cheese, including fat, moisture and protein, type of starter cultures used, use of fat substitutes, pH at

whey draining, trace minerals (particularly calcium), salting and storage conditions. Although several of these factors have been discussed previously, the sections to follow will address the contributing factors in more detail.

2.3 Role of Fat in Mozzarella Cheeses

The fat content of Mozzarella cheese is determined by the casein to fat ratio of the milk, and traditional Mozzarella cheese is made with 45% fat in dry matter (FDM) (Scott, 1986). The FDM for full fat cheeses in Australia is typically greater than 40%. As the fat content of Mozzarella cheese increases, the softness of the cheese increases and vice-versa. Cheeses that are too soft become difficult to handle and shred (McMahon *et al.*, 1993). An FDM content that is too high also results in excessive free oil release during baking, however, the meltability of these cheeses increases (Tunick *et al.*, 1991).

2.3.1 Low fat Mozzarella cheeses

Several attempts have been made to study the manufacture of low fat high-moisture Mozzarella cheeses with limited success. Fat is important in allowing moisture to be retained in the cheese as well as providing a lubricating effect during heating. When the fat is either partially or completely omitted from foods, the properties of the product alter and several undesirable physical and textural characteristic changes occur, specifically related to a loss in ability to retain moisture. The textural characteristics of low fat Mozzarella cheese are adversely affected by reduction in fat level, and flavour profiles change. Reducing the fat content below 10% to make low fat Mozzarella cheese without modifications of the regular process causes a reduction in the functionality demanded by the pizza industry (McMahon *et al.*, 1996). Specific changes occur in the overall composition and structure, including a lower ratio of moisture to protein, and a reduction in the proteolytic activity during refrigerated storage. There is a lower amount of free oil release during pizza baking and this allows the casein matrix to dehydrate,

leading to excessive browning and limited meltability causing an overall reduction in the pizza baking performance. As a result of the fat removal, the protein content becomes higher in low fat Mozzarella cheeses (reduction in the moisture to protein ratio), and the cheeses becomes harder, they have a rubbery texture and there is an increase in springiness and chewiness (Fife _et al._, 1996; Low _et al._, 1998; McMahon _et al._, 1996; Paulson _et al._, 1998; Perry _et al._, 1997). McMahon and Oberg (1998a) have stated that the status of the protein matrix is the most important parameter to controlling functional characteristics of Mozzarella cheese. Scanning electron micrographs showed that fat globule cavities appeared to be smaller in low fat cheeses, with concomitant denser casein structure (Tunick _et al._, 1993a). With fewer fat globules present in low fat cheeses, casein strands increase coalescence during the cooking and stretching stages of the curd (parallel protein strands become more tightly packed), thereby increasing the rate of syneresis. This results in an inferior quality cheese, specifically with poor melt and stretch properties.

Several published reports have shown that low fat Mozzarella cheeses (_ca._ 5 to 10% fat) have inferior melting (_ie._ have less tendency to melt) and browning characteristics (_ie._ greater cook colour) than high fat cheeses (_ca._ 15 to 25% fat) manufactured in an identical manner (Rudan _et al._, 1998; Fife _et al._, 1996). The stretchability of low fat Mozzarella cheese was observed to be tougher, more brittle and less pliable (Fife _et al._, 1996). Because both fat and unbound water act as a lubricant or plasticiser and increase the ability of cheese particles to flow, the moisture content of low fat Mozzarella cheese in relation to the protein content and cheese functionality has been investigated. According to McMahon and Oberg (1998a), the most important approach for improving the functional properties of low fat cheese is to increase the moisture content to create a moisture to protein ratio that is equal or greater than that observed in full fat Mozzarella cheeses. In low fat Mozzarella cheeses, increasing the moisture content can be achieved by increasing the water binding capacity of the proteins so that they become more hydrated or by increasing the size of the serum channels between protein strands. An increase in

the moisture retention in low fat cheeses assists in decreasing hardness and creating improved melting properties (Petersen _et al._, 2000; Low _et al._, 1998). Too high moisture content in low fat Mozzarella cheese, however, tends to exhibit increasing stickiness when masticated and becomes difficult to shred below a fat content of 15% (Olson and Johnson, 1990). It was found that by increasing the moisture to protein ratio, cheeses can be made softer and their meltability and general functionality can be improved making the product more pliable (Fife _et al._, 1996; McMahon _et al._, 1996).

It is expected that the addition of microbial exopolysaccharide (EPS) producing starter cultures, fat replacers and whey proteins will increase moisture retention to counteract the effects resulting from the loss of fat. Consequently, a decrease in chewiness and improved meltability and textural characteristics of low fat Mozzarella cheese are likely to occur.

Low fat Mozzarella cheese with increased meltability has been manufactured using homogenised milk (Tunick _et al._, 1993b). It was believed that there was a reduction in the rigidity of the casein matrix by smaller fat globules (after homogenisation) acting as co-polymers with the casein, or as a result of increased water retention by the cheese due to transfer of casein from the bulk casein matrix to the newly formated fat globule membrane. These results contradict those identified in full fat Mozzarella cheeses, where meltability was found to reduce after homogenisation of cheese milk (Tunick _et al._, 1995).

Techniques which have been used to manufacture low fat Mozzarella cheese include altering the chemical composition of the protein matrix, use of bacterial starter cultures that produce exopolysaccharides or of highly proteolytic strains, and by incorporation of micro-particulated proteins in milk that is used for cheese making.

2.4 Effects of Baking on Full Fat Mozzarella Cheeses

Full fat Mozzarella cheeses when used as a pizza ingredient undergo significant changes during pizza baking as the temperature increases. Full fat cheeses show complete melt and shred fusion and have desirable browning characteristics. The fat, moisture and protein affect the melting and browning properties of Mozzarella cheese during pizza baking. The area of fat at the surface of the shreds of full fat cheeses is sufficient to melt and becomes liquid as the temperature is raised. It expands as the temperature continues to increase and creates a surface layer of fat that coats the exposed protein and moisture surface regions. As the cheese temperature further increases, the free oil release prevents surface moisture evaporation and the cheese shreds collapse, fuse with adjacent shreds and flow under gravity. At surface temperatures above 100°C, steam is formed. The steam is trapped between cheese shreds creating bubbles to form blisters, which thin out at the top to lose the protection of the fat coating and browns to give a typical pizza bake appearance.

2.4.1 Effects of baking low fat Mozzarella cheeses

Unlike the full fat variety, low fat Mozzarella cheeses have a high protein content and undergo case hardening of the shred surface when baked. Because there is a low amount of fat on the shred surface, there is low amount of free oil released during heating insufficient to create a protective barrier of oil on the surface of cheese shreds. The surface moisture rapidly evaporates and individual cheese shreds dehydrate resulting in the unwanted case hardening. This leads to scorching, which gives the pizza an atypical burnt appearance with intact cheese shreds. Although the inner section of the shred is adequately melted, the cheese shreds are not allowed to melt, fuse and flow adequately due to the hardening and scorching of the shred surface (Rudan and Barbano, 1998b).

The lack of the protective free oil release of low fat Mozzarella cheeses can, however, be artificially introduced to allow the adequate melt and fusion of such cheeses. Rudan and Barbano (1998b) showed that the application of a hydrophobic material (vegetable oil applied at 0.9 g of oil per 100 g of cheese) to completely layer the surface of shredded low fat Mozzarella cheese prevented surface dehydration during baking. This assisted in lubricating the surface of the shreds to prevent case hardening and shreds were able to fuse, melt and flow adequately. Surface scorching was absent and low fat cheeses were comparable to the full fat counterpart.

Under typical pizza baking conditions at 232°C for 5 min in a commercial forced-air pizza oven, the cheese used as a topping reaches a temperature of 60 to 70°C, and cools to approximately 40 to 50°C when consumed (Kindstedt *et al.*, 1989b). The wide temperature range during baking and subsequent cooling influences the appearance of Mozzarella cheese. The whiteness changes during heating and cooling of cheeses after pizza baking, measured by the Hunter L,a,b system indicate reversible, heat induced interactions. Increases in whiteness were observed during heating, and a decrease in whiteness was observed during cooling (Metzger *et al.*, 2000a). This was less apparent in low fat cheeses and the changes in the serum phase were reported as the possible cause for this.

2.5 Role of Moisture in Mozzarella Cheeses

The moisture content in cheeses is affected by the rate and extent of syneresis, which in turn is controlled by the milk pH and composition, especially, calcium concentration, fat and protein contents, temperature and time of cooking, rate and duration of curd stirring and cheddaring (Holsinger *et al.*, 1995). Various studies have found that the moisture content of Mozzarella cheese affects its texture and functionality. Too high moisture content gives the cheese a pasty and sticky feel and such cheeses are difficult to manage. A decrease in moisture content of Mozzarella cheese causes a decrease in meltability, cohesiveness and an increase in

hardness, springiness, chewiness and crumbliness (Tunick *et al.*, 1991). Low fat Mozzarella cheeses have lower moisture to protein ratio than the full fat variety and are excessively hard. The moisture level allows control over functional characteristics, and by controlling the moisture content of the product, the cheese manufacturing procedure becomes economically viable due to the increase in yield. In order to achieve an increase in moisture content in low fat Mozzarella cheeses, two main avenues were considered, namely use of exopolysaccharide producing starter cultures and fat replacers.

2.5.1 Role of microbial exopolysaccharides on moisture retention in low fat Mozzarella cheeses

Microbial EPS producing starter cultures have been used to increase the moisture retention of low fat Mozzarella cheeses. EPS in their natural environment are believed to protect the cell against desiccation, phagocytosis and phage attack, antibiotics or toxic compounds, predation from protozoans, osmotic stress, adhesion to solid surfaces and in cellular recognition. Microbial exopolysaccharides have many established applications but are relatively underexploited in the food industry. Some EPS with industrial importance include dextrans, xanthan, gellan, pullulan, yeast glucans and bacterial alginates (Table 2.2). Factors limiting the industrial use of EPS are their production, which requires a thorough knowledge of EPS biosynthesis, and an adapted bioprocess technology.

LAB are a diverse group of Gram-positive, non-sporing microorganisms used for starter culture applications based on their ability to ferment lactose and production of lactic acid. Among these strains of LAB exist thermophilic exopolysaccharide producing starter cultures (Table 2.3). Certain strains of EPS producing LAB have been successfully used to manufacture low fat Mozzarella cheeses with improved moisture retention to create a high moisture to protein ratio, thereby enhancing cheese yield and improving its functional properties (Petersen *et al.*, 2000; Perry *et al.*, 1997).

Table 2.2. Established applications of microbial exopolysaccharides.

	Use	Polymer
Biological properties:	Antitumour agents	β-D-Glucans
Chemical properties:	Oligosaccharide preparation	Curdland, pullulan, scleroglucan
Physical properties:		
Emulsion stabilisation	Food	Xanthan
Film formation	Food coatings	Pullulan
Flocculant	Water clarification	Various
Foam stabilisation	Beer	Xanthan
Gelling agents	Food	Curdland, gellan
Inhibitor of crystal formation	Frozen foods, sugar syrups	Xanthan
Suspending agent	Food	Xanthan

Source: Sutherland (1998).

Table 2.3. Microorganisms used for milk fermentations and EPS production.

Mesophiles	Thermophiles
Lactococcus lactis ssp. _lactis_	_Lactobacillus delbrueckii_ ssp. _bulgaricus_
Lactococcus lactis ssp. _cremoris_	_Streptococcus thermophilus_
Leuconostoc sp.	_Lactobacillus helveticus_
Lactobacillus kefir	_Lactobacillus acidophilus_
Lactobacillus casei	_Bifidobacterium_ sp.
Saccharomyces cerevisiae	
Candida kefir	

Source: Marshall and Tamime (1997).

Microbial exopolysaccharides are extracellular, long-chained and high molecular mass polymers ranging from 4.0×10^4 to 6.0×10^6 (branched, containing α- and β-linkages) of either homopolysaccharide (single type of sugar monomer) or heteropolysaccharide (several types of sugar monomers) in nature that bind water and retard whey expulsion (De Vuyst and Degeest, 1999). The chemical composition of EPS from LAB differs among microorganisms, however, their monomer composition is similar, with D-galactose and D-glucose being predominant in different ratios (Table 2.4).

Table 2.4. Examples of the EPS composition of selected LAB.

Microorganism	Monomers in EPS								
	Gal	Gluc	Fru	Rha	Man	Xyl	Ara	GalA	Neu
S. thermophilus	+	+	-	-	-	-	-	-	-
S. thermophilus	+	+	-	-	-	-	-	+	-
L. delbrueckii ssp. _bulgaricus_	+	+	-	-	+	-	+	-	-
L. delbrueckii ssp. _bulgaricus_	+	+	-	-	-	-	-	-	-
Lc. lactis ssp. _cremoris_	+	+	-	+	-	-	-	-	-

Gal, galactose; Gluc, glucose; Fru, fructose; Rha, rhamnose; Man, mannose; Xyl, xylose; Ara, arabinose; GalA, galactosamine; Neu, neuramic acid.

Source: Cerning (1990).

Microorganisms can produce exopolysaccharides in two distinct forms:

i.   capsular EPS, where the bacterially metabolised extracellular material remains adhered to the cell surface thus creating a discrete covering, and

ii.   ropy EPS, where bacterial strains generate loose slime that is excreted and the polysaccharides part freely from the unicellular microorganisms.

Many capsular strains of bacteria have been shown to produce loose slime in addition to their capsules (Duguid, 1951). Heteropolysaccharides, commonly synthesised by LAB, are generated by polymerising repeating unit precursors formed in the cytoplasm that are the building blocks of the final composition of EPS. Biosynthesis and secretion of EPS from LAB occur during different growth phases and the amount of polymer is influenced by growth conditions. LAB, in order to produce a substantial amount of EPS, have fastidious nutritional requirements and need controlled growth conditions such as pH and temperature to optimise EPS synthesis. A growth medium rich in a source of carbon and nitrogen, as well as minerals and vitamins is necessary. The fortification of the growth medium with a nitrogen source such as yeast extract and whey protein concentrates (WPC) (De Vuyst and Degeest, 1999; De Vuyst _et al._, 1998; Marshall _et al._, 1995) or supplementation with a carbon source in the form of simple carbohydrates (Degeest _et al._, 2001; Gamar _et al._, 1997; Shah, 2002) is shown to increase the EPS production. Milk is a highly nutritious medium and glucose or the glucose moiety from lactose hydrolysis appears to be the source of sugar for EPS biosynthesis in LAB (Figure 2.6) (De Vuyst and Degeest, 1999). These factors make EPS synthesising starter cultures suitable for use in dairy applications.
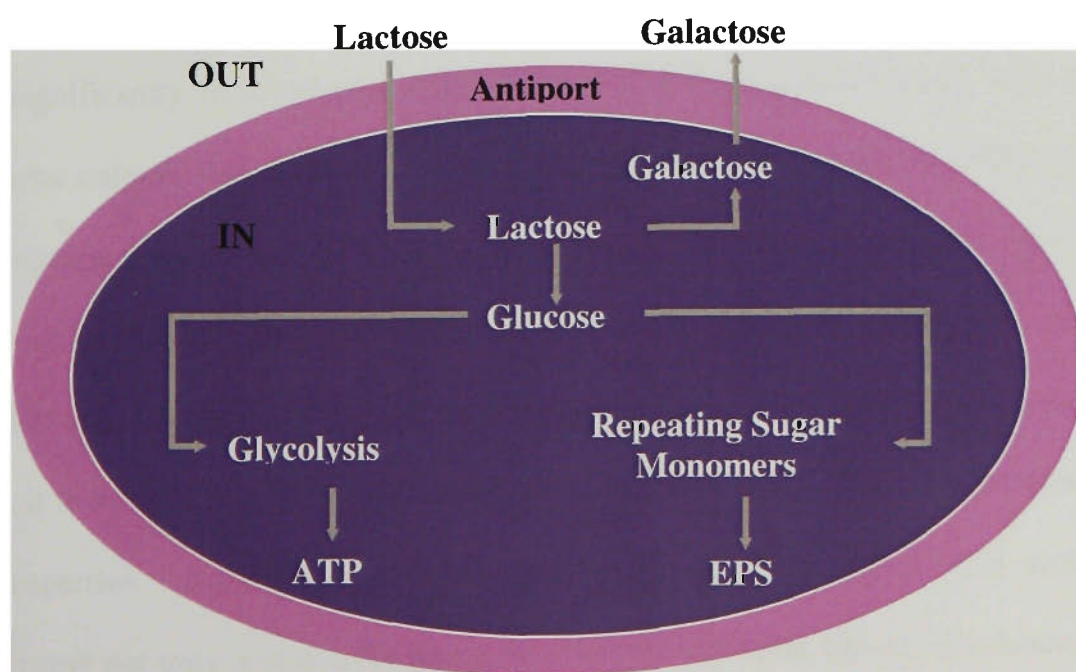
Figure 2.6. Lactose utilisation for EPS biosynthesis by _S. thermophilus_.
Source: Zisu and Shah (2003b).

Freeze drying is a common method used to preserve viable microorganisms in a dormant state by controlling the microbial growth through lowering the surrounding water activity. Traditional non-EPS starter bacteria are often used as rapid set cultures in the form of freeze dried LAB. Although bacterial cells are frozen with a cryoprotectant and the moisture is sublimed under vacuum, the implications of the freeze drying procedure and its effects on EPS production are unknown.

Isolation of ropy EPS secreted into culture media by centrifugal forces presents few problems due to the lack of physical attachment between the polysaccharide and the cell. Capsular EPS must, however, be detached and released from the cell surface. The capsules are readily removed from some strains of bacteria by centrifugation but not from others. Stirring, mixing or homogenisation of the suspension medium generally suffices followed by repeated ethanol precipitation. Crude EPS isolated from more complex media such as milk must be purified. This requires the removal of contaminating proteins and sugars (lactose) by precipitation, enzyme treatment and prolonged dialysis. The purified EPS is then quantified, often by the phenol-sulphuric method (Dubois *et al.*, 1956).

Whey recovered from cheeses manufactured with ropy EPS producing cultures, however, tends to be significantly more viscous than that expelled from cheeses made with non-EPS producing starter culture (Petersen *et al.*, 2000). The magnitude and ability of EPS to affect the viscosity is dependent on the molecular mass of the polysaccharide and varies between bacterial strains (Faber *et al.*, 1998). This undesirable effect poses problems by delaying the membrane processing efficiency of whey and thereby its utilisation for processing whey protein concentrate. Furthermore, it is thought that EPS expelled into these products may cause an alteration in their functional properties. Recent studies have concluded that encapsulated EPS synthesising bacteria (not ropy) not only increase the moisture retention of low fat Mozzarella cheese (Low *et al.*, 1998; Petersen *et al.*, 2000; Perry *et al.*, 1997), but also show no significant increase in whey

viscosity when compared with regular, non-EPS producing starter strains (Petersen *et al.*, 2000). This is due to the metabolic EPS remaining firmly attached to the cell surface. As a result of the increase in moisture retention, the moisture to protein ratio of low fat curd increases. Hassan and Frank (1997) showed that curd tension and firmness of non-fat curd was reduced and was comparable to a full fat rennet curd (containing 40 g of fat/L) by using EPS capsule forming *S. thermophilus* culture. Low fat Mozzarella cheeses made with EPS producing starter cultures exhibiting a higher moisture content than the non-EPS control cheeses also had higher meltability (Perry *et al.*, 1997; Perry *et al.*, 1998; Petersen *et al.*, 2000). Furthermore, EPS producing starter cultures that form large capsules around the bacterial cells tend to aggregate and form clusters large enough to interrupt the protein fusion that occurs during cooking and stretching thus forming more fat-serum channels within the protein matrix. This then results in more moisture being retained in the cheese and functions in a similar way to some fat replacers (section 2.5.2). The ability of encapsulated non-ropy cultures to bind water in comparison to non-EPS producing cultures has also been described in yoghurt studies where the texture of the product was improved (Hassan *et al.*, 1996b).

2.5.2 Role of fat replacers in moisture retention in low fat Mozzarella cheeses

To compensate for the loss of fat in low fat Mozzarella cheeses, fat replacers can be used. The composition of these ingredients varies widely, and fat replacers may be derived from carbohydrates, proteins, or lipids. Many fat replacers are commercially available and examples of such are provided in Table 2.5.

Table 2.5. List of commercially available fat replacers.

Name/Type	Uses/Applications
Simplesse® (protein)	Yoghurt, cheese spread, cream cheese, sour cream, salad dressing, mayonnaise, margarine.
Trail Blazer® (protein)	Frozen desserts.
Olestra® (synthetic- sucrose polyester)	Frozen desserts, table spreads, salad dressings, cheese bakery items, shortenings, cooking oils.
EPG (synthetic- esterfied propoxylated glycerol)	Frozen desserts, table spreads, salad dressings, cheese bakery items.
DDM (synthetic- dialkyl dihexa decymalonate), TATCA (synthetic-trialkoxy tricarbollate)	Mayonnaise, margarine, cooking oils.
Gums (carbohydrate- hydrocolloids)	Salad dressings, formulated foods.
Polydextrose (carbohydrate)	Candy, chewing gum, candy coatings, dry cakes/cookie mixes, frozen dairy products, icings, nutritional bars, puddings, frostings.
Maltrin® M040 and M100 (carbohydrate- maltodextrins)	Frozen desserts, table spreads, salad dressings, margarine, imitation sour cream.
Tapioca Dextrins® (carbohydrate)	Frozen desserts, table spreads, salad dressings, margarine, imitation sour cream, puddings, microwavable cheese sauce.
Paselli SA2 (carbohydrate- potato starch/maltodextrin)	Salad dressings, frostings, frozen desserts, dips, bakery products, mayonnaise, table spreads, meat products, confections.
StaSlim® 143 (carbohydrate-modified potato starch)	Pourable and spoonable salad dressings, soups, cheese cakes, imitation cream cheese.
Prolestra® (sucrose polyester)	Ice-cream, salad oils, mayonnaise, sauces, snacks, table spreads, baked products.
Nutrifat® (hydrolysed starch)	As above.
Finesse® (piezo-proteins), Colestra (low calorie Olestra)	As above.

Source: Summerkamp and Hisser (1990).

Fat replacers convey fat-like sensory properties in foods to some degree, but they never possess full functional equivalency to fat. Fat replacers have been successfully used to manufacture bakery products, confectionery, salad dressings and processed cheese. Adding a fat replacer to milk can also increase cheese moisture content but whether this will provide increased functionality depends on its characteristics. Of importance is the level of microparticulation, particle size, interaction with casein, and distribution between protein matrix and serum within the cheese curd. The melting properties of non-fat Mozzarella cheeses have been shown to improve with the use of maltodextrin (Maltrin® M100) (Stevens and Shah, 2002). Modified starches, in particular can be used to provide desired simulated fat properties by contributing to sensory properties arising from bulking and moisture retention. The fat replacer Salatrim® (fatty acid) has also been used to yield a higher moisture content with improved meltability in Mozzarella cheeses containing 9% fat compared to those made without the fat replacer (Rudan _et al._, 1998).

Natural low fat Mozzarella cheeses (< 6% fat) were made by McMahon _et al._ (1996) using fat replacers aimed at increasing the moisture content and consequently improving the functional properties. By addition of protein based fat replacers, Simplesse® D100 and Dairy-Lo® and carbohydrate based fat replacers, Stellar® 100X and Novagel™ RCN-15 into milk, the cheese moisture content was increased. The meltability was also increased, however, this was dependent on the individual nature of each fat replacer. Of particular significance was the fat replacers morphology (_ie._ microparticulation and particle size), casein interaction and dispersal within the protein matrix and the serum of the curd particles, all of which affected the final cheeses microstructure, moisture content and functionality. In contrast with the previous findings, the apparent viscosity used as a measure of cheese stretch was not significantly affected (McMahon _et al._, 1998a; 1996; Rudan _et al._, 1998).

Fife _et al._ (1995) also used various commercially available fat replacers to increase the moisture content of Mozzarella cheeses. The carbohydrate based fat replacer, Stellar® 100X

showed greatest melt, however, these cheese became sticky and difficult to handle with storage time. The protein based Simplesse® D100 also showed improved meltability and remained manageable with time. At a microstructural level, the latter fat replacers were found in the pockets of fat and serum between strands of casein. Novagel™ RCN-15 (carbohydrate based) and Dairy-Lo® (protein based) fat replacers did not show a higher moisture retention compared to control cheeses, however, the cheeses showed low meltability. These particles were observed to be embedded in the casein matrix as well as in serum pockets. Because McMahon _et al._ (1996) and Fife _et al._ (1995) used identical fat replacers and achieved moisture contents that were contradictory, the method of dispersion and preparation before cheese making must be considered. The heat treatment of carbohydrate based fat replacers is particularly important to allow the gelatinising of starch granules which begin to re-associate and form water holding gels upon cooling.

Small particle sized fat replacers are easily distributed between the protein matrix and the serum channels and have little effect on increasing the openness of the cheese microstructure. Such examples include the microparticulated whey proteins. On the contrary, fat replacers made from microcrystalline cellulose have particle sizes that are larger than individual protein strands and cannot be embedded into the matrix resulting in large serum channel formation in cheeses where more water may be embedded (McMahon _et al._, 1996).

The particulate nature of fat replacers also acts to scatter light and increases the opaqueness of low fat Mozzarella cheese to counteract the more translucent appearance of such cheeses. However, because of the protein or carbohydrate base of the fat replacers, they do not behave like a fat, particularly when heated. The pizza bake performance for such cheeses was evaluated by Rudan _et al._ (1998) and obtained poor results in regards to melting and fusion of shredded cheese used as a pizza toping as well as a high degree of surface scorching.

Although not classified as a fat replacer, WPC form heat induced gels as temperatures above 70°C denature whey proteins. In part, this is associated with casein micelles involving κ-

casein via hydrophobic interactions and intermolecular disulphide bonds (Fox and McSweeney, 2003) and improving their water holding ability. These changes play a role in the textural and rheological properties of foods (Fennema, 1996). Current WPC made from whey typically have variable composition and functionality because of different milk/whey sources, processing equipment and manufacturing methods. The significance and the impact of WPC in a cheese system is highly dependent on the latter variables. If they are added to food on a solid basis, there will be a large difference in functionality due to the differences in protein content (typically ranging from 35 to 95% protein) and solubility. The solubility profiles of native whey proteins at acid and neutral conditions makes them unusual, however, the solubility of commercial WPC has been known to be highly variable and may require pre-treatment to achieve optimum functional capability. WPC are also rich nutritional ingredients that may support or promote the growth of starter cultures and may promote the synthesis of microbial EPS.

2.6 Role of Calcium in Mozzarella Cheeses

The role of calcium has been discussed throughout the cheese making procedure, where calcium exists in milk in the form of bound calcium phosphate with casein and keeps the casein in a colloidal phase. It is important for the structure, texture and functionality of Mozzarella cheese. The firmness of cheese is associated with its calcium content, with firmness increasing with calcium content and vice versa. During the cheese making process, the decrease in the pH of the system (either by the starter cultures or by direct acidification) plays an important role in solubilising the colloidal calcium phosphate from the casein matrix, and calcium is removed from the curd into whey. At optimum pH, the curd calcium content decreases by approximately 75% as compared to the initial content. The removal of calcium causes dissociation of caseins, which becomes available for emulsification of fat and results in less oiling off when melting. The firmness of the cheese influences the shredding quality, and it is important to retain

sufficient calcium so a full fat cheese does not become too soft. However, an excessive calcium content, creates cheeses that are too firm and this also creates problems.

2.6.1 Role of pre-acidification in low fat Mozzarella cheeses

Because calcium plays an essential function in Mozzarella cheese texture by cross linking with protein, the amount of calcium present in the cheese influences its texture (Metzger *et al.*, 2000a). The calcium content in low fat Mozzarella cheese is known to be greater than in low moisture part skimmed cheese when identically manufactured, resulting in harder cheeses. Theoretically, by removing some of the calcium it is possible to manufacture a softer cheese as a result of reduced cross-linking between casein polymers.

The calcium content of Mozzarella cheese can be manipulated, and has been successfully controlled by modifying cheese manufacturing conditions. The effect of milk pre-acidification has been found to affect the composition and yield of low fat Mozzarella cheese. The technique of pre-acidification involves a rapid addition of a food grade acid to milk to lower the pH prior to enzymic coagulation thus causing solubilisation in portions of the micellar calcium. This has been shown to reduce the calcium content in the cheese. Although the remaining micellar calcium is retained in the cheese, nonmicellar calcium is further removed during the whey draining stage (Metzger *et al.*, 2000a).

Pre-acidification studies examining the effects of using various acids and various pH values of the milk at coagulation have shown to affect the calcium content in cheeses. By using citric acid or acetic acid to pre-acidify milk, it has been observed that acidifying to a lower pH causes a greater calcium loss. Pre-acidification with citric acid showed greatest calcium by forming soluble complexes that are removed in whey (Keller *et al.*, 1974; Metzger *et al.*, 2000a). Citric acid is a tricarboxylic acid with three available carboxylic acid groups to donate a pair of electrons to calcium making it a strong chelating agent. It has three pK_a values, 3.06, 4.60 and 6.40 and is able to form inert, water soluble complexes with calcium that are not easily

dissociated. Oberg *et al.* (1993) reported that a pH of 5.6 obtained by acidification using citric acid gave similar calcium curd concentrations as with lowering to pH to 5.2 by other acids because of the chelating effect of citric acid causing greater demineralisation, especially calcium (Keller *et al.*, 1974).

As a result of pre-acidification, the water-holding capacity of the low fat mozzarella cheese is altered. Guo *et al.* (1998) showed that cheeses made with milk pre-acidified to pH 6.0 averaged 5.6 g of expressible serum, whereas the control (no pre-acidification) showed an average loss of 15.7 g per 100 g of cheese after three days of storage. During the first weeks after manufacture, Mozzarella made using pre-acidification generally had a softer body and greater melt characteristics than cheeses of similar age made without acid treatment (Joshi *et al.*, 2003).

Mozzarella cheeses made by pre-acidification were, however, found to recover a lower yield of fat and solids-non-fat when compared with cheeses made using bacterial starter cultures only. Furthermore, mineral losses, in particular calcium are greater with faster and more extensive acidification at the time of rennet addition. A lower pH at the time of rennet addition, therefore, results in higher calcium loss in the whey at the time of whey draining. The pH at whey draining, besides contributing to the calcium content, has also been reported to influence proteolysis. A lower pH enhances the susceptibility of casein proteolysis and contributes to the textural and functional properties of the cheese (Joshi *et al.*, 2003). Low fat Mozzarella cheeses made by pre-acidification have shown improved meltability and reduced hardness (Fife *et al.*, 1996). McMahon and Oberg (1998b) increased cheese meltability by > 300% when the calcium content was reduced from 0.6% to 0.3% in low pH non-fat Mozzarella cheeses. The cheese hardness decreased as the calcium and pH of the acid treatment were lowered and adhesiveness was increased. Microstructural examination of low calcium content cheeses by electron microscopy showed that they had poor fusion of the protein network and large serum pockets (openness) were evident throughout the protein matrix.

Successful plasticisation may be achieved at a high curd pH (> 5.2), *eg.* 5.6 to 5.8, if the total concentration of calcium in the curd is sufficiently low. This has been achieved by direct acidification with an acid in the absence of starter bacteria. Such cheeses also had a protein network with a higher water holding capacity than the conventionally produced Mozzarella curd at pH 5.2 (Kindstedt and Guo, 1997).

The calcium content in dairy products has been estimated by various methods. Atomic absorption spectroscopy has been used with some success to examine the mineral content of low fat Mozzarella cheese made by pre-acidification (Metzger *et al.*, 2001; Metzger *et al.*, 2000b).

CHAPTER 3.0

Selection of EPS Producing Strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*

3.1 INTRODUCTION

Lactic acid bacteria (LAB) are a diverse group of Gram-positive microorganisms that are used extensively by the dairy industry to manufacture fermented products. Thermophillic strains of _S. thermophilus_ and _L. delbrueckii_ ssp. _bulgaricus_ are commonly used to manufacture Mozzarella cheeses. The production of Mozzarella cheeses in Australia has steadily increased since 1990 (Rowney _et al._, 1999). Similarly, an increase in the demand for low fat products has been observed. Low fat Mozzarella cheeses, however, exhibit poor textural and functional characteristics and cannot be made commercially viable without modification to the manufacturing procedure. This has prompted research on identifying LAB with functional characteristics that expand beyond lactic acid production.

Identification of LAB that produce homo- and hetero-polysaccharides is an example of such initiatives. Many strains of LAB including _S. thermophilus_ and _L. delbrueckii_ ssp. _bulgaricus_ have the ability to synthesis these exopolysaccharides (EPS) (Ariga _et al._, 1992; Bouzar _et al._, 1996; De Vuyst and Degeest, 1999). Microbial exopolysaccharides are extracellular, high molecular mass polymers, which have the ability to bind water and retard the expulsion of whey (De Vuyst and Degeest, 1999). Two forms of EPS exist, either in a capsular form whereby the polysaccharide metabolite remains attached to the cell surface to create a discrete covering, or as ropy EPS, which is secreted into the immediate environment. In certain cases, the same microbe may produce both capsular and ropy EPS (Cerning, 1990). The synthesis of EPS is complex and requires the action of highly specific enzymes to act on specific substrates. Nutritional rich cultivation media (rich in carbon/nitrogen energy sources, vitamins and minerals) and optimal growth conditions must be provided for strains to produce EPS at peak levels. Certain strains of bacteria have shown instability in their EPS production trait and organisms have been reported to lose this property after frequent transfers (Cerning _et al._, 1990).

Duguid (1951) demonstrated capsular EPS production by using the technique of wet film India ink staining and observation with a light microscope. Ropy EPS tends to increase the viscosity of solutions (Cerning, 1990) and may be identified by viscosity measurements and visually observing the formation of 'ropy' strands when disturbing the cultivation media with a loop or a pipette tip. EPS can be quantified, however, the capsular type must first be detached from the cell surface by centrifugation, stirring, homogenising and/or precipitation with ethanol. This may at times cause problems as capsular EPS are more readily removed from certain strains than from others.

S. thermophilus has a relatively small genome (O'Sullivan and Fitzgerald, 1998). With the increased production of Mozzarella cheeses and the extensive use of starter bacteria comes the increased risk of a possible phage attack. Analysis of EPS producing _S. thermophilus_ by pulsed field gel electrophoresis (PFGE) has the potential to genetically differentiate between strains. PFGE makes it possible to analyse large fragments of DNA and strains can be compared according to the restriction patterns of their chromosomes. These restriction patterns have been shown to be strain specific (Tanskanen _et al._, 1990). This allows the selection of several strains of _S. thermophilus_ with a unique genetic make-up that will be available for selective use in the case of a strain specific phage attack.

Because microorganisms do not grow well at low water activities, the microbial growth can be controlled by lowering the surrounding water activity. The least damaging way of preserving viable microorganisms in a dormant state is by freeze drying where the cells are frozen in the presence of a cryoprotectant and moisture is sublimed under vacuum. The implications of freeze drying on EPS production are, however, largely unknown.

The aims of this study were to identify capsular and ropy EPS producing strains of _S. thermophilus_ and _L. delbrueckii_ ssp. _bulgaricus_. Based on the amount of EPS produced, its impact on the viscosity of milk and the genetic variation of the bacteria, strains of _S. thermophilus_ were selected for use as starter cultures in Mozzarella cheeses making.

3.2 MATERIALS AND METHODS

3.2.1 Starter Cultures

Eighty strains of potential _S. thermophilus_ and twenty strains of _L. delbrueckii_ ssp. _bulgaricus_ were obtained from the Australian Starter Culture Research Center (ASCRC, Werribee, Australia). The organisms were stored at −80°C in 10% (wt/vol) sterile reconstituted skim milk (RSM) containing 20% (vol/vol) glycerol (BDH Chemicals Pty. Ltd., Kilsyth, VIC, Australia). The skim milk powder was obtained from Murray Goulburn Co-Op. (Brunswick, Melbourne, Australia). Working cultures were propagated three times consecutively using a 1% (vol/vol) inoculum in 10% RSM. _S. thermophilus_ strains were incubated at 37°C for 18 h before use and _L. delbrueckii_ ssp. _bulgaricus_ strains were incubated at 42°C. When necessary, bacterial strains were cultivated specifically in M17 broth (for _S. thermophilus_) and MRS broth (for _L. delbrueckii_ ssp. _bulgaricus_) and incubated at 37°C and 42°C, respectively. The broths were obtained from Amyl Media Co. (Dandenong, Melbourne, Australia).

3.2.2 Identification of EPS Capsules

A 1 µL volume of homogenous RSM containing the active starter culture was placed onto a microscope slide. An equal volume of India ink (Pelikan Co., Hannover, Germany) was added to the RSM and the two were blended into a homogenous mixture. A microscope coverslip was gently placed onto the sample and viewed under oil immersion at either 1000 × magnification using a Zeiss Axioskop microscope fitted with a PCO VarioCam digital camera (Carl Zeiss Inc., Thornwood, NY, USA), or at 100 × magnification using a light microscope

(model Ch-2; Olympus Australia Pty. Ltd., Oakleigh, VIC, Australia). EPS capsules appeared as a distinct clear halo surrounding the bacterial cells.

3.2.3 Carbohydrate Fermentations

Strains believed to be _S. thermophilus_ were grown in M17 broth and _L. delbrueckii_ ssp. _bulgaricus_ strains were grown in MRS broth (as per section 3.2.1). The carbohydrate fermentation profiles of the selected strains were analysed using an API 50 CHL kit (Biomerieux, Marcy-l'Etoile, France) according to the manufacturers instructions. When propagated, the fermentation kits were incubated at either 37°C (for _S. thermophilus_) or 42°C (for _L. delbrueckii_ ssp. _bulgaricus_) for 48 h. Carbohydrate fermentation results were analysed using the extensive starter culture collection database established at ASCRC, Werribee, Australia.

3.2.4 Apparent Viscosity

Each strain was activated in RSM (as per section 3.2.1) and propagated in 200 mL of 10% RSM with a 1.5% inoculum and incubation at 37°C for 24 h. The apparent viscosity was measured with a Brookfield viscometer (model DV II; Brookfield Engineering Laboratory, Stoughton, CA, USA) using a Helipath D spindle at 12 rpm. The samples were stirred and agitated to break the curd completely prior to reading the viscosity and the temperature was maintained at ~22°C. A one-minute adjustment time was applied and multiple viscosity measurements were recorded at five second intervals for up to three minutes. Viscosity measurements were replicated three times.

3.2.5 Isolation and Quantification of Exopolysaccharides

3.2.5.1 Isolation of EPS

The isolation and quantification of EPS was performed according to Zisu and Shah (2003a). Strains of _S. thermophilus_ were activated as described in section 3.2.1 and grown in 100 mL of 10% sterile RSM using 5% inoculum and incubated for 24 h. The cultures were stirred using a magnetic stirrer to disrupt the curd and obtain a homogenous mixture. The caseins were precipitated by adding 20 mL of 20% tri-chloroacetic acid (TCA) solution (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia) while stirring. To remove the precipitate and the bacterial cells, the mixture was centrifuged at 10,000 × _g_ for 20 min at 4°C using a Beckman-Coulter centrifuge (model J2-HS; Beckman-Coulter Instruments Inc., Palo Alto, CA, USA). The supernatant was neutralised to pH 6.8 by adding 4 M NaOH (Roche Diagnostics Australia Pty. Ltd., NSW, Australia) and boiled in a water bath (Thermoline Scientific Equipment Pty. Ltd., NSW, Australia) for 30 min. The insoluble proteins were removed by centrifugation at 10,000 × _g_ for 20 min at 4°C. An equal volume of chilled ethanol (BDH Chemicals) was added to the supernatant and agitated overnight in a refrigerated shaker incubator (Innova 4230; New Brunswick Scientific, Edison, NJ, USA) at 100 rpm and 4°C. The precipitate containing EPS was recovered by centrifugation at 10,000 × _g_ for 20 min at 4°C and the supernatant was discarded. The crude polysaccharide pellet was re-suspended in 10 mL of 0.05 M trizma hydrochloride (tris-HCl) buffer (pH 8.0) (Sigma-Aldrich) by vortexing and the contaminating proteins were digested overnight with 0.2 mg/mL of proteinase K (Sigma-Aldrich) at 37°C. The reaction was stopped by heating the suspension at 90°C for 10 min and polysaccharides were precipitated from solution with 15 mL of chilled ethanol followed by centrifugation at 10,000 × _g_ for 20 min at 4°C. The pellet of EPS was suspended in 10 mL of sterile distilled water and to obtain a completely homogenous mixture, sonicated for 120 min (FX 14PH sonication bath; Unisonics Pty. Ltd., Sydney Australia) to soften the pellet and vortexed into an uniform

suspension. The solution was dialysed in dialysis tubing (1 inch width; Carolina® Biological Supply Co., Burlington, N. Carolina) against distilled water for up to one week to remove contaminating lactose with a minimum of two daily changes of water.

3.2.5.2 Quantification of EPS

Microbial EPS was quantified using the phenol-sulphuric method (Dubois *et al.*, 1956) as used extensively by others for this purpose (Bouzar *et al.*, 1996; Gamar *et al.*, 1997; Gamar-Nourani *et al.*, 1998; Gorret *et al.*, 2001; Grobben *et al.*, 2000; Sebastiani and Zelger, 1998; Torino *et al.*, 2001). A 2 mL sample of EPS solution containing 10 to 100 μg of EPS was pipetted into test tubes. A blank solution was prepared by substituting distilled water for the EPS solution. A 1 mL volume of 5% phenol (Roche Diagnostics) was added to each sample and 5 mL of concentrated sulphuric acid (95.5%, sp. gr.1.84) (Roche Diagnostics) was rapidly pipetted into each test tube to obtain adequate mixing. The test tubes were allowed to stand for 10 min before shaking and placing into a water bath at 28°C for 15 min. The blank solution was set as the reference using 2.5 mL cuvettes (PMA #1960; Kartell SPA, Richmond, VIC, Australia) and the absorbency was recorded at 490 nm. The absorbency of each sample was measured in duplicate using a spectrophotometer (Novaspec II, Pharmacia LKB Biochrom Ltd., Science Park, Cambridge, England).

3.2.5.3 Preparation of standards

EPS was expressed as a glucose equivalent. The standards were prepared according to section 3.2.5.2. A standard curve was prepared containing 10 to 100 μL of glucose (Figure 3.1).

3.2.6 Genetic Differentiation of *S. thermophilus* Strains Using Pulsed Field Gel Electrophoresis

3.2.6.1 Preparation of agarose

High purity low-melting-temperature agarose (LMT) (Progen Industries Ltd., Dara, QLD, Australia) (2.5% wt/vol *ie.* 25 mg/mL) was prepared by dissolving the agar in NaCl/EDTA/tris solution (1 M NaCl (BDH Chemicals), 10 mM EDTA (disodium salt) pH 8.0 (Merck), 10 mM tris-HCl pH 8.0 (Sigma-Aldrich), and sterilised at 121°C for 15 min (Getinge autoclave GE150; Getinge Australia Pty. Ltd., Bulimba, QLD, Australia). The solidified agar was heated in a microwave oven (900 W) for 15 sec before use. The volume of agarose prepared was calculated as required (*ie.* 0.1 mL of agarose for each sample), and the final temperature was maintained in a water bath at 50°C for later use.

3.2.6.2 Preparation of block mould strips

Block mould strips (Pharmacia 'insert moulds' 80-1102-55 (12 wells per strip)) were washed in mild detergent and sanitised with 70% ethanol before use. Each block mould strip was sealed off at the back with masking tape and appropriately labeled. Two blocks were allocated per strain.

3.2.6.3 Cell culture preparation

Cell cultures were activated as described in section 3.2.1 and grown in MRS (for *L. delbrueckii* ssp. *bulgaricus*) or M17 (for *Streptococcus thermophilus*) broths with 1% inoculum and incubated for 18 h at 42°C and 37°C, respectively. A 1 mL aliquot of each broth was pipetted into a 1.8 mL Eppendorf tube separately. The tubes were centrifuged for 2 min at 10,000 × g (Eppendorf benchtop centrifuge model 5415C; Eppendorf Netheler-Hinz Gmbh, Hamburg, Germany). The supernatant was removed using a pipette and the cell pellet was

washed by suspending in 750 μL of NaCl/EDTA/tris solution followed by vortexing for sufficient time to obtain an uniform and homogenous cell suspension. The tubes were centrifuged for 1 min at 10,000 × g and the supernatant discarded. Finally, the cell pellet was suspended in 150 μL of the NaCl/EDTA/tris solution and warmed to 45°C.

3.2.6.4 DNA block preparation

To prepare agarose blocks for electrophoresis, a 100 μL volume of the pre-prepared molten LMT agarose (see section 3.6.2.1) was added to the cell suspension in each tube prepared in section 3.2.6.3 followed by mixing gently to achieve a homogenous solution. The cell-agarose suspension was then transferred to appropriate block moulds (see section 3.6.2.2) filled to the brim and allowed to set at room temperature (two moulds were prepared for each strain).

The masking tape was removed from the moulds once the agar was set. Agarose blocks were removed from the moulds with the aid of a sterile spatula and duplicate blocks placed into 1.8 mL Eppendorf tubes. Blocks were submerged in 0.5 mL (per tube) of lysis solution (100 mM EDTA (disodium salt) pH 8.0, 1 M NaCl, 10 mM tris-Cl pH 8.0, 1% lauroyl-sarcosine (Sigma-Aldrich), 10 mg/mL lysozyme (ICN Biochemicals, Aurora, Ohio, USA)) and incubated overnight at 37°C. The lysis solution was removed with a pipette and the blocks were submerged in 0.5 mL (per tube) of proteinase solution (100 mM EDTA (disodium salt) pH 8.0, 1% lauroyl-sarcosine, 1 mg/mL proteinase K (ICN Biochemicals) before incubating at 42°C for 4 d. Proteinase solution was removed with a pipette and the blocks were submerged in 0.5 mL (per tube) of storage solution (100 mM EDTA (disodium salt) pH 8.0, 1% lauroyl-sarcosine) before storing at 4°C. The blocks were used within two days.

Using a sterile razor blade, slices of approximately 1 mm in length were cut from the blocks and placed into separate 1.8 mL Eppendorf tubes. The slices were equilibrated in two steps. The first step involved two changes of 300 μL of T/0.1E (for 1 h each). The second step required two changes of 50 μL of the SURE/cut buffer A (1×) restriction enzyme buffer (45 min

each). The remaining equilibration solution was removed using a pipette and 50 µL of restriction enzyme buffer containing 10 U of *Sma* I enzyme was added to each tube (*Sma* I; 1000U, Roche Diagnostics). Digestion took 3 to 6 h at 30°C.

3.2.6.5 Preparation of gel for electrophoresis

A 1% (wt/vol) (*ie.* 1 mg/mL) gel was prepared by dissolving agarose powder in 0.5× TBE buffer (10× TBE - 108 g tris base (Sigma-Aldrich), 55 g boric acid (Sigma-Aldrich), 40 mL 0.5 M EDTA pH 8.0, 1 L H_2O) and heated in a microwave oven for 40 sec. The molten agarose was poured into the appropriate mould (15 cm^2) with the required comb in position and allowed to cool. The comb was removed when the gel was set.

To load the gel, each digested slice of LMT agarose (prepared in section 3.2.6.4) was cut to a size that would allow insertion into individual wells of the gel. Each slice was carefully inserted into a separate well using small spatulas. The marker gel (PFGE marker; Roche Diagnostics) was cut in a manner identical to the samples and placed in the two outside wells on either side of the gel. A third marker was placed into the middle well. All wells were covered with molten agarose in 0.5× TBE buffer using a pipette and allowed to set.

3.2.6.6 Electrophoresis

The gel was run in a Pharmacia LKB GN controller and tank at 290V (power supply Pharmacia Biotech EPS 3500). A four-phase procedure was used (total running time of 8 h): Phase 1: 1 sec, 6 h; Phase 2: 10 sec, 0 h; Phase 3: 10 sec, 2 h; Phase 4: 20 sec, 0 h. The temperature of the system was maintained at 8°C by externally circulating water with a cooler pump (Pharmacia LKB multiTemp II).

The gels were stained in 300 to 500 mL of 1 mM EDTA with 3 µL of 10 mg/mL ethidiumbromide (Sigma-Aldrich) for 1 h. Stained gels were viewed under UV (UV/White

Darkroom; UVP Laboratory Products, Upland, CA, USA) and analysed with Labworks software (V3.0.02.00, Media Cybernetics). PFGE was replicated four times.

3.2.7 Freeze Drying

3.2.7.1 Sample preparation

S. thermophilus 285 and *S. thermophilus* 1275 were grown in M17 broth as described in section 3.2.1. The active culture were centrifuged at 2714 × g (Sorvall RT7, Newtown, Conn., U.S.A.) at 4°C for 15 min. The cell pellet was collected and washed twice with sterile distilled water, re-suspended by vortexing in 50 mL of 0.1 M phosphate buffer (pH 6.8) (153 mL of 0.2 M monobasic stock (13.9 g sodium phosphate monobasic (Sigma-Aldrich) in 500 mL distilled water) and 147 mL of 0.2 M dibasic stock (53.65 g sodium phosphate dibasic heptahydrate (Sigma-Aldrich) in 500 mL distilled water)), and re-centrifuged at 2714 × g at 4°C for 15 min. The supernatant was discarded and 50 mL of 0.1 M phosphate buffer (pH 6.8) containing 2.0 % (wt/vol) of food grade cryoprotectant, UnipectinTM RS 150 (Savannah Bio Systems, Balwyn East, VIC, Australia) was added. The mixture was vortexed, and the homogenous suspension was poured into large petri dishes and frozen at −18°C for 48 h.

3.2.7.2 Freeze drying

Freeze-drying of the frozen mixture was carried out using a Dynavac FD300 freeze-dryer (Airvac Engineering Pty. Ltd., Rowville, Australia) at -88 °C for 40 h for primary freeze drying and 8 h for secondary freeze drying. After freeze-drying, the hygroscopic cultures were transferred into sterile tubes and stored at -18°C until used.

3.2.7.3 Sampling of freeze dried samples

Freeze dried strains of *S. thermophilus* 285 and *S. thermophilus* 1275 were suspended in 10 mL of 10% RSM (0.1 mg sample and incubated at 37°C) on the day following freeze-drying and after 90 d of storage at –20°C. The reactivated strains were examined for capsular EPS production by wet film India ink staining according to section 3.2.2.

3.2.8 Statistical Analysis

The amount of EPS synthesised by each strain of *S. thermophilus* was quantified in triplicate and each of the replicates was analysed twice. Results were presented as a mean ± standard error of replicates. To find significant differences between analyses, the means were analyzed using one-way analysis of variance (ANOVA) with a 95% confidence interval using Microsoft® Excel StatPro™ (Palaside Corporation, Newfield, NY). ANOVA data with a $P < 0.05$ was classified as statistically significant.

3.3 RESULTS AND DISCUSSION

Eleven strains of EPS producing *S. thermophilus*, two strains of *L. delbrueckii* ssp. *bulgaricus* and one strain of *Lactococcus lactis* ssp. *lactis* were identified. The strains were screened for their potential to be used as a starter culture in the manufacture of low fat Mozzarella cheeses. The capsular EPS producing *S. thermophilus* 285 and the mixed capsular/ropy EPS producing *S. thermophilus* 1275 were ultimately selected for cheese making trials.

3.3.1 Observation of Capsular EPS

Whey medium (used to grow all strains) and MRS or M17 broths (used to grow *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* strains, respectively) were initially used to cultivate bacteria from which cells were collected for capsular EPS examination by wet film India ink staining. No EPS capsules were, however, identified. RSM was then selectively used to grow all strains of LAB bacteria due to its nutritious composition. Of the eighty strains of cocci examined by wet film India ink staining after cultivation in RSM, twelve strains (representing 15% of the LAB examined) were identified to produce capsular EPS. Cocci strains 285, 1275, 820, 753, 1439, 287, 288, 371, 486, 751, 760 and 801 were capsular EPS producers identified by the distinctive clear halo surrounding each cell (Figure 3.3, A to H). Figure 3.2 depicts the absence of a surrounding capsular halo in the non-EPS producing strain 1303. Of the twenty strains of bacilli that were screened, strains 756 (Figure 3.4) and 840 (representing 10% of the LAB examined) were the only lactobacilli identified to produce capsular EPS. Capsular EPS production was evident when LAB were grown in the highly nutritious RSM media and EPS production was absent in the less nutritive media (*ie.* whey media, M17- and MRS- broth). This indicates that EPS synthesis is complex and the cells require complex nutrients for optimum production of EPS.

3.3.1.1 Observation of capsular EPS in freeze-dried cells

We examined the capsular EPS production of *S. thermophilus* MR-1C. This strain was reported to have the ability to produce capsular EPS by a number of authors (Low *et al.*, 1998; Perry *et al.*, 1997; Perry *et al.*, 1998; Petersen *et al.*, 2000). *S. thermophilus* MR-1C was activated in sterile RSM as were other LAB in section 3.3.1. The MR-1C strain was, however, used to propagate RSM in freeze dried form and examined for capsular EPS production. Capsular EPS was not identified. Because the cells of MR-1C were freeze dried, the freeze

drying procedure was identified as a possible cause for the impediment of capsular EPS production. The cocci strains 285 and 1275 were freeze dried and examined for EPS capsules immediately after freeze drying and after 90 d of storage at −20°C to test this hypothesis. Capsular EPS was identified at both time points for both strains. The EPS production of strains 285 (Figure 3.5, A and B) and 1275 was not affected by freeze drying. The destabilisation of EPS production by _S. thermophilus_ MR-1C was unlikely to be caused by freeze drying.

3.3.2 Carbohydrate Fermentation

The carbohydrate utilisation profiles of each strain were consistent with those existing in the ASCRC database (Table 3.1). The EPS producing cocci strains 285, 1275, 753, 1439, 287, 288, 371, 486, 751, 760, 801 and non-EPS 1303 were confirmed as _S. thermophilus_ with a certainty of 99.2%. Strain 820 (EPS) was, however, identified as _Lactococcus lactis_ ssp. _lactis_. The identification degree of certainty was lower than that of _S. thermophilus_ at 73.3%. The EPS producing bacilli strains 840 and 756 were positively identified as _L. delbrueckii_ ssp. _bulgaricus_ with 99.7% certainty.

3.3.3 Apparent Viscosity

Figure 3.6 shows the apparent viscosity of each strain of _S. thermophilus_ after 24 h of fermentation in 10% RSM. A one-minute adjustment time was allowed for each sample before measurements were recorded. After an equilibrium was reached, the majority of capsular EPS producing strains of LAB showed a similar viscosity which ranged from approximately 1000 to 1500 Cps. The non-EPS producing _S. thermophilus_ 1303 also had a viscosity that measured within this range, although its viscosity was one of the lowest. In general, capsular EPS did not increase the viscosity of fermented milk as compared to that fermented with non-EPS culture. _S._

thermophilus 753 was an exception, and exhibited the highest viscosity of any capsular EPS producing strain over the first minute of sampling before reducing to levels exhibited by other capsular strains. The strain 820, which was identified as _L. lactis_ ssp. _lactis_, had a greater viscosity than strains of _S. thermophilus_ of approximately 1700 Cps.

S. thermophilus 1275 showed the greatest viscosity and was believed to produce ropy EPS in addition to capsular EPS. The strain had an initial viscosity at over 2500 Cps before being reduced to approximately 2000 Cps. Because of the higher initial viscosity exhibited by _S. thermophilus_ 1275, the strain showed a reduction in viscosity over time. This was contrary to the lower milk viscosity created by the capsular EPS producing strains where the viscosity remained constant after the stabilisation stage.

Triplicate measurements of the milk viscosity showed similar, but not identical results. The relationship between viscosity and EPS synthesis may vary and has been reported to decrease due to the action of degrading enzymes (Cerning _et al._, 1988; Macura and Townsley, 1984). The amount of variation in milk viscosity was dependent on the bacterial strain.

3.3.4 Quantification of EPS

As indicated in section 3.3.1, capsular EPS were absent when cells were grown in either whey-based media and in M17 and MRS broths. Similarly, EPS quantification yielded no results when starter cultures were grown in the latter media (data not shown).

Table 3.2 shows the results of EPS quantification of selected strains of _S. thermophilus_ immediately after inoculation into RSM at 0 h and after 24 h of fermentation at 37°C. The lactic acid produced by the starter cultures increased acidity and the pH of the milk was reduced from 4.39 to 4.68. At 0 h, immediately after inoculation of the RSM with the starter bacteria, the EPS was quantified at levels between 5 and 10 mg/L. The EPS concentrations at this point were

similar ($P > 0.05$). EPS was detected before fermentation due to carry-over during inoculation and possibly due to small amounts of residual lactose.

As anticipated, non-EPS producing _S. thermophilus_ 1303 showed no increase in EPS concentration after 24 h of fermentation. After 24 h of fermentation, EPS concentrations in RSM increased slightly when fermented with _S. thermophilus_ 287, 288 and 486 to yield 10.97, 17.97 and 19.03 mg/L of EPS, respectively. Cerning (1990) stated that some strains of bacteria produce capsular EPS that is difficult to remove from the cell surface and the application of centrifugal forces alone is not sufficient. This may, in part be responsible for the subtle increases in EPS concentrations observed by the latter strains after 24 h of fermentation. _S. thermophilus_ strains 285, 1439 and 753 had an increase in the EPS concentrations to 83.46, 99.29 and 90.05 mg/L, respectively, after 24 h of fermentation. The synthesis of EPS was similar between the three strains ($P > 0.05$). _S. thermophilus_ 1275 produced the greatest amount of EPS at 360.30 mg/L after 24 h of fermentation ($P < 0.05$).

From the results of EPS quantification, viscosity measurements (section 3.3.3) and visual observation of the formation of 'ropy' strands when disturbing the RSM media, _S. thermophilus_ strain 1275 was identified as mixed capsular/ropy EPS producer.

3.3.5 Genetic Differentiation of _S. thermophilus_ Strains Using PFGE

Eleven strains of _S. thermophilus_ and a strain of _L. lactis_ ssp. _lactis_ were subjected to PFGE to compare each at a genetic level. The PFGE electrophoretic restriction patterns of the chromosomes of selected strains of _S. thermophilus_ are shown in Figure 3.7. PFGE allowed an accurate genetic differentiation between _S. thermophilus_ and revealed seven _Sma_ I fragment patterns from eleven strains studied. There was substantial genetic diversity among strains of _S. thermophilus_. A genetic similarity occurred in two groups. The first of these groups included strains 801, 287, 288, 371 (Figure 3.7, columns 5, 11, 12 and 13, respectively) and the second

group was made up of _S. thermophilus_ 753 and 751 (Figure 3.7, columns 7 and 8). The remaining strains of _S. thermophilus_ (1439, 1275, 760, 486, 285) showed genetic diversity to _L. lactis_ ssp. _lactis_ and between themselves.

3.4 CONCLUSIONS

Eleven strains of _S. thermophilus_ (801, 285, 371, 760, 1275, 751, 753, 1439, 287, 288 and 486) produced capsular EPS. _S. thermophilus_ 1275 was a mixed capsular/ropy EPS producer. Two strains of _L. delbrueckii_ ssp. _bulgaricus_ (756 and 840) a strain of _L. lactis_ ssp. _lactis_ showed the ability to synthesise capsular EPS. PFGE was used to differentiate between the EPS producing strains of _S. thermophilus_ on a genetic level and discriminated 7 different fragment patterns. Two genetically similar groups were identified. The first of the groups consisted of strains 287, 288, 371 and 801 and the second group consisted of strains 751 and 753. The remaining strains of EPS producing _S. thermophilus_ were genetically independent. Ropy EPS production by _S. thermophilus_ 1275 was greatest in milk at 360 mg/L after 24 h of fermentation. Capsular EPS in _S. thermophilus_ 753, 1439, 285 was measured at 90, 99 and 84 mg/L of EPS, respectively, while _S. thermophilus_ 287, 288 and 486 synthesised a more modest EPS at 11, 18 and 19 mg/L of EPS after 24 h. The capsular EPS producing _S. thermophilus_ 285 and the mixed ropy/capsular EPS producing _S. thermophilus_ 1275 were selected for the manufacture of low fat Mozzarella cheese making trials.

Table 3.1. Carbohydrate utilisation and strain identification using an API 50 CH kit.

Reference strain	Significant taxonomy (% Identification)	Significant taxonomy
288	99.2	*Streptococcus thermophilus*
801	99.2	*Streptococcus thermophilus*
285	99.2	*Streptococcus thermophilus*
371	99.2	*Streptococcus thermophilus*
287	99.2	*Streptococcus thermophilus*
486	99.2	*Streptococcus thermophilus*
760	99.2	*Streptococcus thermophilus*
751	99.2	*Streptococcus thermophilus*
1439	99.2	*Streptococcus thermophilus*
753	99.2	*Streptococcus thermophilus*
1275	99.2	*Streptococcus thermophilus*
1303 (non-EPS)	99.2	*Streptococcus thermophilus*
840	99.7	*Lactobacillus delbrueckii* ssp. *bulgaricus*
756	99.7	*Lactobacillus delbrueckii* ssp. *bulgaricus*
820	73.3	*Lactococcus lactis* ssp. *lactis*

Table 3.2 EPS extracted from 10% reconstituted skim milk at 0 h and 24 h of fermentation by strains of *S. thermophilus* at 37°C (n = 6 ± standard error).

S. thermophilus	[1]EPS 0 h (mg/L)	EPS 24 h (mg/L)	pH
1303	5.68 ± 1.07[a]	6.59 ± 2.22[h]	4.65
1275	10.97 ± 5.21[a]	360.30 ± 24.31[a]	4.40
753	5.95 ± 2.53[a]	90.05 ± 12.25[cd]	4.48
1439	6.27 ± 0.52[a]	99.29 ± 21.38[bcd]	4.68
285	9.49 ± 0.70[a]	83.46 ± 17.51[d]	4.56
287	5.26 ± 0.27[a]	10.97 ± 1.54[gh]	4.60
288	6.17 ± 2.78[a]	17.97 ± 2.04[f]	4.56
486	5.57 ± 2.10[a]	19.03 ± 3.82[efg]	4.39

[abc]One-way ANOVA of means in a column with different superscript are significantly different ($P < 0.05$).
[1]EPS = Exopolysaccharides produced by *S. thermophilus* strains.
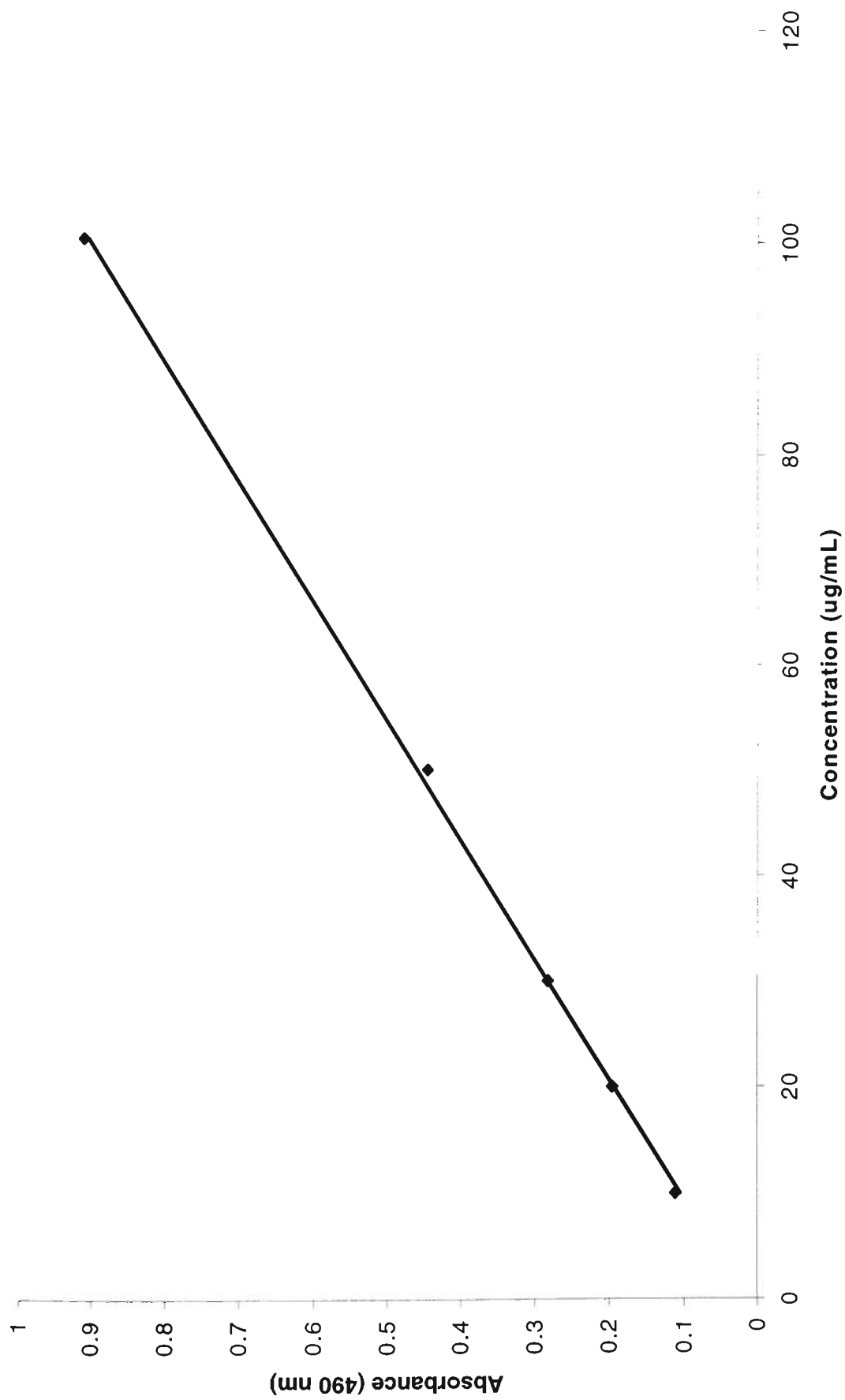
Figure 3.1. EPS standard curve containing 10 to 100 μL of sugar (glucose used as an equivalent to EPS).
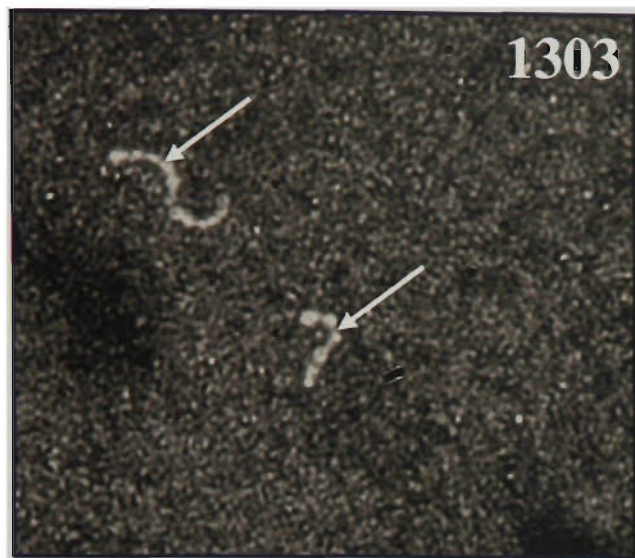
Figure 3.2. *Streptococcus thermophilus* 1303 (non-EPS) viewed at 1000 × magnification using light microscopy after wet film India ink staining.
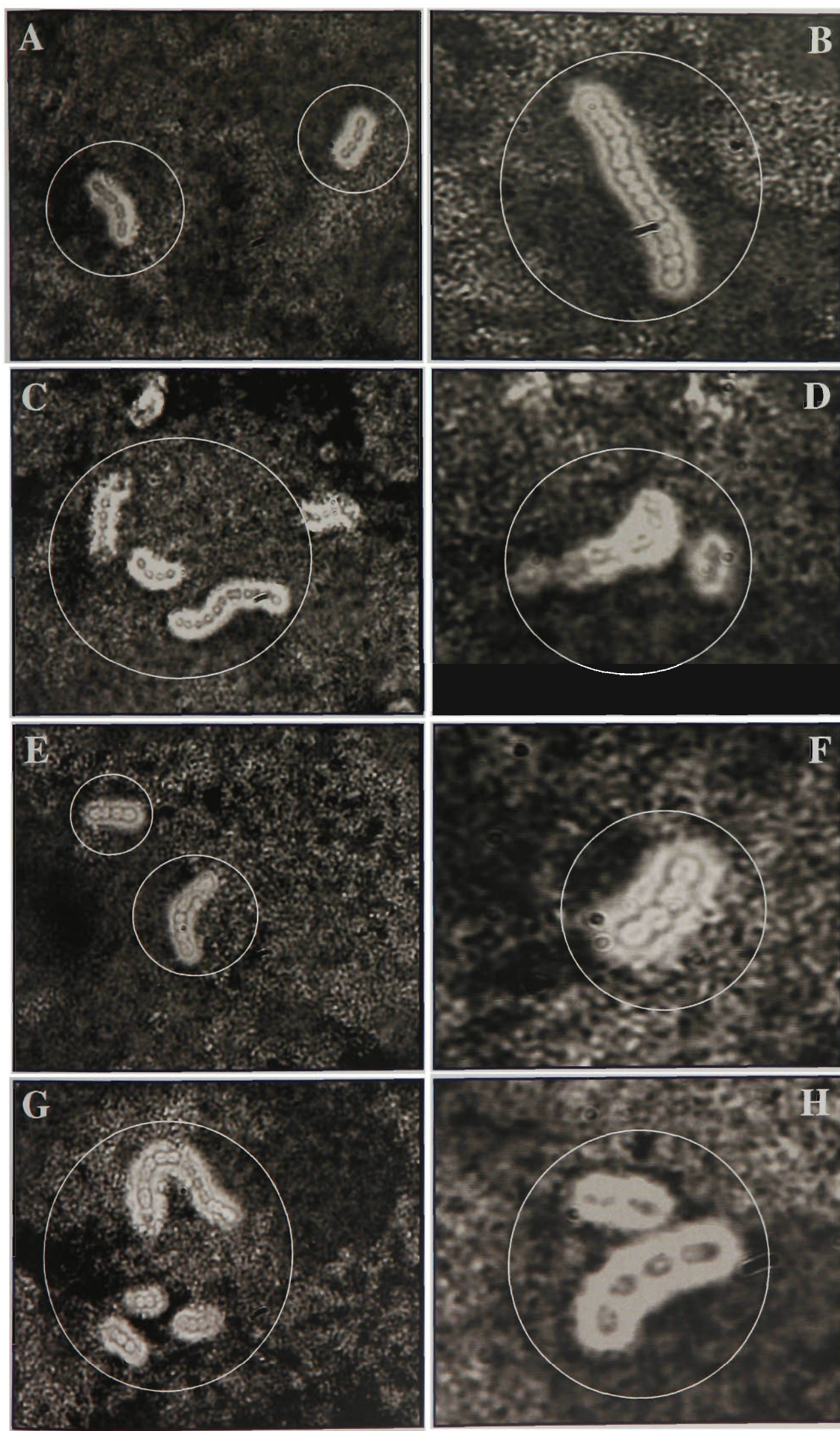
Figure 3.3. Capsular EPS identified as a clear halo surrounding the cells of *Streptococcus thermophilus* 1439 (A), 288 (B), 285 (C), 1275 (D), 288 (E), 753 (F), 287 (G), 486 (H) captured at 1000 × magnification using light microscopy and wet film India ink staining. Images D, F, H and image B were magnified 2 × and
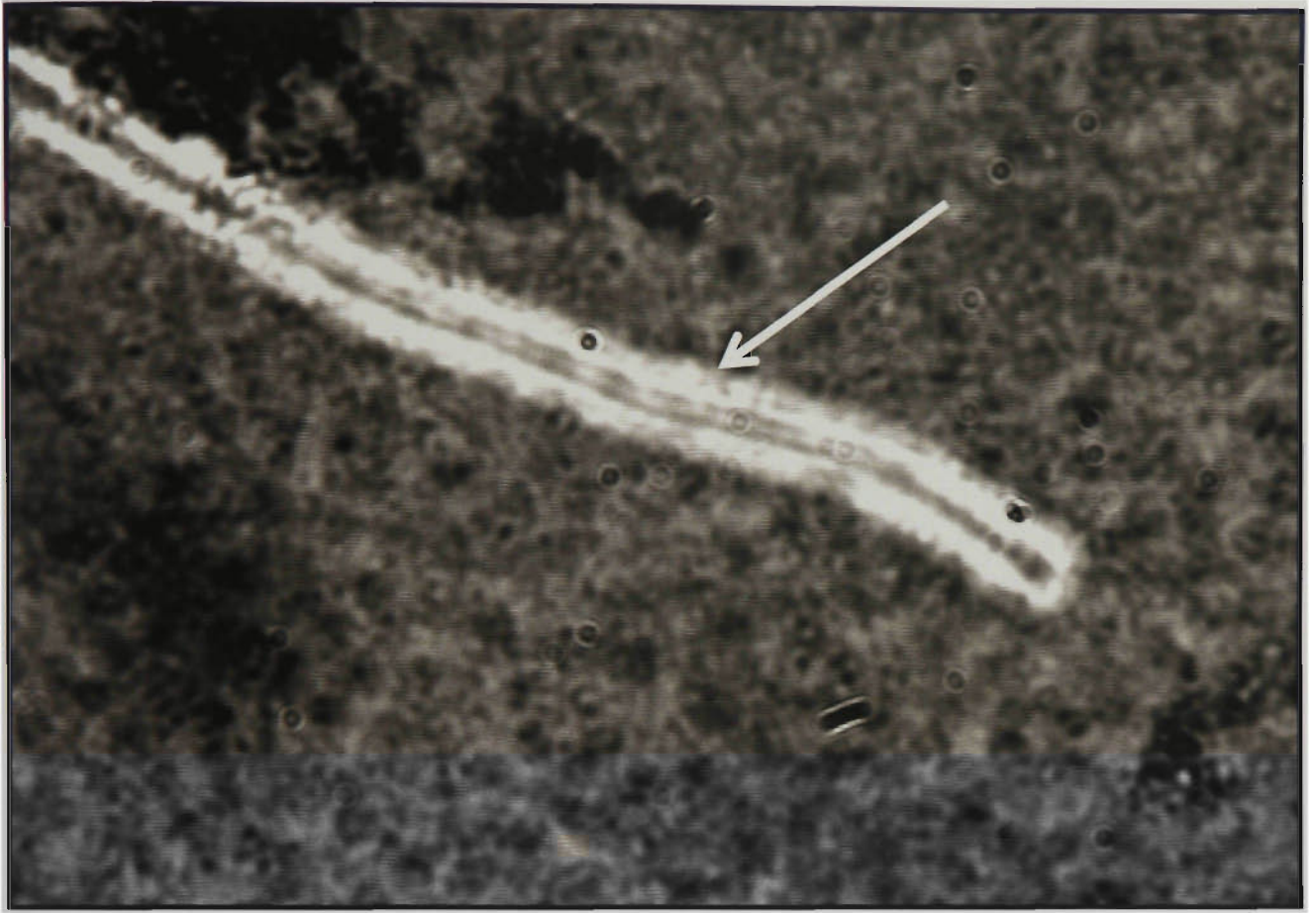
Figure 3.4. Capsular EPS identified as a clear halo surrounding the cells of _Lactobacillus delbrueckii_ ssp. _bulgaricus_ 756 viewed at 1000 × magnification using light microscopy after wet film India ink staining.
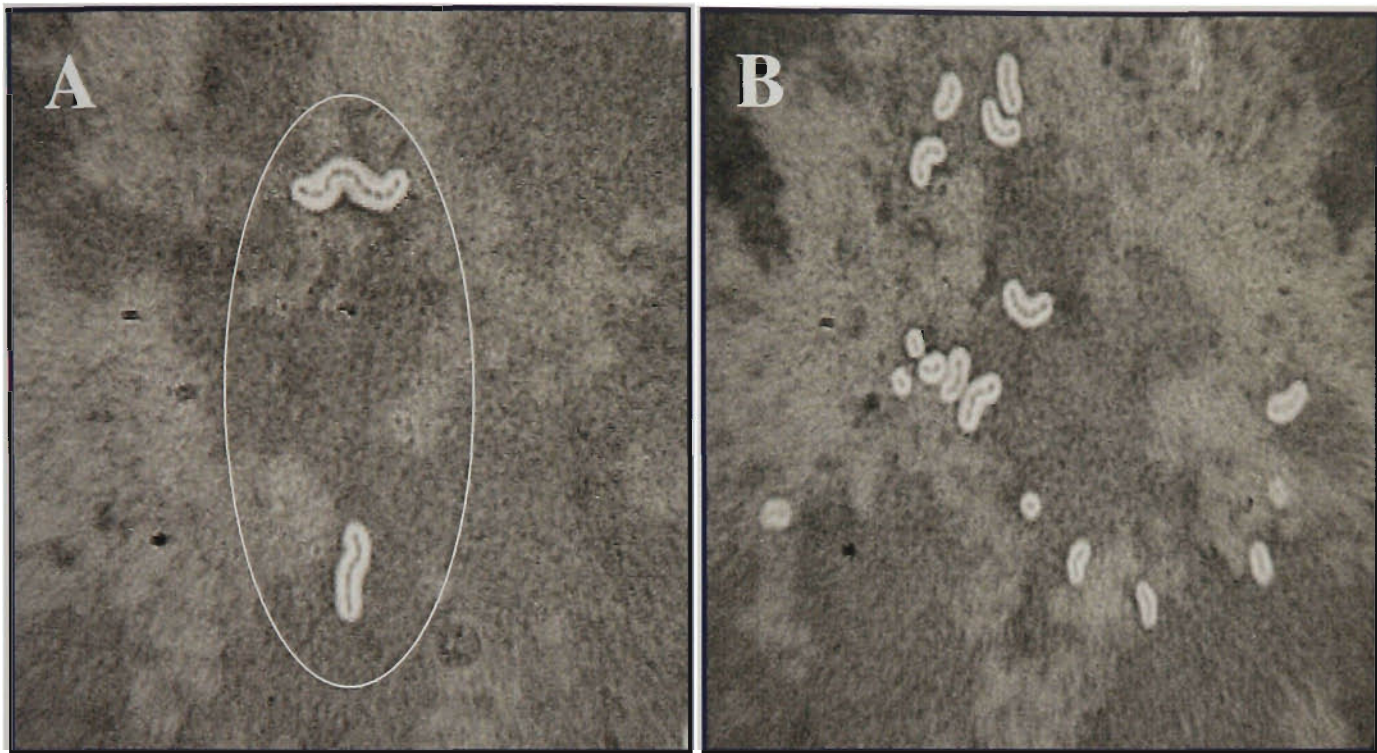
Figure 3.5. Capsular EPS identified as a clear halo surrounding the cells of _Streptococcus thermophilus_ 285 before freeze drying (A) and after freeze drying and storage for 90 d at 4°C (B) viewed at 100 × magnification using light microscopy and wet film India ink staining.
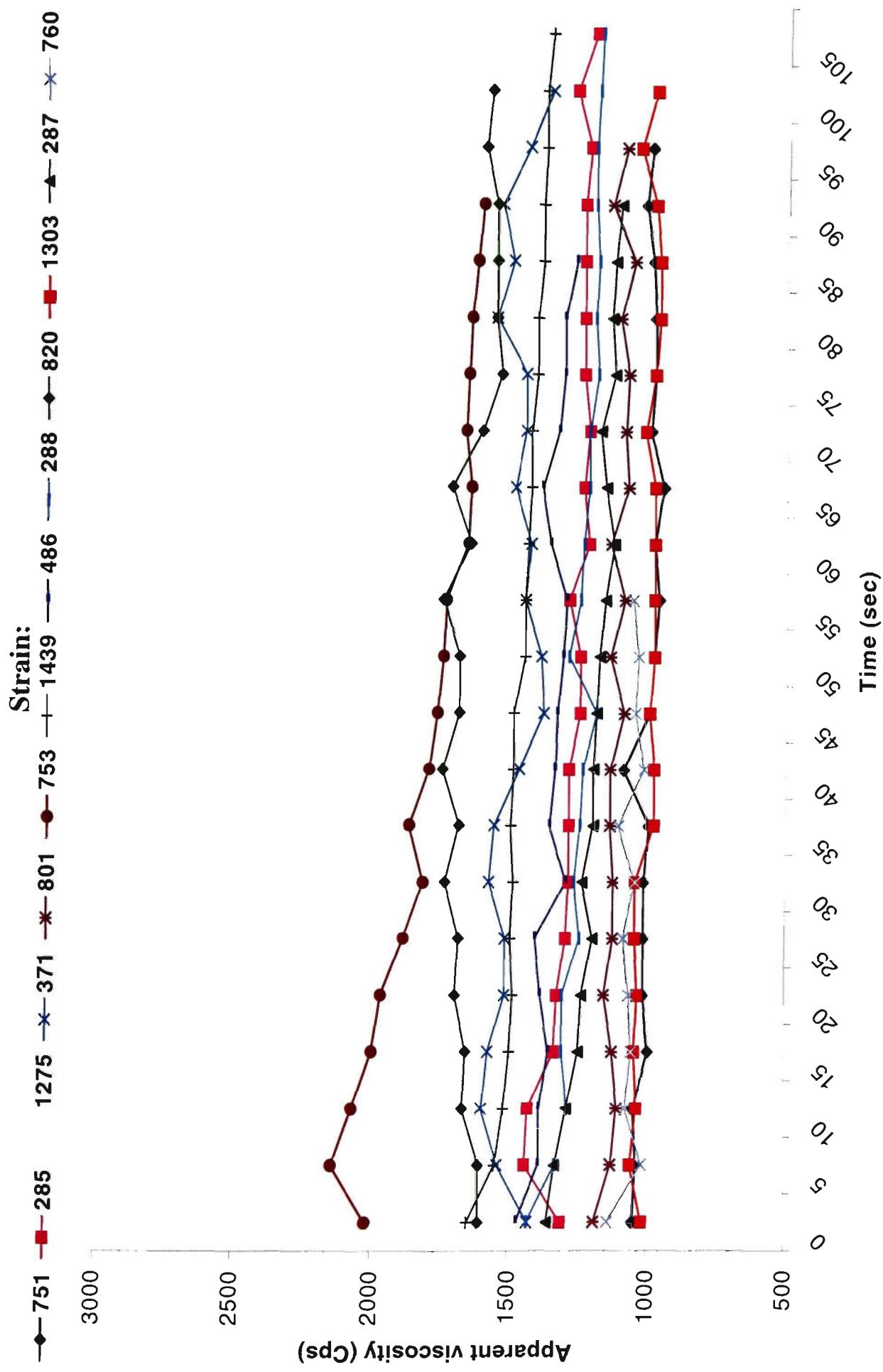
Figure 3.6. Apparent viscosity of selected EPS producing strains of *Streptococcus thermophilus* ($n = 3$).

66
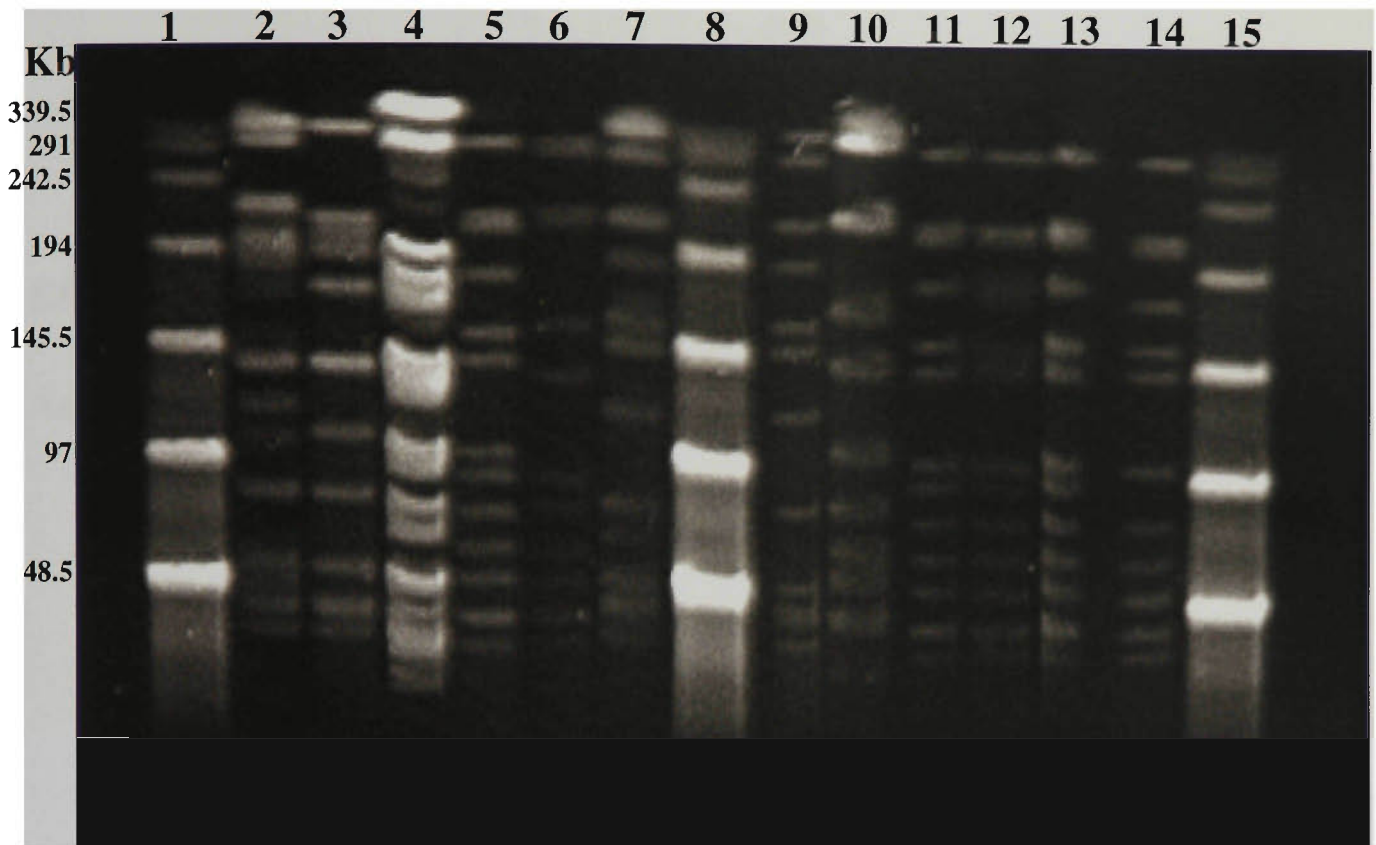
Figure 3.7. Pulsed field electrophoretic restriction patterns of 12 EPS producing strains of LAB. DNA digested with S*ma* I endonuclease enzyme. Lanes: 1. PFGE ladder; 2. *S. thermophilus* 1439; 3. *S. thermophilus* 1275; 4. *L. lactis* ssp. *lactis* 820; 5. *S. thermophilus* 801; 6. *S. thermophilus* 760; 7. *S. thermophilus* 753; 8. PFGE ladder; 9. *S. thermophilus* 751; 10. *S. thermophilus* 486; 11. *S. thermophilus* 371; 12. *S. thermophilus* 288; 13. *S. thermophilus* 287; 14. *S. thermophilus* 285; 15. PFGE ladder.

CHAPTER 4.0

Effects of pH, Temperature, Supplementation with WPC, and Adjunct Cultures on the Production of Exopolysaccharides by

Streptococcus thermophilus 1275

4.1 INTRODUCTION

Polysaccharides are used extensively by the food industry to thicken, gel, emulsify and stabilise suspensions. Most polysaccharides are of plant and algae origins with the exception of a few microbial derived exopolysaccharides (EPS), chiefly xanthan gum and gellan (Coultate, 1996).

Many food grade microorganisms have the ability to synthesise EPS and the potential to exploit such bacteria has recently been recognised. EPS producing lactic acid bacteria (LAB) are commonly used, particularly in the dairy industry for the manufacture of fermented milks. EPS plays a key role in the rheological behaviour, mouthfeel and texture of fermented products without the use of additives.

Microorganisms produce EPS in two distinct forms: ropy EPS or loose slime that is excreted into the surroundings, and capsular EPS that remain adhered to the cell surface creating a discrete covering (Broadbent *et al.*, 2003). Many capsular strains of bacteria have been shown to produce loose slime in addition to capsules (Chapter 3.0; Broadbent *et al.*, 2003; Cerning, 1990; Duguid, 1951). The term EPS is generally used to describe all forms of bacterial polysaccharides found outside the cell wall. The role of EPS is not clearly defined. Capsular EPS play a part in cell protection from the immediate environment when conditions are unfavourable (Cerning, 1990). Microbial EPS are extracellular, long-chained and high molecular mass polymers (branched, containing α- and β-linkages) that are either homopolysaccharide or heteropolysaccharide in nature (De Vuyst and Degeest, 1999). The carbohydrate composition of EPS is unique to different strains of bacteria and may vary depending on the growth conditions; however, glucose and galactose in particular are frequently detected in the EPS composition of many bacterial species (Ariga *et al.*, 1992; Bouzar *et al.*, 1996; Castern *et al.*, 1998; Cerning, 1990; De Vuyst and Degeest, 1999; Knoshaug *et al.*, 2000; Ricciardi *et al.*, 2002; Roberts *et al.*, 1995; Uemura *et al.*, 1998).

The amount and the composition of the EPS produced by thermophillic LAB are strongly influenced by culture and fermentation conditions and are growth associated. Production of EPS is dependent on the temperature and pH of the medium as well as composition of the medium in terms of carbon and nitrogen source, and mineral and vitamin contents (De Vuyst _et al._, 1998; Gamar-Nourani _et al._, 1998; Gorret _et al._, 2001; Grobben _et al._, 2000). The effect of temperature and pH on EPS production is highly variable and is dependent on the strain used and the experimental conditions. Some workers have found EPS production to be optimal at low temperatures (Cerning _et al._, 1992; Gamar _et al._, 1997; Kojic _et al._, 1992; Marshall _et al._, 1995; van den Berg _et al._, 1995), while others have shown EPS production to be favoured at much higher temperatures (De Vuyst _et al._, 1998; Grobben _et al._, 1995). The optimum pH for EPS production generally ranges between 5 and 7. De Vuyst _et al._ (1998) have shown that optimal EPS production and growth for _Streptococcus thermophilus_ LY03 were at 42°C and pH 6.2. The composition of the medium, nitrogen source (De Vuyst and Degeest, 1999; De Vuyst _et al._, 1998; Marshall _et al._, 1995) and carbon source supplementation (Degeest _et al._, 2001; Gamar _et al._, 1997) are shown to increase EPS production (Chapter 3.0). Partial replacement of skim milk powder (SMP) with WPC results in a higher buffering capacity as compared to that provided by SMP alone due to the contribution of whey proteins, salts such as citrates, phosphates and lactates (Kailasapathy _et al._, 1996).

No previous work has speculated on the possibility of culturing a non-EPS producing _S. thermophilus_ strain with a second EPS producing strain of _S. thermophilus_. By growing a non-EPS producing _Lactobacillus delbrueckii_ ssp. _bulgaricus_ strain in the presence of an EPS producing _S. thermophilus_ strain, it was however found that EPS production was stimulated. The level of EPS produced had increased to reach approximately 800 mg/L (Cerning _et al._, 1988; Cerning _et al._, 1990).

EPS degradation has been linked to the presence of depolymerizing enzymes (Cerning, 1990; Cerning _et al._, 1992; De Vuyst _et al._, 1998; Pham _et al._, 2000). Many studies have shown

the EPS content to decline with prolonged fermentation times (Cerning, 1990; Cerning *et al.*, 1992; De Vuyst *et al.*, 1998; Gorret *et al.*, 2001; Pham *et al.*, 2000). Pham *et al.* (2000) studied the possible relationship between cell extract enzymes of *Lactobacillus rhamnosus* R and EPS yield and found the presence of various glycohydrolases, including both α- and β-glucosidase and galactosidase, which were responsible for lowering polymer viscosity and liberating reducing sugars.

S. thermophilus produces β-D-galactosidase (Shah and Jelen, 1990), which breaks down the disaccharide lactose to its sugar monomers, glucose and galactose. *S. thermophilus* is homofermentative and produces lactic acid from glucose fermentation. Galactose is collected in the surrounding environment as these bacteria do not metabolise this sugar (Walstra and Jenness, 1984). The objective of this study was to examine the EPS production by *S. thermophilus* 1275 in skim milk medium under varying environmental conditions to understand the relationship between pH, growth temperature and supplementation with nutrients and non-EPS adjunct cultures in order to improve EPS synthesis and recognise factors which limit their production.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial Strains

The mixed capsular/ropy EPS producing *S. thermophilus* strain 1275 and non-EPS 1303 were used in this study. Working cultures were prepared according Chapter 3.0, section 3.2.1.

4.2.2 Fermentation Experiments and Culture Media

Skim milk powder was acquired from Murray Goulburn Co-Op. (Brunswick, Melbourne, Australia) and dissolved in distilled water to make RSM (10%, wt/vol). The RSM was used as

the fermentation medium. The RSM medium was sterilised separately at 121°C for 15 min and aseptically transferred into the sterile fermentation vessel. A 2 liter Biostat® B fermenter (B. Braun Biotech International, Melsungen, Germany) was used for growing _S. thermophilus_ 1275 in 10% RSM separately at various pH (4.5, 5.5 and 6.5) and temperatures (30, 37, 40 and 42°C) for 24 h. Samples were collected at 0 h and thereafter at 6 h intervals for quantification of EPS, cell count, and concentrations of lactose, glucose galactose and lactic acid. The fermenter together with the corrective solutions (50% (wt/vol) citric acid (Ajax Chemicals, Sydney, Australia) and 3 M NaOH) were sterilised _in situ_ at 121°C for 30 min. A 1% inoculum of 18 h grown culture was added to 1.8 liters of the medium. The initial pH of the medium was adjusted with 50% (wt/vol) citric acid and maintained at the necessary pH (4.5, 5.5 and 6.5) with automatic addition of 3 M NaOH required to neutralise lactic acid produced by the microorganisms. The fermenter was operated with agitation (100 rpm) to maintain homogeneity of the medium. The required temperatures (30, 37, 40 and 42°C) were maintained automatically.

4.2.3 Sampling

Three hundred milliliter samples were aseptically withdrawn every six hours and immediately cooled on ice before freezing at −20°C to determine the amount of EPS, and concentrations of lactic acid, lactose, glucose and galactose at a later date. A fresh sample was also taken for immediate enumeration of the bacterial population.

4.2.4 Enumeration of Bacterial Cells

A 1 mL aliquot of inoculated RSM was sampled and plated every 6 h using the pour plating technique and M17 agar containing lactose (Amyl Media Co., Dandenong, Melbourne, Australia). Solidified agar plates were incubated aerobically at 37°C for 48 h.

4.2.5 Effects of pH on Cell Growth and EPS Production

Effects of pH on EPS production were examined separately at pH 4.5, 5.5 and 6.5 at 37°C. The inoculation was carried out as previously mentioned in section 4.2.2. The desired pH was maintained automatically over 24 h using 3 M NaOH and 50% (wt/vol) citric acid. Samples were aseptically removed at 0 h and thereafter every 6 h. Additionally, the effect of pH on EPS production was examined by removing pH automation and allowing the pH to drop freely during fermentation for 24 h at 37°C.

4.2.6 Effects of Temperature on Cell Growth and EPS Production

Fermentations were conducted separately at 30°C, 37°C, 40°C and 42°C at pH 5.5. Optimum pH was found to be pH 5.5. Temperature and pH were maintained automatically as described earlier (section 4.2.2) over 24 h and samples were aseptically withdrawn at 0 h and thereafter every 6 h.

4.2.7 Effects of WPC on Cell Growth and EPS Production

Whey protein concentrate (WPC) 392 containing 80.4% protein (New Zealand Dairy Board, Wellington, New Zealand) was used as an additional nitrogen source. WPC was supplemented to RSM at 0.5% (wt/vol) prior to sterilisation. Preliminary studies showed that a concentration of 0.5% (wt/vol) was adequate for improved growth. Higher concentrations would not be cost-effective in food applications. The fermentation was conducted at pH 5.5 and 37°C, as this pH and temperature combination was found to be ideal. Furthermore, the effects of pH

and supplementation with WPC 392 on EPS production were also examined by removing the automation and allowing the pH to drop freely during fermentation for 24 h at 37°C.

4.2.8 Effects of Co-culturing with non-EPS *S. thermophilus* Adjunct Culture on Cell Growth and EPS Production

Effect of co-culturing with non-EPS producing *S. thermophilus* strain 1303 as an adjunct culture on the EPS production was studied. Two combinations of *S. thermophilus* 1275 and *S. thermophilus* 1303 (75:25 (0.75% and 0.25%) and 50:50 (0.50% and 0.50%)) were examined based on the initial medium volume. Bacterial cultures were grown separately at the conditions described earlier (section 4.2.2) and an appropriate quantity of each strain was transferred into the fermentation vessel. Fermentations were conducted for 24 h at pH 5.5 and 37°C.

4.2.9 Isolation and Quantification of Exopolysaccharides

A 100 mL aliquot of the frozen RSM sample was thawed overnight at 4°C. EPS was isolated and quantified from samples according to Chapter 3.0, section 3.2.9.

4.2.10 Determination of Lactic Acid, Lactose, Glucose and Galactose

High performance liquid chromatography (HPLC) was used to determine the concentrations of lactic acid, lactose, glucose and galactose. The HPLC consisted of a Varian 9100 autosampler, equipped with a Varian 9012 solvent delivery system, a Varian Star 9040 refractive index detector, and a SSII 505 LC column oven (Varian Inc., Palo Alto, CA, USA). Data was collected using Varian software (Varian Inc., Version 5.51).

4.2.10.1 Lactic acid

Seventy microliters of concentrated nitric acid and 5930 µL of 0.009 N H_2SO_4 were added to 5 mL of homogenous sample for protein digestion, from which a 1.6 mL aliquot was micro-centrifuged (Eppendorf 5415C at maximum speed for 10 min) and filtered through a 0.20 µm syringe filter (FP3/0.0.2 µm CA-S; Schleider and Schuell GmbH, Dassel, Germany). A working volume of 50 µL was injected into an Aminex HPX-87H column (Bio-Rad Laboratories) using 0.009 N H_2SO_4 as the mobile phase, held at 65°C using a flow rate of 0.6 mL/min. Lactic acid (Sigma) standard was prepared in 0.009 N H_2SO_4 at appropriate concentrations and run in triplicate at identical conditions. A standard curve for the determination of lactic acid is shown in Figure 4.1.

4.2.10.2 Lactose, glucose and galactose

Samples were prepared as with lactic acid (section 4.2.10.1), with the exception of replacing sulpuric acid with a mixture of acetonitrile/water (75:25) as the sample diluent. A working volume of 25 µL was injected into an Aminex amino column (Supelco, Australia) using acetonitrile/water (75:25) as the mobile phase, held at 35°C using a flow rate of 1.5 mL/min. Lactose was from BDH Laboratory supplies (Pode, England) and glucose and galactose were obtained from Ajax Chemicals (Sydney, Australia). Appropriate concentrations for each sugar were prepared as a standard using the acetonitrile/water (75:25) mixture and analysed in triplicate at identical conditions. A standard curve for the determination of lactose and galactose is shown in Figure 4.2, A and B. Typical chromatograms for the determination of lactic acid, lactose, glucose and galactose are shown in Figure 4.3, A and B.

4.2.11 Statistical Analysis

Each fermentation experiment was performed in duplicate. For EPS quantification and bacterial counts, samples were withdrawn in duplicate and each sample was analysed twice and results are presented as a mean ± standard error of 8 analyses. Samples for analysis of concentration of lactic acid, lactose, glucose and galactose were withdrawn in triplicate and each sample analysed twice and are presented as means of 12 values. Measurements of pH during fermentation and bacterial counts were taken in duplicate and are presented as means of 4 determinations. To find significant differences in EPS production and lactose, glucose, galactose and lactic acid concentrations, the means were analysed using one-way analysis of variance (ANOVA) with a 95% confidence interval using Microsoft® Excel StatPro™. ANOVA data with a $P < 0.05$ was classified as statistically significant.

4.3 RESULTS

4.3.1 Effect of pH of the Growth Medium on Bacterial Growth and EPS Production

Table 4.1 shows the influence of pH on EPS production, bacterial counts as well as the concentrations of lactic acid, lactose, glucose and galactose during fermentation of RSM with *S. thermophilus* 1275. *S. thermophilus* 1275 was grown in 10% (wt/vol) RSM at 37°C over 24 at a constant pH of 4.5, 5.5 or 6.5. There appeared to be a relationship between pH, EPS production and the bacterial growth. There was a significant difference in EPS production between pH 4.5, 5.5 and 6.5 after 24 h of fermentation ($P < 0.05$). EPS production ceased at pH 4.5 and this pH created unfavourable growth conditions for the bacterial population. At pH 5.5, EPS production was favoured by *S. thermophilus* 1275 yielding 458 mg/L in 24 h. The maximum amount of EPS was produced at 24 h, although the death phase followed after 12 h. The growth phase of

cells was maintained for a longer period of time before reaching the death phase. Other workers have found pH 6.2 as the optimum pH for _S. thermophilus_ (De Vuyst _et al._, 1998). On the contrary, pH 6.5 resulted in less EPS production than that at pH 5.5, yielding a maximum of 255 mg/L. At this pH, the cell growth was accelerated over the first 6 h of the exponential growth phase. The amount of EPS production declined after the first 12 h. The decline in the EPS content observed between 6 h (205 mg/L) and 12 h (158 mg/L) of fermentation may be due to enzymic degradation.

Hence, the pH of 5.5 was chosen for the rest of the experiments for examining other variables such as temperature.

The lactose content in the RSM was 4.9%. At pH 4.5, a small amount of lactose (3.4%) was utilised and consequently a low level of galactose accumulated. At pH 5.5, 82% of the lactose was utilised, whereas at pH 6.5, all the lactose was utilised after 18 h of fermentation. Highest level of lactic acid was produced at pH 5.5. At pH 6.5, there was an increase in lactose catabolism during the initial stages of the fermentation and the glucose moiety was possibly used in EPS production and consequently resulting in a small amount of lactic acid production with a continued accumulation of the galactose moiety. EPS biosynthesis from the catabolism of lactose by _S. thermophilus_ via a lactose and galactose antitransport system was described by De Vuyst _et al._ (1999). No glucose was detected in samples in our study, suggesting that this sugar was metabolised completely.

When the pH was allowed to drop freely due to acid production by the fermenting organism (Table 4.2), EPS production continued over the 24 h reaching a maximum of 406 mg/L. Cell numbers were lower than those observed when the pH was maintained at pH 5.5. It is interesting to note that higher amount of EPS was produced and more lactose was utilised at pH 4.08 when the pH was allowed to drop freely than at a constant pH 4.5. At pH 4.5, _S. thermophilus_ 1275 did not grow out of the lag phase, whereas with a free drop in pH starting

from pH 6.37, the *S. thermophilus* 1275 population was able to increase before reaching unfavourable pH levels.

4.3.2 Effect of Temperature on Growth and EPS Production

Table 4.3 shows the effects of temperature on the growth of *S. thermophilus* 1275 and EPS production. *S. thermophilus* 1275 was grown in RSM at 30, 37, 40 and 42°C, while the pH was kept constant at 5.5. As shown in the table, the optimum temperature for EPS production was 40°C, yielding 622 mg/L of EPS at 24 h of fermentation. De Vuyst *et al.* (1998) found 42°C as the optimum temperature for the growth and EPS production by *S. thermophilus*. However, mesophilic microorganisms showed greater EPS production at much lower temperatures (Cerning *et al.*, 1992; Gamar *et al.*, 1997; Kojic *et al.*, 1992; van den Berg *et al.*, 1995).

At 24 h, EPS production was significantly less at 30°C and 42°C than the amount of EPS quantified at 40°C ($P < 0.05$). At 30°C, EPS production was slow at 6 h and a maximum of 390 mg/L was produced at 24 h. Cell counts were also much lower at this temperature. Thus a temperature of 30°C appeared to be suboptimal for both cell growth and EPS production. EPS production was also lower at 42°C yielding a maximum of only 322 mg/L. The amount of lactose utilised was highest (86%) at 42°C as compared to 82% at 37°C and 63% at 40°C. Galactose accumulated over time. It is interesting to note that the highest amount of lactose was metabolised at 42°C, however, the maximum amount of EPS was produced at 40°C.

4.3.3 Influence of Supplementation with WPC on Cell Growth and EPS Production

Table 4.4 shows the growth of *S. thermophilus* 1275 and EPS production as affected by the addition of WPC 392 (0.5% wt/vol) at pH 5.5 and 37°C. EPS production increased uniformly over the 24 h of fermentation at a greater rate when supplemented with WPC as

compared to that with RSM. After 24 h of fermentation, the amount of EPS produced significantly increased ($P < 0.05$) from 458 mg/L (without WPC) to a remarkable amount of 1029 mg/L with WPC supplementation. Cell counts were lower with WPC supplementation than those with RSM, suggesting that the nutrients from WPC were used in the synthesis of EPS rather that for cell growth. A similar level of lactose consumption and galactose accumulation was evident between the two variables, however, lactic acid production was lower when RSM was supplemented with WPC. The addition of WPC when fermenting at pH 5.5 and 37°C (Table 4.2) resulted in producing 12.33 g/L of lactic acid by _S. thermophilus_ 1275, the amount significantly less ($P > 0.05$) than that resulted from fermentation of RSM in which 27.12 g/L of lactic acid was produced at 24 h. The rate of lactose hydrolysis and the formation of galactose were similar in both instances.

There was a rapid increase in the amount of EPS with uncontrolled pH in the first 12 h of fermentation, then the EPS production slowed as the pH dropped, reflecting the impact of acid on EPS production (Table 4.5). After 24 h, EPS production was less than half as compared to that produced at an optimal pH of 5.5. There was little difference in the amount of EPS produced after 24 h of fermentation upon supplementation with WPC (491 mg/L) or without WPC (406 mg/L). The final pH reached was higher (pH 4.21) and 2.24 g/L of lactic acid was produced with WPC addition as compared to pH of 4.08 and 3.09g/L of lactic acid with RSM (Table 4.2). This was possibly due to the buffering effects of WPC. Kailasapathy _et al._ (1996) have shown that protein and phosphates from the addition of WPC improved buffering capacity. There was less lactose hydrolysed in batches with uncontrolled pH, even with supplementation with WPC (Table 4.5), suggesting the role of acid in EPS production.

4.3.4 Effect of non-EPS _S. thermophilus_ Adjunct Culture on Growth and EPS Production

Effects of fermentation with a combination of EPS and non-EPS producing _S. thermophilus_ on production of EPS is shown in Table 4.6. Two combinations were examined, a 50% mixture of each strain, and a mixture consisting of 75% of _S. thermophilus_ 1275 and 25% of _S. thermophilus_ 1303. Fermentations were performed at pH 5.5 and 37°C. The presence of adjunct _S. thermophilus_ 1303 showed a significant increase ($P < 0.05$) in EPS production after 24 h. Cell counts were lower in batches containing mixtures of EPS producing _S. thermophilus_ and non-EPS _S. thermophilus_, however, the enumeration technique used was unable to ascertain the proportion of EPS and non-EPS strains. The 50% mixture of each strain showed the slowest response in EPS production after 6 h ($P > 0.05$) due to the reduced numbers of the EPS producing bacteria, however, after 12 h the cell numbers reached high levels producing 676 mg/L of EPS after 24 h. A 75% EPS strain and 25% non-EPS _S. thermophilus_ mixture showed the highest quantity of EPS production of 832 mg/L at 24 h ($P < 0.05$). Unlike the 50% combination, the 75% EPS mixture showed rapid EPS production in the initial stages of the fermentation. Reducing the numbers of EPS bacteria had adverse effects on EPS production in the initial stages as well as on the ultimate amount of EPS produced. Overall, it appears that adjunct non-EPS starter cultures have the potential to increase production of EPS and may be utilised as a cheap alternative to nutrient supplementation.

4.4 DISCUSSION

Excretion of microbially derived exopolysaccharides varies greatly between different strains of LAB and also within species. Cerning _et al._ (1990) reported EPS production of 337 mg/L by a strain of _S. thermophilus_, while De Vust _et al._ (1998) showed EPS production of 546 mg/L by another strain of _S. thermophilus_. In our study, _S. thermophilus_ 1275 was found to

produce 406 mg/L of EPS without pH control and 458 mg/L when an optimum pH 5.5 was maintained at 37°C.

Both the ropy and capsular constituents of EPS produced by _S. thermophilus_ 1275 were quantified as a whole and reported as the total concentration of EPS. Capsular EPS was removed from the cell surface after precipitation of proteins with TCA and collected in the supernatant along with the ropy EPS using high-speed centrifugation. Although both types of EPS were produced by the strain being investigated, it was the ropy form that had greatest impact on the amount of EPS produced as it was directly secreted into the medium where it accumulated. Our previous work with both capsular and ropy strains of _S. thermophilus_ yielded largest amounts of ropy EPS that coincided with altered physical properties of the medium, while only moderate EPS increases were detected with capsular strains without obvious changes in physical properties of the medium (Chapter 3.0, section 3.3.4). Since the capsular component remains attached to the cell surface, this form of EPS is likely to alter slightly with time and is proportionate to the number of bacterial cells present. It is therefore expected that any increase of capsular EPS would occur in the early stages of fermentation as the number of bacteria increase in the exponential growth phase.

The composition of the medium is vital to the production of EPS; it affects the amount of EPS produced from individual strains. In a separate study, M17 medium used to specifically culture _S. thermophilus_, although supported cell growth, failed to sustain EPS production (Chapter 3.0, section 3.3.4). Similar findings have been reported by De vuyst _et al._ (1998). A favourable carbon/nitrogen ratio complemented by vitamins and minerals such as that found in milk media, when further supplemented with WPC resulted in an enhanced EPS production, reaching 1029 mg/L after 24 h when a desired pH 5.5 was maintained, representing one of the highest yields ever achieved by a _S. thermophilus_ strain. It was also shown that without pH control, the yield of EPS was much lower, hence high yields of EPS under practical applications such as that used in the fermentation of food may not be achieved.

EPS production is influenced by pH, however, the optimum pH may vary. Most researchers have shown the optimum pH for EPS synthesis to be around pH 6. In our study, a lower pH of 5.5 resulted in an increase in EPS production by increasing the time the culture remains in the exponential growth phase as well as in the stationary phase.

EPS production was shown to be mainly growth associated, hence the greatest rate of EPS production occurred under conditions optimal for growth (temperature and pH). EPS biosynthesis begun simultaneously with cell growth and was greatest over the first 6 to 12 h of fermentation during the exponential growth phase. This trend indicated primary metabolite kinetics, however, there was no significant statistical difference in EPS production between 18 h and 24 h of fermentation within variables with the exception of the co-cultured (75:25) (Table 4.6) and 30°C (Table 4.3) variables. Many workers have published similar findings (Kojic _et al._, 1992; Manca de Nadra _et al._, 1985). As the cell population entered the stationary phase the production of EPS slowed, however, there was an increase in EPS production over the entire 24 h, even when the cells had reached the death phase. This trend in EPS production was evident when conditions were favourable such as fermentation at pH 5.5 and 40°C, and with the addition of WPC while pH 5.5 was maintained. Similar fermentation patterns were demonstrated by De Vuyst _et al._ (1998) with _S. thermophilus_ LY03. It was suggested that slowing of EPS production over time occurred due to a possible enzymic degradation of EPS in the medium (De Vuyst _et al._, 1998; Degeest _et al._, 2001).

It has been shown that enhanced EPS production is achieved for other strains including _S. thermophilus_ when increasing the nitrogen availability in the medium, generally by means of yeast extract and/or peptone addition (De Vuyst _et al._, 1998; Gorret _et al._, 2001). In our experiment, supplementation with WPC 392, provided an additional nitrogen source by providing peptides and amino acids made readily available for cell metabolism throughout the 24 h experiment and showed a gradual increase in EPS production. It was shown that the availability of a nitrogen source was more important for increasing the total yield of EPS

produced as _S. thermophilus_ has the capability of catabolising larger proteins. The simpler peptides and amino acid constituents of WPC that are responsible for increased EPS synthesis and are indirectly responsible to sustain catabolism of lactose to glucose and galactose and EPS biosynthesis as depicted by De Vuyst and Degeest (1999). Glucose resulting from lactose hydrolysis is used in the structuring of the backbone of the EPS molecules. After 24 h, there was a 55% increase in EPS production when supplementing with WPC. On the contrary, in pure RSM with the gradual depletion of these vital nutrients, the rate of EPS synthesis slowed after 6 h of fermenting.

Limited information can be found in literature relating to EPS production as influenced by the presence of adjunct, non-EPS producing LAB. EPS produced by _S. thermophilus_ increase when grown in the presence of _L. delbrueckii_ ssp. _bulgaricus_ (Cerning _et al._, 1988; Cerning _et al._, 1990). _L. delbrueckii_ spp. _bulgaricus_, when used to ferment foods, has an optimum working pH range lower than that for _S. thermophilus_. In our study, we used a bacterial species that was most metabolically active at a similar pH range as the EPS producer. The objective was to find if supplementation with non-EPS _S. thermophilus_ could further stimulate the synthesis of EPS by functioning optimally in the same pH range, thus depicting the early stages of bacterial fermentation in foods such as the production of yogurt before the pH drops to levels favoring other bacterial species such as the lactobacilli. When _S. thermophilus_ 1275 was grown in the presence of a non-EPS producing _S. thermophilus_ 1303 adjunct culture, the amount of EPS increased to 832 mg/L at 24 h. The increase in the synthesis of EPS due to the addition of _S. thermophilus_ 1303 may be an indication of a complementary relationship that existed between the two strains of _S. thermophilus_. Both combinations of co-culturing showed a sustained increase in EPS production over 24 h of fermentation to closely resemble the pattern observed while supplementing with WPC, unlike that of the pure strain where the rate of EPS synthesis slowed after 6 h as a result of essential nutrient depletion. This suggests that there was a gradual release of peptides and amino acids that were readily available to the bacterial cells, similar to

that observed with the symbiotic relationship that exists between *S. thermophilus* and *L. delbrueckii* spp. *bulgaricus* in yogurt preparation that has been found to improve the growth of the bacterial population (Adams and Moss, 1997). Furthermore, the same circumstances were also shown to increase growth of probiotic bacteria (Dave and Shah, 1998). In our experiments, *S. thermophilus* 1303 not only provided essential nutrients to sustain cell metabolism, but also sustained EPS synthesis. However, the beneficial effects of co-culturing on EPS production can only be achieved to such a magnitude if the strain is a ropy EPS producer. As previously described, the amount of capsular EPS synthesised is related to the number of bacterial cells present as the capsules remain cell bound. EPS concentrations will therefore be lower when co-culturing with capsule producing strains, if these bacterial cells lack the ability to release ropy EPS due to the lower initial inoculum concentrations. After 24 h of fermentation of *S. thermophilus* 1275 in conjunction with *S. thermophilus* 1303, there was a 45% increase in the EPS production with the 75% EPS co-culture combination and a 32% increase with the 50% EPS co-culture combination.

The fermentation pattern of *S. thermophilus* 1275 is homofermentative. The gradual decrease in the concentration of lactose in the fermentation medium as observed in Tables 4.1 to 4.6 was due to the activity of β-D-galactosidase produced by the fermenting bacteria, where lactose is hydrolysed to glucose and galactose. Glucose not used in EPS biosynthesis was completely converted to lactic acid by glycolytic degradation and galactose was accumulated into the medium. *S. thermophilus* lacks the ability to metabolise galactose (De Vuyst and Degeest, 1999). Lactose was completely degraded at pH 6.5, which corresponded to high lactic acid production and rapid growth. At pH 4.5 (Table 4.1), there was little metabolic activity and comparatively a small amount of lactose was converted to lactic acid.

4.5 CONCLUSIONS

EPS production by *S. thermophilus* 1275 was shown to be growth associated. The optimum temperature for the production of EPS by *S. thermophilus* 1275 was 40°C and pH 5.5. Fermentation under these conditions yielded 458 mg/L of EPS. EPS production, cell growth, metabolism of lactose and lactic acid production by *S. thermophilus* 1275 were influenced by the pH of the medium, temperature of incubation, supplementation with WPC and non-EPS producing adjunct *S. thermophilus* culture. Addition of WPC and adjunct starter cultures showed improved production of EPS. Supplementation with WPC at optimum temperature and pH yielded 1029 mg/L of EPS, whereas co-culturing with non-EPS *S. thermophilus* at optimum temperature and pH yielded 832 mg/L of EPS. The use of non-EPS producing adjunct *S. thermophilus* could be used as a cheaper alternative to supplementation with WPC as a means of increasing the production of EPS.

Table 4.1. Bacterial counts, amount of EPS[1], and concentrations of lactic acid, lactose, glucose and galactose produced during fermentation of RSM[2] by *Streptococcus thermophilus* 1275 at 0, 6, 12, 18 and 24 h at various pH at 37°C (mean ± standard error).

pH	Fermentation time (h)	EPS (mg/L; n = 8)	Bacterial counts ($\log_{10}$ cfu/ml; n = 4)	Lactic acid (g/L; n = 12)	Lactose (g/L; n = 12)	Glucose (n = 12)	Galactose (g/L; n = 12)
4.5	0	15 ± 0.5^{lmno}	5.91 ± 0.03	[3]ND^{lmno}	49.05 ± 0.60^{a}	ND	ND^{klm}
	6	17 ± 1.4^{ijklmno}	5.98 ± 0.04	ND^{mno}	48.48 ± 0.60^{a}	ND	0.55 ± 0.02^{jklm}
	12	19 ± 2.6^{hijklmno}	6.23 ± 0.01	0.54 ± 0.01^{k}	49.03 ± 0.46^{a}	ND	0.93 ± 0.01^{ijklm}
	18	16 ± 0.6^{jklmno}	6.04 ± 0.03	0.64 ± 0.04^{jk}	48.37 ± 0.56^{a}	ND	1.19 ± 0.06^{hijklm}
	24	15 ± 2.0^{klmn}	5.89 ± 0.05	0.73 ± 0.05^{ij}	47.36 ± 0.38^{a}	ND	1.82 ± 0.06^{ghijklm}
5.5	0	13 ± 1.0^{n}	6.00 ± 0.02	ND^{no}	47.70 ± 1.39^{a}	ND	ND^{lm}
	6	327 ± 7.4^{c}	8.39 ± 0.09	8.40 ± 0.09^{d}	36.19 ± 0.43^{b}	ND	7.67 ± 0.08^{f}
	12	342 ± 15.0^{bc}	8.61 ± 0.01	19.37 ± 0.03^{c}	20.32 ± 0.26^{c}	ND	14.33 ± 0.11^{e}
	18	390 ± 8.4^{ab}	8.56 ± 0.09	24.62 ± 0.14^{b}	11.84 ± 0.14^{d}	ND	15.94 ± 0.33^{de}
	24	458 ± 7.1^{a}	8.44 ± 0.00	27.12 ± 0.22^{a}	8.50 ± 0.08^{f}	ND	17.63 ± 0.26^{bcd}
6.5	0	15 ± 0.0^{mn}	6.09 ± 0.01	ND^{o}	48.46 ± 0.39^{a}	ND	ND^{m}
	6	205 ± 25.0^{fg}	8.42 ± 0.01	2.14 ± 0.01^{h}	9.03 ± 0.06^{e}	ND	16.48 ± 0.12^{cd}
	12	158 ± 9.5^{g}	8.19 ± 0.02	2.80 ± 0.01^{g}	0.07 ± 0.02^{ghi}	ND	19.06 ± 0.26^{ab}
	18	227 ± 15.0^{e}	8.11 ± 0.03	3.02 ± 0.02^{c}	0.00 ± 0.00^{hi}	ND	20.37 ± 0.09^{a}
	24	255 ± 5.3^{de}	8.00 ± 0.05	2.84 ± 0.01^{fg}	0.00 ± 0.00^{i}	ND	20.68 ± 0.21^{a}

[a,b,c]One-way ANOVA of means in the same column with different superscript are significantly different ($P < 0.05$).

[1]EPS = Exopolysaccharides.

[2]RSM = Reconstituted skim milk.

[3]ND = Not detected.

Table 4.2. Bacterial counts, amount of EPS[1], and concentrations of lactic acid, lactose, glucose and galactose produced during fermentation of RSM[2] at 0, 6, 12, 18 and 24 h by *Streptococcus thermophilus* 1275 at 37°C (mean ± standard error).

Fermentation time (h)	pH (n = 4)	EPS (mg/L; n = 8)	Bacterial counts ($\log_{10}$ cfu/ml; n = 4)	Lactic acid (g/L; n = 12)	Lactose (g/L; n = 12)	Glucose (n = 12)	Galactose (g/L; n = 12)
RSM							
0	6.37 ± 0.01	12 ± 1.5^d	6.02 ± 0.04	$^3ND^e$	51.50 ± 0.40^a	ND	ND^d
6	4.97 ± 0.04	171 ± 16.0^c	8.01 ± 0.02	1.55 ± 0.01^d	42.05 ± 0.46^{bcd}	ND	6.36 ± 0.11^c
12	4.37 ± 0.04	201 ± 20.0^b	7.99 ± 0.02	2.33 ± 0.00^c	41.07 ± 0.69^{cd}	ND	8.30 ± 0.14^b
18	4.17 ± 0.01	342 ± 29.5^a	7.97 ± 0.01	2.82 ± 0.01^b	38.96 ± 0.57^d	ND	9.51 ± 0.30^{ab}
24	4.08 ± 0.005	406 ± 13.3^a	8.06 ± 0.03	3.09 ± 0.02^e	35.35 ± 0.42^e	ND	9.61 ± 0.09^a

[abc]One-way ANOVA of means in the same column with different superscript are significantly different ($P < 0.05$).

[1]EPS = Exopolysaccharides.

[2]RSM = Reconstituted skim milk.

[3]ND = Not detected.

Table 4.3. Bacterial counts, amount of EPS[1], and concentrations of lactic acid, lactose, glucose and galactose produced during fermentation of RSM[2] by *Streptococcus thermophilus* 1275 at 0, 6, 12, 18 and 24 h at pH 5.5 and various temperatures (mean ± standard error).

Temperature	Fermentation time	EPS	Bacterial counts	Lactic acid	Lactose	Glucose	Galactose
(^{o}C)	(h)	(mg/L; n = 8)	($\log_{10}$ cfu/ml; n = 4)	(g/L; n = 12)	(g/L; n = 12)	(n = 12)	(g/L; n = 12)
30	0	18 ± 0.5^{pq}	5.81 ± 0.00	[3]ND^{opqr}	48.34 ± 0.54^{ab}	ND	ND^{opqr}
	6	46 ± 4.5^{o}	7.21 ± 0.02	1.36 ± 0.01^{n}	45.53 ± 0.30^{b}	ND	2.73 ± 0.01^{n}
	12	204 ± 5.7^{mn}	7.94 ± 0.01	4.93 ± 0.04^{m}	38.06 ± 0.66^{de}	ND	6.45 ± 0.20^{l}
	18	302 ± 10.5^{jkl}	7.99 ± 0.03	7.57 ± 0.24^{k}	27.74 ± 0.58^{ghij}	ND	8.55 ± 0.22^{jk}
	24	390 ± 13.2^{efg}	7.93 ± 0.01	9.78 ± 0.09^{i}	26.55 ± 0.38^{hij}	ND	10.53 ± 0.25^{hi}
37	0	13 ± 1.0^{r}	6.00 ± 0.02	ND^{p}	47.70 ± 1.39^{ab}	ND	ND^{pqr}
	6	327 ± 7.4^{hijk}	8.39 ± 0.09	8.40 ± 0.09^{j}	36.19 ± 0.43^{e}	ND	7.67 ± 0.08^{jk}
	12	342 ± 15.0^{ghij}	8.61 ± 0.01	19.37 ± 0.03^{f}	20.32 ± 0.26^{kl}	ND	14.33 ± 0.11^{d}
	18	390 ± 8.4^{fg}	8.56 ± 0.09	24.62 ± 0.14^{d}	11.84 ± 0.14^{no}	ND	15.94 ± 0.33^{bcd}
	24	458 ± 7.1^{cdef}	8.44 ± 0.00	27.12 ± 0.22^{ab}	8.50 ± 0.08^{p}	ND	17.63 ± 0.26^{d}
40	0	9 ± 0.0^{s}	5.94 ± 0.00	ND^{q}	51.64 ± 0.03^{a}	ND	ND^{qr}
	6	394 ± 14^{defghi}	8.42 ± 0.01	5.65 ± 0.02^{l}	39.58 ± 0.08^{cd}	ND	4.77 ± 0.00^{m}
	12	499 ± 11^{bc}	8.39 ± 0.01	10.82 ± 0.03^{h}	29.85 ± 0.17^{fg}	ND	7.31 ± 0.17^{kl}
	18	584 ± 10^{a}	8.29 ± 0.10	14.49 ± 0.03^{no}	24.25 ± 0.13^{j}	ND	11.65 ± 0.20^{f}
	24	622 ± 11.5^{a}	8.17 ± 0.01	18.58 ± 0.07^{g}	18.92 ± 0.15^{lm}	ND	12.24 ± 0.12^{ef}
42	0	16 ± 1.5^{q}	6.03 ± 0.01	ND^{r}	50.60 ± 0.09^{a}	ND	ND^{r}
	6	199 ± 9.0^{n}	8.53 ± 0.01	14.17 ± 0.03^{g}	24.73 ± 0.02^{ij}	ND	10.80 ± 0.06^{gh}
	12	270 ± 20.5^{lm}	8.63 ± 0.01	21.68 ± 0.09^{e}	18.91 ± 0.01^{m}	ND	14.68 ± 0.04^{cd}
	18	290 ± 12.5^{kl}	8.69 ± 0.06	24.70 ± 0.16^{cd}	11.01 ± 0.02^{o}	ND	16.43 ± 0.06^{ab}
	24	322 ± 27.0^{ijkl}	8.44 ± 0.02	26.68 ± 0.16^{b}	7.09 ± 0.00^{q}	ND	17.27 ± 0.08^{a}

[abc] One-way ANOVA means in the same column with different superscript are significantly different ($P < 0.05$).

[1] EPS = Exopolysaccharides.

[2] RSM = Reconstituted skim milk.

[3] ND = Not detected.

Table 4.4. Bacterial counts, amount of EPS[1], and concentrations of lactic acid, lactose, glucose and galactose produced by *Streptococcus thermophilus* 1275 in RSM[2] supplemented with WPC[3] 392 at 0, 6, 12, 18 and 24 h at pH 5.5 and 37°C (mean ± standard error).

Growth media	Fermentation time (h)	EPS (mg/L; n = 8)	Bacterial counts (log_{10} cfu/ml; n=4)	Lactic acid (g/L; n = 12)	Lactose (g/L; n = 12)	Glucose (n = 12)	Galactose (g/L; n = 12)
RSM	0	13 ± 1.0[i]	6.00 ± 0.02	[4]ND[ij]	47.70 ± 1.39[a]	ND	ND[gh]
	6	327 ± 7.4[g]	8.39 ± 0.09	8.40 ± 0.09[g]	36.19 ± 0.43[b]	ND	7.67 ± 0.08[f]
	12	342 ± 15.0[fg]	8.61 ± 0.01	19.37 ± 0.03[c]	20.32 ± 0.26[de]	ND	14.33 ± 0.11[d]
	18	390 ± 8.4[ef]	8.56 ± 0.09	24.62 ± 0.14[b]	11.84 ± 0.14[g]	ND	15.94 ± 0.33[cd]
	24	458 ± 7.1[de]	8.44 ± 0.00	27.12 ± 0.22[a]	8.50 ± 0.08[hi]	ND	17.63 ± 0.26[abc]
RSM + 0.5% WPC 392	0	15 ± 2.0[hi]	5.79 ± 0.05	ND[j]	49.10 ± 0.43[a]	ND	ND[h]
	6	489 ± 29.0[cde]	8.07 ± 0.01	4.44 ± 0.11[h]	33.59 ± 0.29[c]	ND	11.39 ± 0.28[e]
	12	745 ± 11.4[b]	8.14 ± 0.01	9.13 ± 0.04[f]	18.09 ± 0.45[e]	ND	16.36 ± 0.08[bc]
	18	930 ± 27.9[a]	8.22 ± 0.03	10.82 ± 0.12[e]	12.02 ± 0.38[fg]	ND	18.16 ± 0.18[ab]
	24	1029 ± 14.[la]	8.12 ± 0.05	12.33 ± 0.05[d]	8.24 ± 0.39[i]	ND	18.63 ± 0.09[a]

abcOne-way ANOVA of means in the same column with different superscript are significantly different ($P < 0.05$).

[1]EPS = Exopolysaccharides.

[2]RSM = Reconstituted skim milk. .

[3]WPC = Whey protein concentrate.

[4]ND = Not detected.

Table 4.5. Bacterial counts, amount of EPS[1], and concentrations of lactic acid, lactose, glucose and galactose produced by *Streptococcus thermophilus* 1275 during fermentation of RSM[2] supplemented with WPC[3] 392 at 0, 6, 12, 18 and 24 h at 37°C (mean ± standard error).

Fermentation time (h)	pH (n = 4)	EPS (mg/L; n = 8)	Bacterial counts (log$_{10}$ cfu/ml; n = 4)	Lactic acid (g/L; n = 12)	Lactose (g/L; n = 12)	Glucose (n = 12)	Galactose (g/L; n = 12)
RSM + 0.5% WPC							
0	6.39 ± 0.02	12 ± 0^c	6.07 ± 0.03	[4]ND^e	48.96 ± 0.84	ND	ND^e
6	4.94 ± 0.02	292 ± 0^b	8.17 ± 0.00	1.02 ± 0.02^d	39.14 ± 0.01	ND	5.91 ± 0.09^d
12	4.54 ± 0.01	438 ± 11.0^a	8.11 ± 0.01	1.75 ± 0.02^c	35.97 ± 0.37	ND	6.33 ± 0.02^{cd}
18	4.34 ± 0.005	455 ± 8.3^a	8.05 ± 0.01	2.08 ± 0.03^b	32.69 ± 0.33	ND	8.73 ± 0.31^b
24	4.21 ± 0.015	491 ± 10.3^a	8.05 ± 0.02	2.24 ± 0.03^a	31.03 ± 0.93	ND	11.05 ± 0.15^a

^{abc}One-way ANOVA of means in the same column with different superscript are significantly different ($P < 0.05$).

[1]EPS = Exopolysaccharides.

[2]RSM = Reconstituted skim milk.

[3]WPC = Whey protein concentrate.

[4]ND = Not detected.

Table 4.6. Bacterial counts, amount of EPS[1], and concentrations of lactic acid, lactose, glucose and galactose produced by *Streptococcus thermophilus* 1275 and *S. thermophilus* 1303 in RSM[2] at 0, 6, 12, 18 and 24 h of fermentation at pH 5.5 and 37°C (mean ± standard error).

Strain ratio	Fermentation time (h)	EPS (mg/L; n = 8)	Bacterial counts (log$_{10}$ cfu/ml; n = 4)	Lactic acid (g/L; n = 12)	Lactose (g/L; n = 12)	Glucose (n = 12)	Galactose (g/L; n = 12)
100% *S. thermophilus* 1275	0	13 ± 1.0^{no}	6.00 ± 0.02	[3]ND^{mno}	47.70 ± 1.39^{a}	ND	ND^{ijk}
	6	327 ± 7.4^{kl}	8.39 ± 0.09	8.40 ± 0.09^{k}	36.19 ± 0.43^{b}	ND	7.67 ± 0.08^{h}
	12	342 ± 15.0^{ikl}	8.61 ± 0.01	19.37 ± 0.03^{f}	20.32 ± 0.26^{e}	ND	14.33 ± 0.11^{e}
	18	390 ± 8.4^{ij}	8.56 ± 0.09	24.62 ± 0.14^{d}	11.84 ± 0.14^{h}	ND	15.94 ± 0.33^{cde}
	24	458 ± 7.1^{ghi}	8.44 ± 0.00	27.12 ± 0.22^{c}	8.50 ± 0.08^{i}	ND	17.63 ± 0.26^{abc}
50% *S. thermophilus* 1275; 50% non-EPS *S. thermophilus* 1303	0	12 ± 2.5^{o}	5.37 ± 0.01	ND^{no}	48.82 ± 0.22^{a}	ND	ND^{ijk}
	6	317 ± 16.6^{l}	7.96 ± 0.02	3.83 ± 0.07^{l}	31.59 ± 0.47^{d}	ND	10.13 ± 0.14^{fg}
	12	550 ± 9.0^{f}	8.04 ± 0.02	8.84 ± 0.04^{j}	12.83 ± 0.16^{gh}	ND	16.55 ± 0.20^{bcd}
	18	613 ± 6.4^{e}	7.98 ± 0.02	11.38 ± 0.09^{h}	4.90 ± 0.08^{kl}	ND	17.99 ± 0.13^{a}
	24	676 ± 17.^{lcdeb}	7.75 ± 0.01	12.41 ± 0.08^{g}	2.66 ± 0.14^{m}	ND	18.00 ± 0.08^{a}
75% *S. thermophilus* 1275; 25% non-EPS *S. thermophilus* 1303	0	15 ± 1.5^{mno}	5.53 ± 0.03	ND^{o}	49.79 ± 0.36^{a}	ND	ND^{k}
	6	391 ± 31.5^{hij}	8.10 ± 0.01	10.68 ± 0.37^{i}	32.70 ± 0.12^{cd}	ND	9.07 ± 0.18^{g}
	12	645 ± 13.0^{de}	8.13 ± 0.02	23.22 ± 0.35^{e}	13.33 ± 0.33^{fg}	ND	15.66 ± 0.20^{de}
	18	720 ± 3.7^{b}	7.99 ± 0.04	29.04 ± 0.14^{b}	6.79 ± 0.28^{j}	ND	17.98 ± 0.13^{a}
	24	832 ± 12.8^{a}	7.97 ± 0.01	31.41 ± 0.07^{a}	3.66 ± 0.09^{lm}	ND	18.21 ± 0.11^{a}

[abc]One-way ANOVA of means in the same column with different superscript are significantly different ($P < 0.05$).

[1]EPS = Exopolysaccharides.

[2]RSM = Reconstituted skim milk.

[3]ND = Not detected.

Figure 4.1. Standard curve for the determination of lactic acid produced by *Streptococcus thermophilus* 1275 by HPLC.
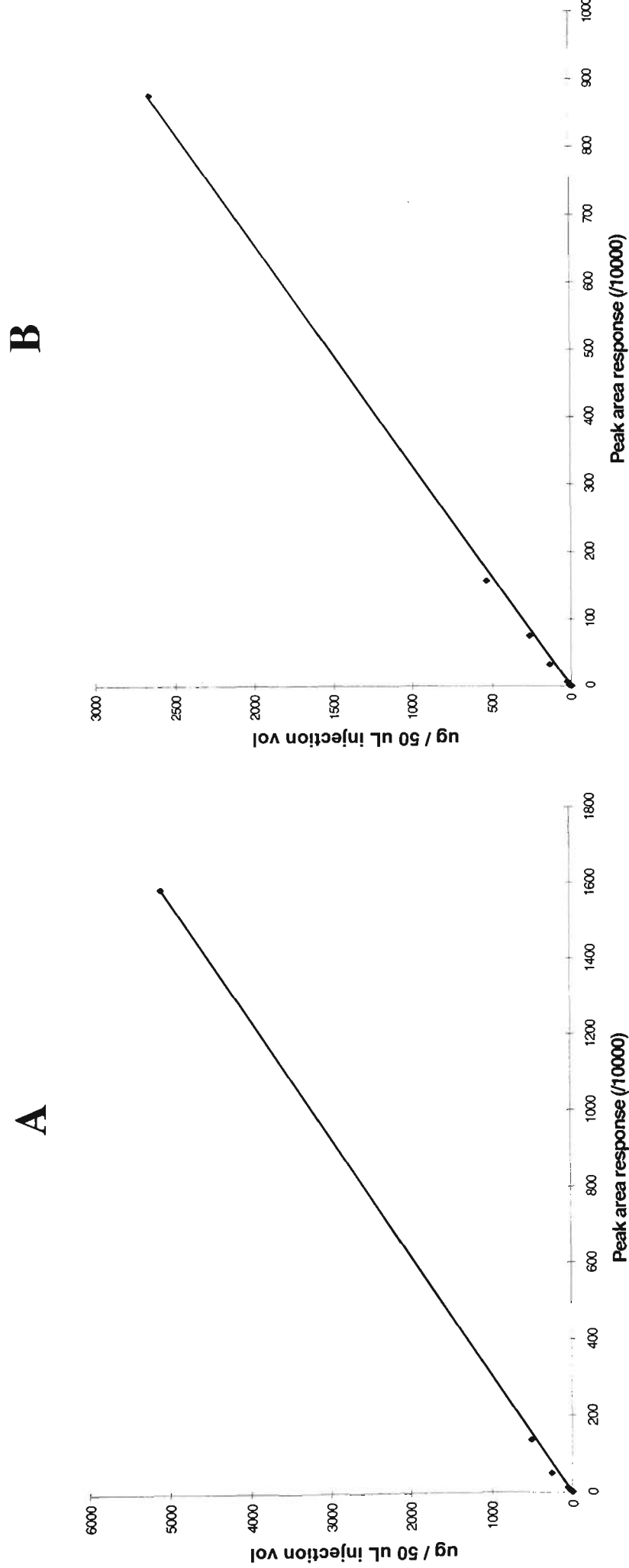
Figure 4.2. Standard curve for the determination of lactose (A) and galactose (B) catabolised by *Streptococcus thermophilus* 1275 by HPLC.

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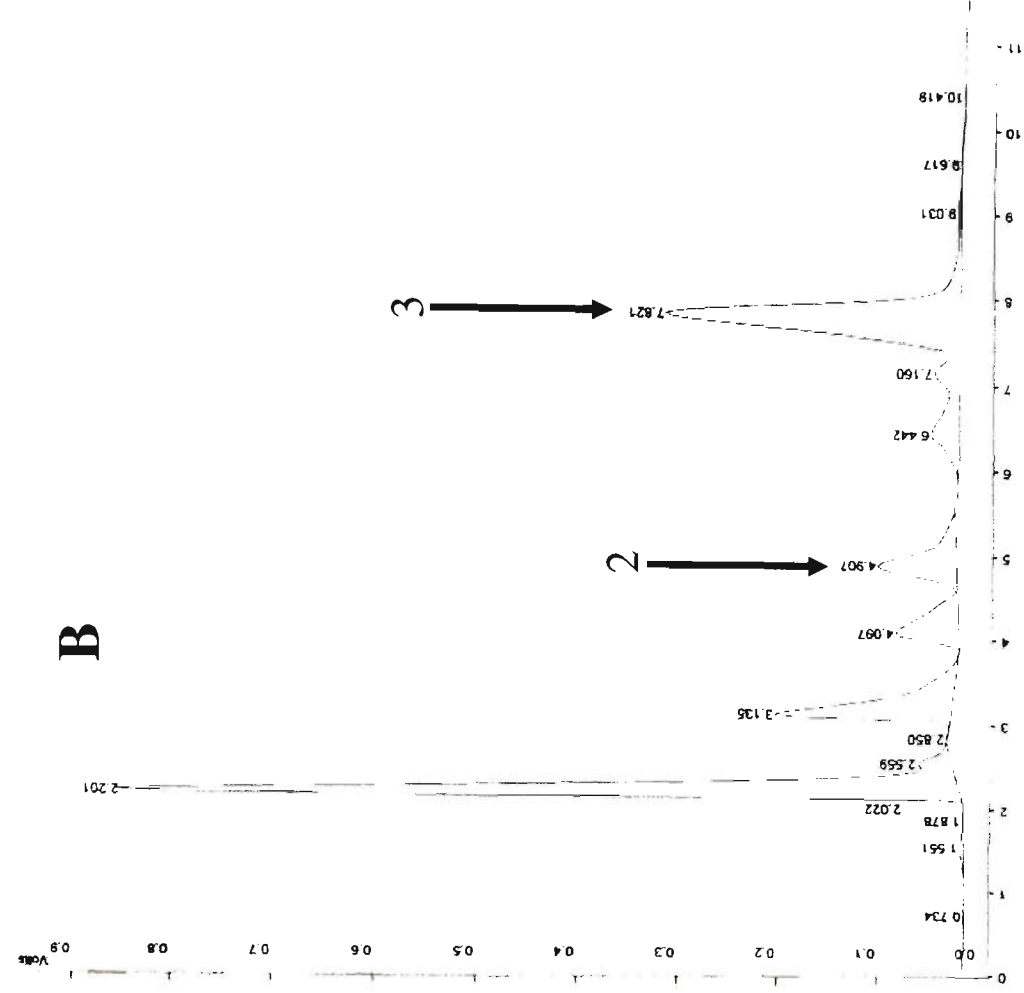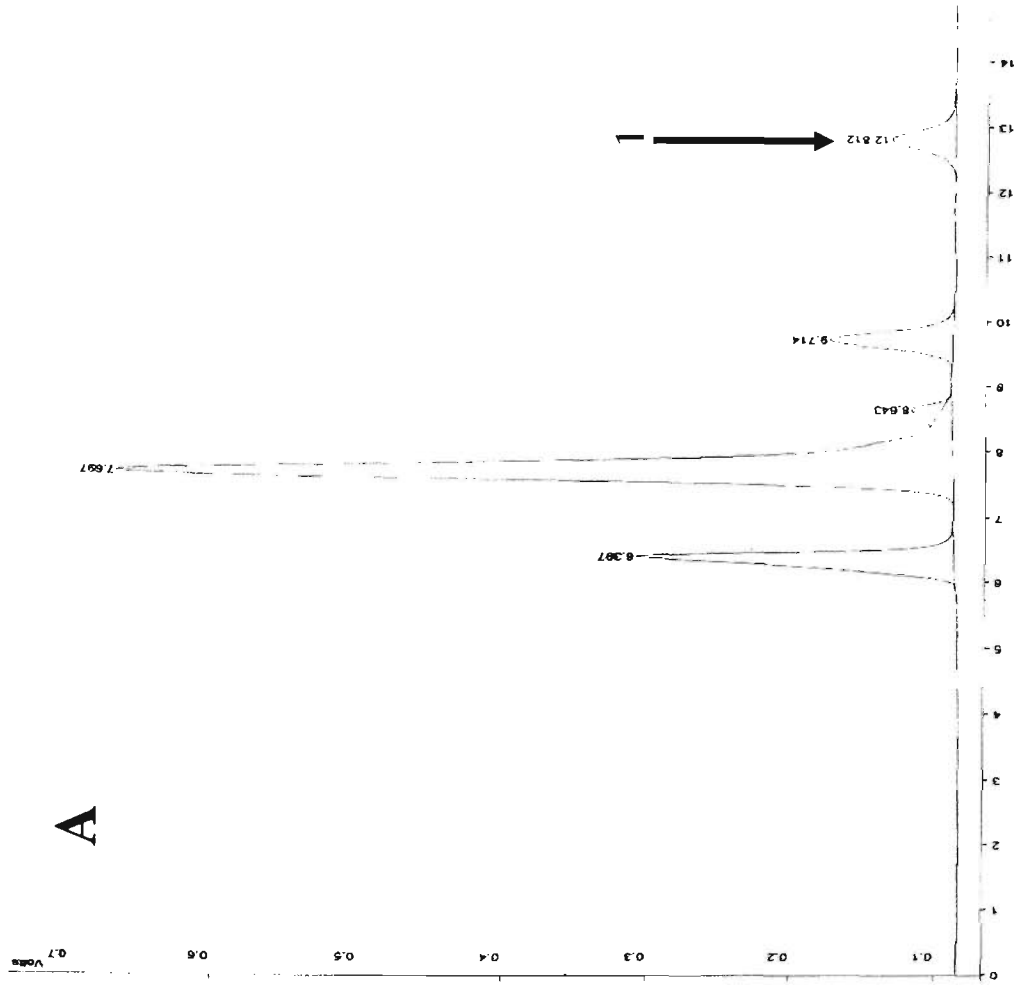
Figure 4.3. Typical chromatogram of organic acids (A) and carbohydrates (B) from RSM fermented by *Streptococcus thermophilus* 1275 and detected by HPLC with a refractive index detector. Compounds identified were lactic acid (1), galactose (2) and lactose (3).

CHAPTER 5.0

Role of Microbial Exopolysaccharides on Moisture Retention and

Texture and Functionality of Low Fat Mozzarella Cheeses

5.1 INTRODUCTION

Reduced fat cheeses are becoming increasingly popular. Removing fat from Mozzarella cheeses creates physical changes that lead to loss in functionality. Low fat cheeses become hard, rubbery and show poor stretch, melt and flow when heated (McMahon and Oberg, 1998a; McMahon *et al.*, 1993; Mistry and Anderson, 1996). Due to reduced fat content, there is a lack of free oil release and low fat Mozzarella cheeses exhibit excessive scorching and poor bake characteristics when used as a pizza topping (Fife *et al.*, 1996; Rudan and Barbano, 1998b).

Moisture increases the ability of cheese particles to flow when heated and has been associated with improved texture and performance in low fat cheeses. With a moisture to protein ratio equal to, or greater than that observed in full fat Mozzarella cheeses, low fat varieties were made with improved melt and cook colour (Fife *et al.*, 1996; Low *et al.*, 1998; McMahon *et al.*, 1996; McMahon and Oberg, 1998a; Petersen *et al.*, 2000).

Many strains of *Streptococcus thermophilus* have the ability to synthesize exopolysaccharides (EPS). The long-chained, highly branched and high molecular mass character of EPS polymers has the ability to entrap moisture and retard whey expulsion. Microbial exopolysaccharides have been used successfully to promote the water holding capacity of low fat Mozzarella cheeses (Low *et al.*, 1998). Several studies report specific improvement in the functional properties of low fat cheeses made with EPS cultures (Perry *et al.*, 1997; Perry *et al.*, 1998; Petersen *et al.*, 2000). EPS also contributes to the rheological behaviour and texture of fermented milk products (Hassan *et al.*, 2002a; Hassan *et al.*, 1996a; Hassan *et al.*, 1996b; Marshall and Rawson, 1999; Rawson and Marshall, 1997; Rohm and Kovak, 1993). Capsular EPS forming lactic cultures were shown to produce a more open casein network (Hassan and Frank, 1997). Microorganisms can synthesise EPS as either ropy or loose slime that is excreted into the surroundings, or as a capsule that remains adhered to the cell surface to create a distinct covering (Broadbent *et al.*, 2003). The ability to secrete ropy EPS in

addition to producing capsular EPS is shared by certain strains (Chapter 3.0; Broadbent *et al.,* 2003; Cerning, 1990; Duguid, 1951; Zisu and Shah, 2003a), including *S. thermophilus* 1275 used in this study. Capsular EPS is of particular importance in our research as it has been reported to show minimal adverse effect during the processing of whey (Low *et al.,* 1998; Perry *et al.,* 1997; Petersen *et al.,* 2000).

EPS synthesis is dependent on the starter cultures used, and growth conditions and is growth associated (De Vuyst *et al.,* 1998; Gamar-Nourani *et al.,* 1998; Gorret *et al.,* 2001; Grobben *et al.,* 2000; Zisu and Shah, 2003a). In an earlier study, optimum EPS production by *S. thermophilus* 1275 in milk was achieved at pH 5.5 and 40°C (Chapter 4.0; Zisu and Shah, 2003a). These growth conditions are reproducible during cheese making.

The effects of capsular and ropy EPS on texture and functionality of low fat Mozzarella cheeses remain largely unknown. Similarly, little is known about EPS synthesis in cheeses by either ropy or capsular EPS producing strains. The aims of this study were to examine effects of capsular and ropy EPS producing strains of *S. thermophilus* on EPS production, moisture retention, texture and functionality of low fat Mozzarella cheeses.

5.2 MATERIALS AND METHODS

5.2.1 Starter Cultures

S. thermophilus strains 1275 (a mixed capsular/ropy EPS producer), 285 (a capsular EPS producer) and 1303 (non-EPS) and *L. delbrueckii* ssp. *bulgaricus* strain 1368 (non-EPS) were used in this study. The strains were described in Chapter 3.0. Working cultures were prepared as described in Chapter 3.0, section 3.2.1.

5.2.2 Cheese Making

Each batch of cheese was made with a total of 10 L of milk in 30 L capacity custom made, temperature controlled cheese vats. A milk blend containing 0.5% fat was made with pasteurised Pura Light Start milk (containing 1% fat; National Foods Ltd., Melbourne, VIC, Australia) and Skinny milk (containing 0.1% fat; Parmalat Foods Australia Pty. Ltd., Rowville, VIC, Australia). This was used to make cheeses with a final fat content of 6% as indicated below:

Batch 1: _S. thermophilus_ 1303 (non-EPS) + _L. delbrueckii_ ssp. _bulgaricus_ 1368 (non-EPS) (Control),

Batch 2: _S. thermophilus_ 285 (capsular EPS) + _L. delbrueckii_ ssp. _bulgaricus_ 1368,

Batch 3: _S. thermophilus_ 1275 (ropy EPS) + _L. delbrueckii_ ssp. _bulgaricus_ 1368.

Milk was inoculated with 1.6% of _S. thermophilus_ and 0.8% of _L. delbrueckii_ ssp. _bulgaricus_ and tempered to 35°C for half an hour. Single strength chymosin (Chymax; Chr. Hansen, Pty. Ltd., Bayswater, VIC, Australia) was added at the rate of 4 mL per 10 L of milk. The milk was coagulated in 25 min, and curd was cut using a cheese knife (1 cm^2 wire grid) based on setting time and subjective assessment of the coagulum with a knife. After cutting, curd was cooked for 20 min, gradually increasing the temperature to 40°C. Whey was drained at pH 6.2 and the curd was cheddered into slabs before milling at pH 5.2. Salt was added to the curd at 1.5% (wt/wt) and held for 30 min. A curd sample (40 g) was collected for later analysis and stored at –20°C. The remaining salted curd was stretched for 7 min in hot water maintained at 75°C. The volume of stretching water was 2.5 times the weight of the curd and contained 3% salt (wt/wt).

Cheese blocks were immediately placed into barrier bags and vacuum sealed (using a Multivac A300/16 machine, Multivac Sepp Haggenmuller KG, Wolfertschwenden, Germany) before storing at 4°C in a fan forced cold room.

5.2.3 Cheese Analysis

An appropriate aliquot of cheese was removed from the cheese blocks on the day following production. Each cheese was analysed for fat (Babcock method), moisture (atmospheric oven method) and protein (Kjeldahl method) contents in accordance with the Association of Official Analytical Chemists (AOAC) methods (AOAC, 1999). Textural characteristics were analysed at d 7 and d 28. A simulated pizza bake, stretch and meltability test for each cheese was performed at d 28.

5.2.4 Isolation and Quantification of Exopolysaccharides

A 100 mL volume of distilled water was mixed with 15 g of finely chopped curd and homogenised completely at 24,000 rpm for 2 min using a ultra turrax homogeniser (T25, Janke & Kunkel GmbH & CoKG, 1KA Labortechnik, Staufen, Germany). The homogenous mixture was sonicated for 90 min using an FX 14PH sonication bath (Ultrasonics Pty. Ltd., Sydney, Australia). EPS was extracted and quantified from the homogenous mixture according to the procedure described in Chapter 3.0, section 3.2.5.

5.2.5 Cheese Melt

Meltability of Mozzarella cheeses was determined using 250 mm long glass tubes with a diameter of 24 mm and thickness of 3 mm (R.B. Instruments, Mt. Eliza, VIC, Australia) by a method similar to that used by Poduval and Mistry (1999). A plunger was used to pack 10 g of finely grated cheese into the glass tubes that were previously sealed at one end with a rubber stopper. The unsealed end was closed off with a perforated rubber stopper to enclose the

sample. The length of the compressed cheese sample was measured using a Vernier caliper and stored at 4°C for 4 h to allow an even temperature distribution within samples. The tubes were placed horizontally into an oven pre-heated to 110°C and the cheese samples were melted for 100 min. Cheeses were allowed to cool for 30 min and the length of the melted cheese was recorded. The difference in initial and final lengths was presented as the melt distance. This test was sensitive enough to distinguish between the meltability performance of low fat Mozzarella cheeses processed by various techniques.

5.2.6 Cheese Stretch

Cheese stretch was measured using an Instron Universal Testing Machine (UTM) (Model 5564; Instron Ltd., London, England) fitted with a 100 N load cell by a method similar to that described by Bhaskaracharya and Shah (2002). A cross bar spindle was placed at the bottom of a beaker and covered with 50 g of shredded cheese sample, which was compressed with a rubber head plunger. The beaker was covered with an aluminium foil to avoid evaporation and heated in a water bath (Thermoline Scientific Equipment Pty. Ltd., NSW, Australia) at 62°C for 1 h until the cheese sample was uniformly melted. The cross bar spindle was attached to the UTM immediately upon removal from the water bath and stretched at 300 mm/min. The melted samples were pulled vertically through the 300 mm over 1 min and the stretch distance (the distance cheese was pulled vertically before the sample broke), and resistance force (N) recorded as the sample was stretched) were recorded simultaneously using Merlin software (Merlin II, version 5.31.14).

5.2.7 Texture Profile Analysis

Textural characteristics of cheeses including hardness (the peak force of the first compression), adhesiveness (the negative force (area 3) of the first compression), cohesiveness (area 2 / area 1 of the compression cycle), springiness (the height that the cheese recovers between the first and second compression) and chewiness (hardness × cohesiveness × springiness) were analysed with the UTM at room temperature (~22°C). These parameters have been described in detail by Pons and Fiszman (1996). Cheese samples were taken using a cylindrical probe/corer with a 20 mm diameter. These samples were cut to approximately 20 mm in length using a specifically designed cutting device that accommodated for the specific shape and size of the sample. Each sample was allowed to equilibrate to room temperature for 10 min after removing from refrigerated storage (4°C). A 500 N load cell was used with a flat plunger and samples were compressed to 50% of their original size at a speed of 50 mm/min using a two-cycle compression test. A 50% compression was selected for TPA analysis in order to allow deformation to occur without breaking of the sample. Compression data was collected using Merlin software (Bhaskaracharya and Shah, 1999).

5.2.8 Pizza Bake

Shredded Mozzarella cheese (300 g) was used as a topping on a pizza base (Don Emilio's, Freshwell Foods, Coolaroo, VIC, Australia) covered with a thin layer of tomato paste (Leggos, Simplot Australia Pty. Ltd., Cheltenham, VIC, Australia). Pizzas were baked at 232°C for 7 min in a Lincoln Impinger 1304-4 conventional pizza oven (Lincoln Foodservice Products Inc., Fort Wayne, IN, USA). The appearance of the cheese on the pizza was visually evaluated for shred fusion and melt, colour and the extent of blistering. The Hunter L,a,b system was used to describe the colour profiles of cheeses before cooking and immediately after baking with a

Minolta chroma-meter (CR-300, Minolta Corporation, Ramsey, New Jersey, USA) at d 28 of storage at 4°C.

5.2.9 Statistical Analysis

Each batch of cheese was made in duplicate and results are presented as means ± standard error of replicates. Moisture, hardness, adhesiveness, cohesiveness, springiness, chewiness and stretch data are an average of 6 replicates, while the results of fat, protein, melt and EPS are representative of 4 analyses. Pizza bake was replicated three times. Hunter L,a,b values obtained from the pizza bake are representative of 12 readings. To find significant differences between data, the means were analyzed using one-way analysis of variance (ANOVA) with a 95% confidence interval using Microsoft® Excel StatPro™ (Palaside Corporation, Newfield, NY). ANOVA data with a $P < 0.05$ was classified as statistically significant.

5.3 RESULTS AND DISCUSSION

5.3.1 Yield and Composition

Table 5.1 shows the approximate processing time of low fat Mozzarella cheeses and the average composition of cheeses and curds. The time employed to manufacture low fat Mozzarella cheeses was longest for control cheeses made with non-EPS starter, taking approximately 5 h and 7 min. This was followed by EPS cheeses made with capsular producing *S. thermophilus* 285, taking 4 h and 45 min. Cheeses made with ropy *S. thermophilus* 1275 showed a manufacturing time of 4 h and 13 min. Longer fermentation times were expected to result in greater syneresis of whey from the curd particles and produce a cheese with lower moisture

content. It is likely that starter cultures with the ability to synthesise EPS produced lactic acid at a greater rate than that of the non-EPS counterpart as a result of an increase in moisture retention. Higher moisture content in curd after the draining of the whey accelerates growth rates and dilutes fermentation by-products. Consequently, the fermentation steps following the draining of whey were shortened. A moisture content of 53.77% (57.20% as moisture in non-fat substance (MNFS) and a moisture to protein (M:P) ratio of 1.41 were recorded in control cheeses. Manufacturing cheeses with capsular and ropy EPS producing strains of _S. thermophilus_ significantly increased the cheese moisture content and M:P ratio to 57.15% (60.73% as MNFS; M:P ratio of 1.65) and 58.39% (61.98% as MNFS; M:P ratio of 1.77), respectively ($P < 0.05$). Cheeses made with ropy EPS showed a significantly higher moisture content as compared to those made with capsular EPS ($P < 0.05$). It should be noted that the commercial milks used to make cheese were potentially homogenised in addition to pasteurisation. Homogenisation has been reported to increase the moisture retention and meltability of low fat Mozzarella cheeses (Tunick _et al._, 1993b). The milk used to make cheeses in Chapters 6 to 9 was not homogenised. The relation between EPS content and moisture retention was further highlighted in the composition of curds. EPS was not detected in control curds, but was present in the EPS curd at a concentration of 31.09 mg/g ($P < 0.05$) and at a significantly greater concentration of 51.65 mg/g in curds made with ropy EPS ($P < 0.05$). Although EPS production by the ropy culture was higher than that of the capsular type and an increase in moisture retention occurred, this did not result in a significant increase in the cheese yield ($P > 0.05$). The yield was, however, higher in cheeses made with ropy cultures (11.57 kg/100 kg of milk) as compared to the capsular type (10.35 kg/100 kg of milk). All EPS containing cheeses had a significant increase in yield over the control cheeses (9.82 kg/100 kg of milk). Yield was reported as the amount of cheese per 100 kg of milk used based on the actual amount of curd produced from 10L of milk.

It was anticipated that ropy EPS production would be greater than the capsular type (Zisu and Shah, 2003a). Capsular EPS remained attached to the bacterial cells and was thereby dependent on the presence of cell numbers. In earlier work, ropy EPS produced by _S. thermophilus_ 1275 was isolated in greater amounts in comparison to the capsular type by _S. thermophilus_ 285 in culture media (Chapter 3.0, section 3.3.4) and in fermentation data obtained from experiments with _S. thermophilus_ 1275 (Chapter 4.0; Zisu and Shah, 2003a).

The fat content was similar between cheese types on a wet basis as well as fat in dry matter ($P > 0.05$) and was not expected to influence textural and functional patterns. The protein content was influenced by the increase in moisture content. A greater moisture content had a dilution effect resulted in a lower protein content with a greater M:P ratio and vice versa.

5.3.2 Cheese Melt

The melt distance recorded for cheeses heated to 110°C for 100 min at d 28 is given in Figure 5.1 and the meltability of cheeses is shown in Figure 5.2. Control cheeses made with non-EPS starters had an average melt distance of 39 mm (Figure 5.2, A). EPS cheeses showed a slightly better flow distance of 44 mm, however, this was not significant ($P > 0.05$) (Figure 5.2, B). The melt distance recorded for cheeses made with ropy EPS was significantly greater, reaching 76 mm ($P < 0.05$) (Figure 5.2, C). Perry _et al._ (1997, 1998) and Petersen _et al._ (2000) reported increased melt in low fat Mozzarella cheese made from EPS cultures as influenced by an increase in moisture content. Cheeses made with capsular EPS produced by _S. thermophilus_ 285 also showed increased moisture content but this did not influence melt distance. Cheeses made with ropy EPS showed moisture seepage into the test tubes during heating and this may have acted as a lubricant on the surface of the test tube assisting cheese to melt and flow further down the tube.

5.3.3 Cheese Stretch

Figure 5.3 shows the stretch distance of cheeses heated to 62°C after 28 d of storage at 4°C. Figure 5.4 (A to C) shows the stretch characteristics observed for each cheese type during the stretching process. Control cheeses showed the least amount of stretch of approximately 222 mm. Figure 5.4 (A) shows the inability of control cheeses to stretch to a maximum of 300 mm due to brittleness. Cheeses made with capsular and ropy EPS producing starters showed a significantly greater stretch ($P < 0.05$). Both types of EPS containing cheeses achieved a maximum stretch distance of 300 mm, and a fibrous and smooth stretch was observed (Figure 5.4, B and C). To differentiate between the two EPS cheeses, the maximum stretch distance allowed by the test method must be increased.

Cheeses made with ropy EPS, however, expelled an excessive amount of unbound moisture when heated causing a build-up of moisture at the bottom of the beaker. The surface of cheeses was also coated with a sticky layer of slime. The water holding capacity of the ropy EPS was low particularly during heating, causing seepage of unbound moisture from cheeses prior to hydration of the casein micelles. The nature of ropy EPS caused it to behave in a unique manner in the final product and was not acceptable in cheeses. Ropy EPS created problems during the handling of cheeses and degraded the overall quality. EPS in the resulting whey is also likely to interfere with the processing of whey.

5.3.4 Texture Profile Analysis

5.3.4.1 Cheese hardness

Figure 5.5 shows the changes in cheese hardness between d 7 and d 28 of storage at 4°C. Control cheeses were the hardest ($P < 0.05$) and appeared to soften between d 7 and d 28 of storage, although this was not significant ($P > 0.05$).

Using EPS producing starter bacteria significantly reduced the overall hardness at both time points ($P < 0.05$), due to higher moisture retention and lower protein content. This may have contributed to a decrease in the cross-linking of the casein matrix. Unlike the control cheeses, those made with EPS showed a significant softening between d 7 and d 28 due to increased hydration of the protein microstructure and proteolysis ($P < 0.05$). The higher moisture content was likely to promote greater enzyme activity. Hassan and Frank (1997) found that using capsular EPS producing starter cultures, they were able to reduce the tension and firmness of non-fat rennet curd to similar levels in curd standardised to 40 g fat/L. Cheeses made with capsular EPS were found to be harder than those made with ropy EPS at d 7 and d 28 ($P < 0.05$). The most desirable softness of cheeses was detected in those made with capsular EPS. Ropy EPS containing cheeses were too soft at d 28 and they were also covered in an external layer of surface slime. The slime was more apparent at d 7. Moisture seepage was also evident when samples were subjected to pressure applied by the UTM

5.3.4.2 Cheese adhesiveness

Adhesiveness of cheeses between 7 and 28 d of storage at 4°C is given in Figure 5.6. Adhesiveness was absent in control cheeses at d 7 and increased slightly at d 28. This increase, however, was not significant ($P > 0.05$). Adhesion was significantly lower in cheeses made with non-EPS starter cultures ($P < 0.05$). Adhesiveness was of particular interest in cheeses made with EPS producing _S. thermophilus_ due the higher moisture content associated with these

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cheeses as well as the moisture seepage and the formation of the sticky coating of slime associated with cheeses made with ropy EPS. Adhesive forces of capsular- and ropy- EPS cheeses were significantly greater than those recorded for control cheeses at d 7 and d 28 ($P < 0.05$). Hardness of EPS cheeses was concurrently lower and cohesiveness was greater. Cheeses made with EPS starter cultures exhibited similar adhesion between the capsular and ropy types at both time points ($P > 0.05$). Although adhesiveness showed a tendency to increase between d 7 and d 28 within each variable, this was not significant ($P > 0.05$). The external coating of slime produced by _S. thermophilus_ 1275 did not contribute to adhesiveness.

5.3.4.3 Cheese cohesiveness

Figure 5.7 shows the cohesiveness of cheeses between d 7 and d 28 of storage at $4^{\circ}C$. Control cheeses showed the least cohesiveness at d 7 and d 28 ($P < 0.05$). These were also the hardest cheese, exhibited no adhesive forces and had low moisture and high protein contents. The strength of the internal bonds of the protein matrix that make up the body of the cheese reduced between d 7 and d 28 and resulting cohesiveness dropped significantly ($P < 0.05$).

Cheeses made with capsular and ropy EPS displayed greater cohesiveness than those recorded for the control cheeses at d 7 and d 28 ($P < 0.05$). As was observed in the pattern exhibited by cheese hardness, cohesiveness results reveal that cheeses made with capsular EPS show lower cohesiveness than those made with ropy EPS at both time points ($P < 0.05$). Likewise, a significant decrease occurred between d 7 and d 28 in both variables ($P < 0.05$).

5.3.4.4 Cheese springiness

Figure 5.8 presents the springiness of cheeses between d 7 and d 28 of storage at $4^{\circ}C$. Control cheeses showed the highest springiness overall ($P < 0.05$). No significant deformation occurred between d 7 and d 28 ($P < 0.05$) and the protein matrix was able to return to its original state after compression. Cheeses made with EPS producing strains of _S. thermophilus_ resulted in

a product exhibiting a lower degree of springiness ($P < 0.05$). Capsular and ropy EPS containing cheeses showed a similar springiness at d 7 and d 28 ($P > 0.05$). As was observed with the control cheeses, no significant deformation occurred between d 7 and d 28 ($P < 0.05$) in ropy cheeses, however, capsular EPS cheeses had a significant increase in springiness ($P < 0.05$).

5.3.4.5 Cheese chewiness

Because samples were not destroyed during compression, the secondary TPA parameter of chewiness was defined by the primary parameters of hardness × cohesiveness × springiness (Figure 5.9). Chewiness was shown to closely relate to hardness of the cheese. Non-EPS control cheeses showed most chewiness ($P < 0.05$) and there was no significant reduction between d 7 and d 28 ($P > 0.05$). EPS containing cheeses, due to the higher moisture and lower protein contents, demonstrated a lower degree of chewiness ($P < 0.05$). Chewiness was found to be greater in cheeses made with capsular EPS than in those made with the ropy form at d 7 and d 28 ($P < 0.05$). The two varieties of EPS cheeses were influenced by storage time and had reduced chewiness between d 7 and d 28 ($P < 0.05$).

5.3.5 Pizza Bake

Figure 5.10 (A to C) shows the results from a simulated pizza bake test performed at 232°C for 7 min at 28 d of storage. Browning, melt and flow of shredded cheese were evaluated after baking in a similar fashion to that previously performed by other researchers (Fife _et al.,_ 1996; Metzger _et al.,_ 2000a; Rudan and Barbano, 1998a; Rudan and Barbano, 1998b). All cheese types performed poorly, sustaining a high degree of scorching, possibly as a result of high moisture loss from the shred surface in the absence of sufficient free oil release leading to incomplete shred fusion and inadequate melt. Control cheeses (Figure 5.10, A) exhibited most scorching and the highest degree of incomplete shred fusion across the entire surface of the

pizza. EPS (Figure 5.10, B) and ropy (Figure 5.10, C) cheeses showed slight improvement with a reduction in the amount of scorching occurring towards the center of the pizza surface where moisture losses were smallest.

5.3.5.1 Hunter L,a,b values

Table 5.2 shows the Hunter L,a,b values at d 28, before cooking and immediately after baking while cheeses were warm. Due to an increase in moisture and a reduction in protein in EPS cheeses, a significant difference in L-values (used to describe whiteness) was recorded between cheeses prior to cooking ($P < 0.05$). Control cheeses had the lowest L-values followed by the capsular and ropy batches. When cheeses were warm immediately after baking, L-values increased significantly ($P < 0.05$) with the exception of ropy cheeses that showed similar readings ($P > 0.05$). L-values were greater in EPS containing cheeses as compared to the control cheeses but were similar between the two EPS types. Correspondingly, the highest degree of scorching was observed in control cheeses.

Before cooking, Hunter a-values (used to describe green to redness) were negative (closer to green) and were similar between the control and EPS cheeses ($P > 0.05$) but were higher in the ropy cheeses ($P < 0.05$). After baking, a-values increased significantly ($P < 0.05$) towards red in a response to scorching of the cheese shreds. Control cheeses showed most scorching and exhibited the lowest L-values and the greatest a-values ($P < 0.05$), followed by the two EPS cheeses which were similar ($P > 0.05$).

Hunter b-values (used to describe yellowness) were lowest in ropy cheeses before cooking and after baking ($P < 0.05$) and exhibited the highest whiteness. Control and EPS cheeses showed a lower but similar degree of yellowness before and after baking ($P > 0.05$). After baking, b-values increased significantly (*ie.* became more yellow) ($P < 0.05$).

5.4 CONCLUSIONS

The processing time of low fat Mozzarella cheeses made with EPS producing *S. thermophilus* strains 285 and 1275 was shorter than that made with non-EPS producing *S. thermophilus* 1303. Control cheeses made with non-EPS producing *S. thermophilus* 1303 had low moisture retention and poor yield. Moisture retention and yield of cheeses were significantly improved with capsular EPS producing *S. thermophilus* 285 and ropy EPS producing *S. thermophilus* 1275. Ropy EPS was isolated from curds at higher concentration and exhibited slightly greater moisture retention in cheeses than the capsular type, however, this did not increase the yield. EPS reduced the hardness, springiness and chewiness of low fat Mozzarella cheeses and improved the melt and stretch characteristics. Adhesion and cohesiveness were, however, greater in EPS cheeses and ropy EPS showed poor water holding capacity resulting in the seepage of moisture when cheeses were heated. Furthermore, ropy EPS resulted in a sticky layer of slime coated ropy cheeses, but this did not contribute to additional adhesiveness. Due to a lack of free oil release from low fat cheeses during pizza baking, a high degree of scorching occurred. All low fat cheese types continue to show a poor pizza bake performance. As the capsular and ropy forms of EPS provide similar beneficial effects in low fat Mozzarella cheeses, the capsular form of EPS produced by *S. thermophilus* 285 is favoured for use in cheeses due to the problems associated with slime formation with ropy strains. The use of microbial EPS, particularly the capsular type in low fat Mozzarella cheeses showed improvements in textural and functional properties associated with a reduced fat content. Furthermore, EPS producing strains have the potential to improving cheese yield.

Table 5.1. Processing time and the average composition of low fat Mozzarella cheeses and curds (mean ± standard error).

Cheese	Processing time (h) (n = 2)	Moisture (%) (n = 6)	Actual yield (kg/100 kg milk) (n = 2)	EPS in curd (mg/kg) (n = 4)	Fat (%) (n = 4)	[1]MNFS (%) (n = 4)	[2]FDM (%) (n = 4)	Protein (%) (n = 4)	[3]M:P
[4]Control	5.07	53.77 ± 0.39^c	9.82 ± 0.11^b	[7]ND^c	6.00 ± 0.11^a	57.20 ± 0.20^c	12.98 ± 0.10^a	38.18 ± 0.32^a	1.41
[5]EPS	4.45	57.15 ± 0.14^b	10.35 ± 0.01^a	31.09 ± 0.57^b	5.90 ± 0.14^a	60.73 ± 0.14^b	13.77 ± 0.15^a	34.72 ± 0.14^{bc}	1.65
[6]Ropy	4.13	58.39 ± 0.53^a	11.57 ± 0.32^a	51.65 ± 2.55^a	5.78 ± 0.30^a	61.98 ± 0.42^a	13.89 ± 0.31^a	33.07 ± 0.90^c	1.77

[a,b,c]One-way ANOVA of means in a column with different superscript are significantly different ($P < 0.05$).

[1]MNFS = Moisture in non-fat substance.

[2]FDM = Fat in dry matter.

[3]M:P = Moisture to protein ratio based on the final (average) moisture and protein contents.

[4]Control = Cheeses made with exopolysaccharide (EPS) negative cultures.

[5]EPS = Cheeses made with capsular EPS producing *S. thermophilus* 285.

[6]Ropy = Cheeses made with ropy EPS producing *S. thermophilus* 1275.

[7]ND = Not detected.

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Table 5.2. Mean Hunter L,a,b values (n = 12 ± SE) for whiteness measurements of low fat Mozzarella cheeses for given variables at d 28.

	[1]Control		[2]EPS		[3]Ropy	
	[4]BC	[5]Warm	BC	Warm	BC	Warm
L	63.55 ± 0.27[d]	71.45 ± 1.08[bc]	69.15 ± 0.27[c]	75.24 ± 0.64[a]	74.97 ± 0.56[a]	75.52 ± 0.57[a]
a	-4.89 ± 0.06[ef]	-0.08 ± 0.71[a]	-4.96 ± 0.05[f]	-2.16 ± 0.39[bc]	-4.61 ± 0.07[d]	-3.08 ± 0.32[c]
b	18.04 ± 0.25[d]	26.72 ± 0.66[a]	18.61 ± 0.29[cd]	26.04 ± 0.46[a]	16.45 ± 0.24[e]	24.16 ± 0.44[b]

[abc]One-way ANOVA of means across a row with different superscript are significantly different ($P < 0.05$).
[1]Control = Cheeses made with exopolysaccharide (EPS) negative cultures.
[2]EPS = Cheeses made with capsular EPS producing *S. thermophilus* 285.
[3]Ropy = Cheeses made with ropy EPS producing *S. thermophilus* 1275.
[4]BC = Cheese measurements before cooking.
[5]Warm = Cheese measurements immediately after baking at 232°C.

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Figure 5.1. Cheese melt of control, [1]EPS (cheeses made with capsular EPS producing *S. thermophilus* 285) and [2]ropy (cheeses made with ropy EPS producing *S. thermophilus* 1275) cheeses after 28 d of storage at 4°C.

[abc]One-way ANOVA of means (n = 6) between cheese types with different superscript are significantly different ($P < 0.05$).

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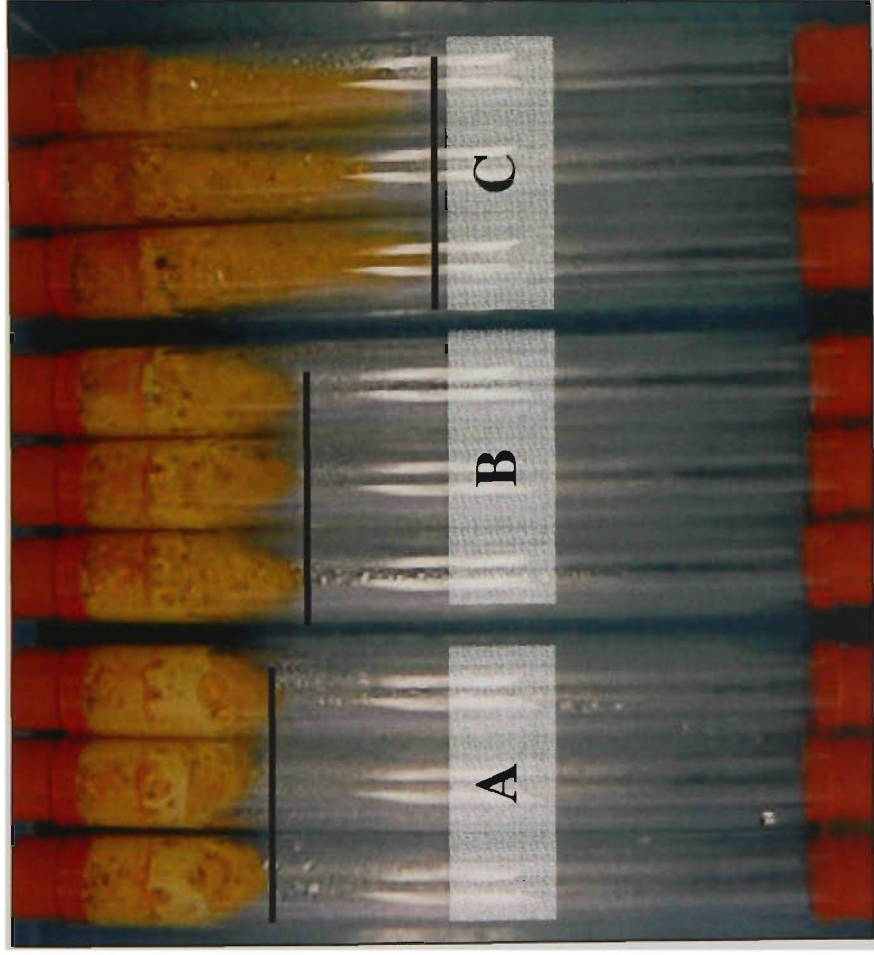
Figure 5.2. Approximate flow distance after storage for 28 d and melting at 110°C for 100 min of control (A), EPS (cheeses made with capsular EPS producing *S. thermophilus* 285) (B) and ropy (cheeses made with ropy EPS producing *S. thermophilus* 1275) (C) cheeses.
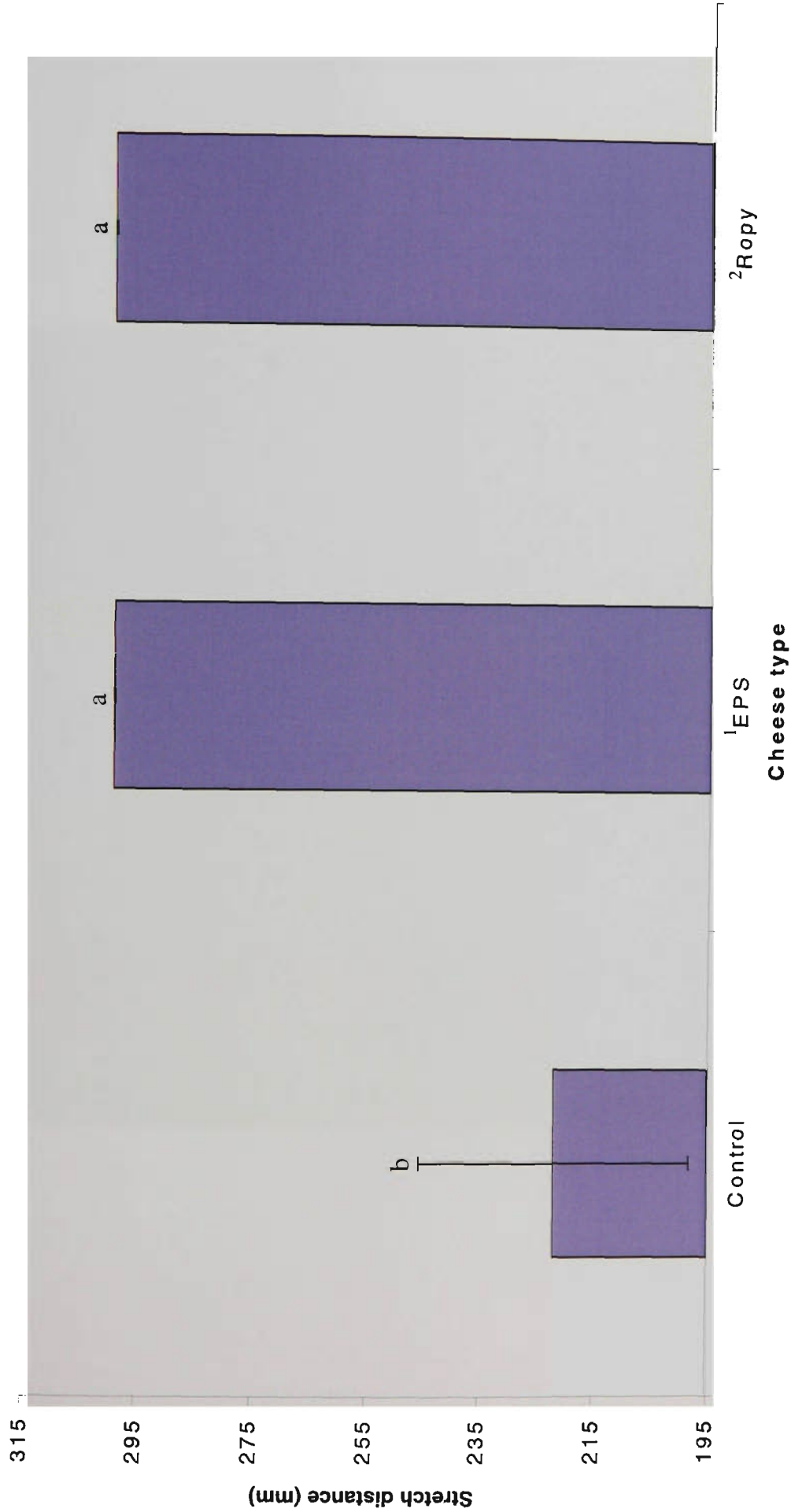
Figure 5.3. Stretch performance at 300 mm/min and 62°C for control, [1]EPS (cheeses made with capsular EPS producing *S. thermophilus* 285) and [2]ropy (cheeses made with ropy EPS producing *S. thermophilus* 1275) cheeses after 28 d of storage at 4°C. [abc]One-way ANOVA of means (n = 6) between cheese types with different superscript are significantly different ($P < 0.05$).
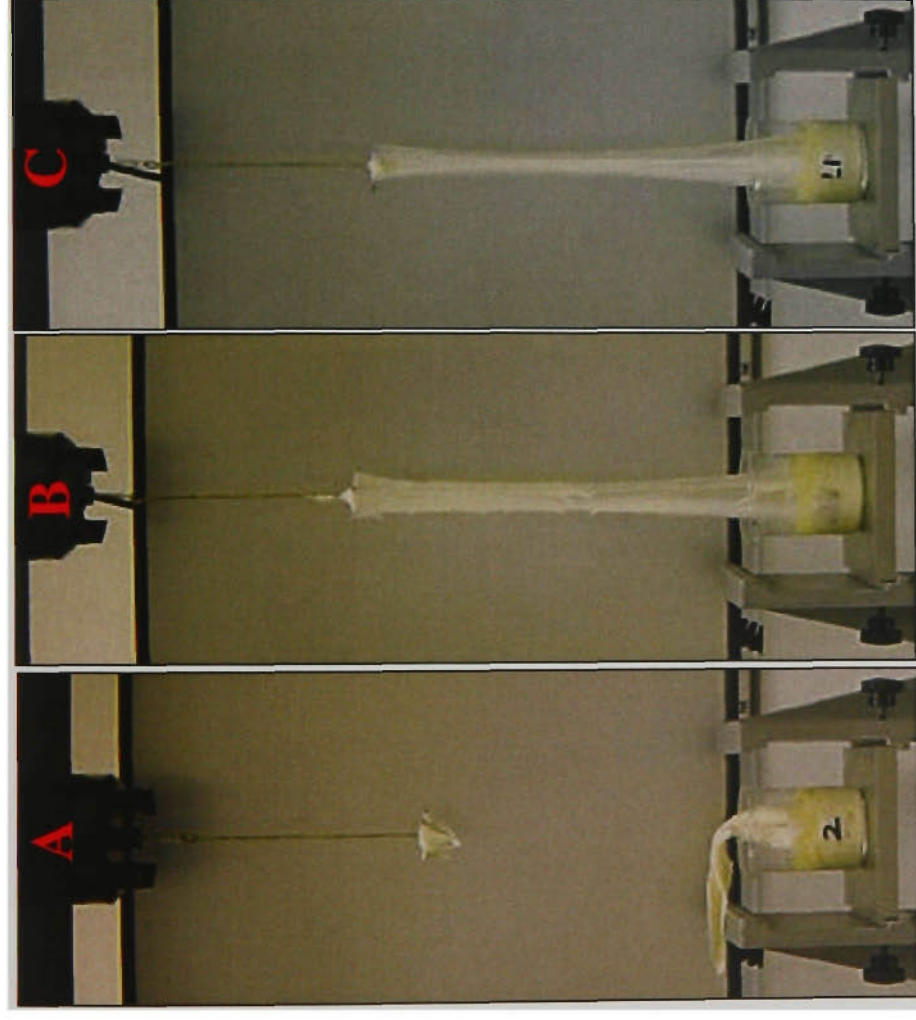
Figure 5.4. Stretch characteristics of control (A), EPS (cheeses made with capsular EPS producing *S. thermophilus* 285) (B) and ropy (cheeses made with ropy EPS producing *S. thermophilus* 1275) (C) cheeses at 62°C after 28 d of storage.
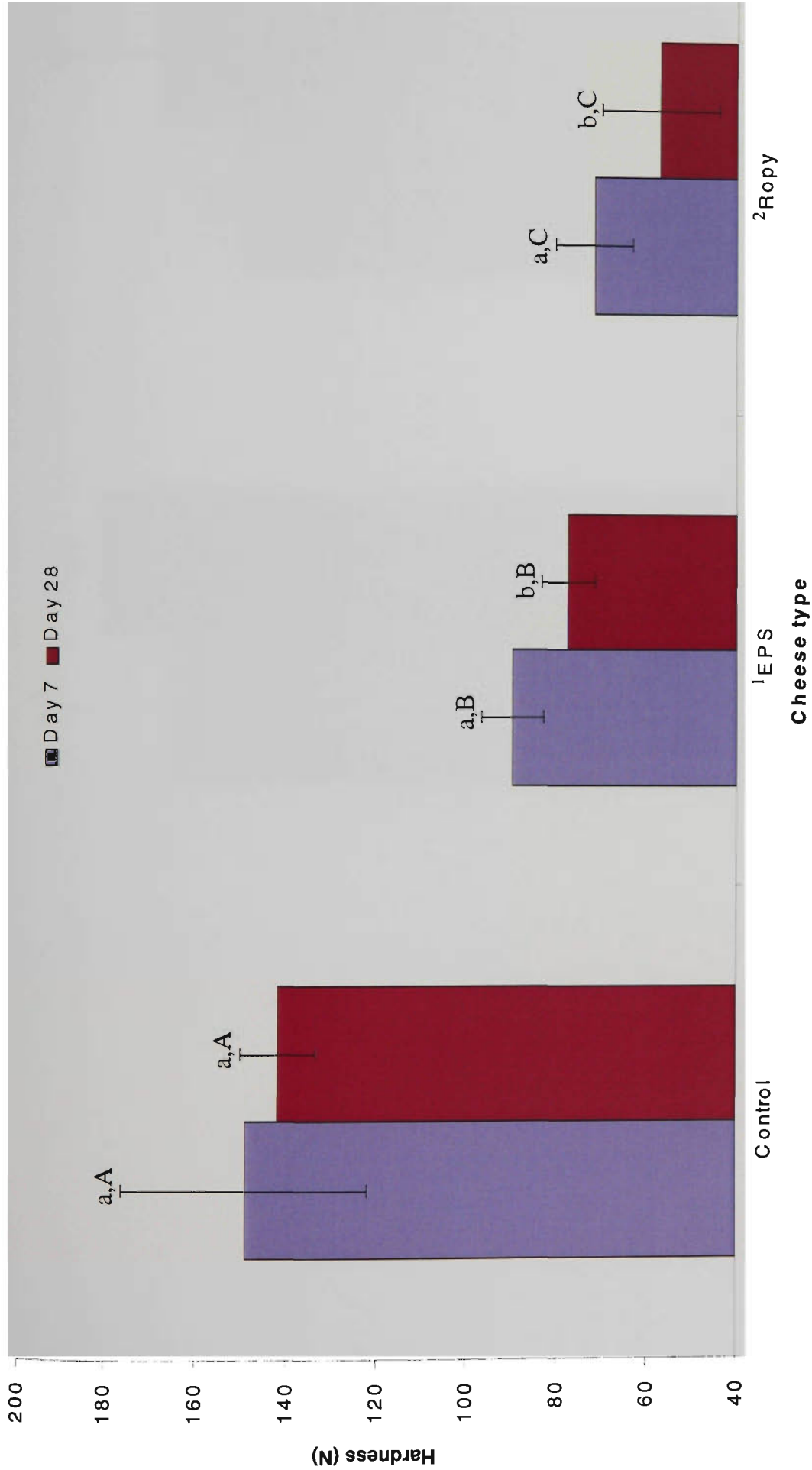
Figure 5.5. Hardness of control, [1]EPS (cheeses made with capsular EPS producing *S. thermophilus* 285) and [2]ropy (cheeses made with ropy EPS producing *S. thermophilus* 1275) cheeses over 28 d of storage at 4°C. [abc]One-way ANOVA of means (n = 6) between d 7 and d 28 within a cheese type and [ABC]one-way ANOVA of means at d 7 and 28 between cheese types with different superscript are significantly different ($P < 0.05$).

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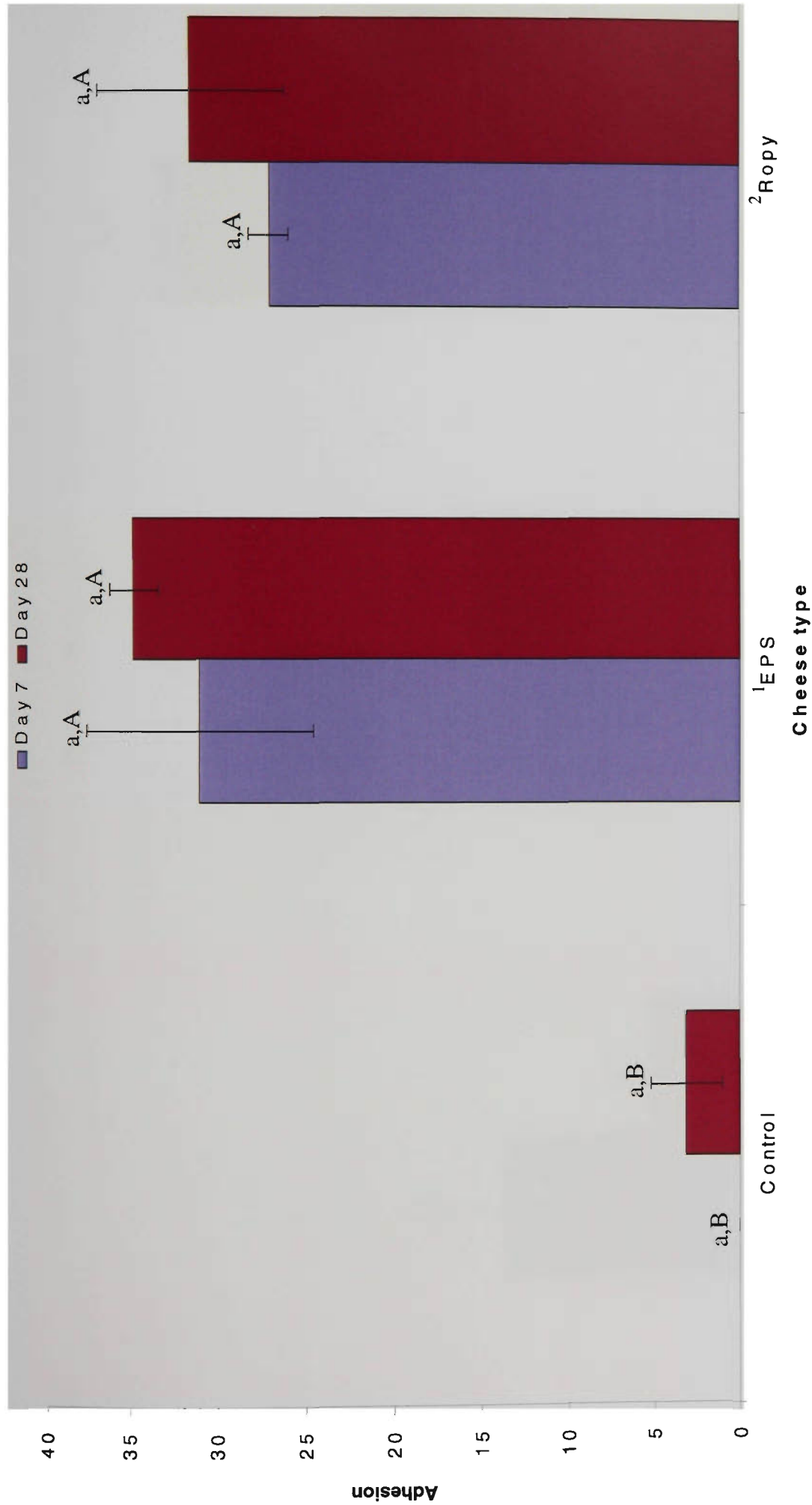
Figure 5.6. Adhesiveness of control, [1]EPS (cheeses made with capsular EPS producing *S. thermophilus* 285) and [2]ropy (cheeses made with ropy EPS producing *S. thermophilus* 1275) cheeses over 28 d of storage at 4°C.

[abc]One-way ANOVA of means (n = 6) between d 7 and d 28 within a cheese type and [ABC]one-way ANOVA of means at d 7 and 28 between cheese types with different superscript are significantly different (*P* < 0.05).

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Figure 5.7. Cohesiveness of control, [1]EPS (cheeses made with capsular EPS producing *S. thermophilus* 285) and [2]ropy (cheeses made with ropy EPS producing *S. thermophilus* 1275) 3 types of cheeses over 28 d of storage at 4°C.

[abc]One-way ANOVA of means (n = 6) between d 7 and d 28 within a cheese type and [ABC]one-way ANOVA of means at d 7 and 28 between cheese types with different superscript are significantly different (*P* < 0.05).

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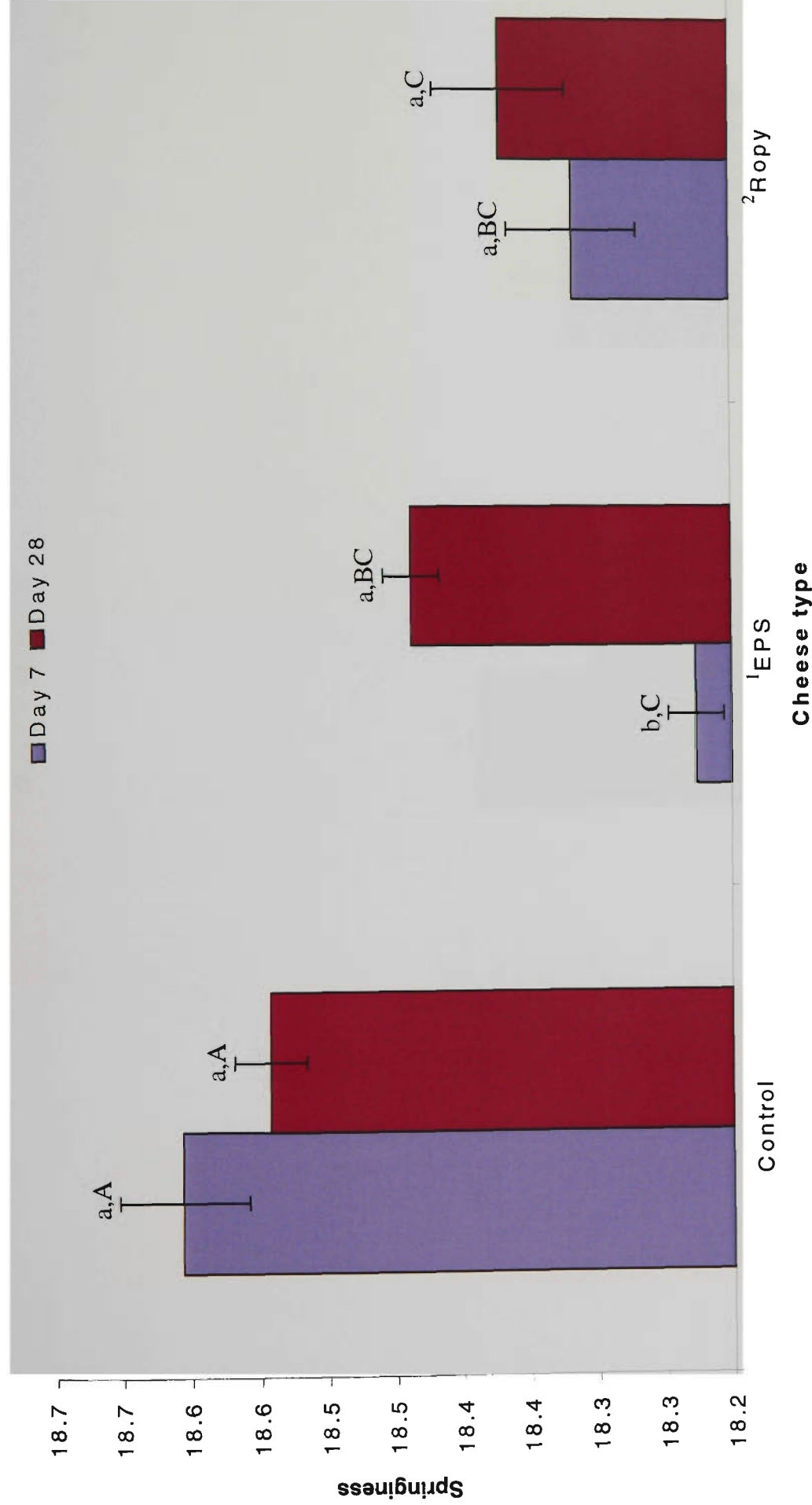
Figure 5.8. Springiness of control, [1]EPS (cheeses made with capsular EPS producing *S. thermophilus* 285) and [2]ropy (cheeses made with ropy EPS producing *S. thermophilus* 1275) cheeses over 28 d of storage at 4°C. [abc]One-way ANOVA of means (n = 6) between d 7 and d 28 within a cheese type and [ABC]one-way ANOVA of means at d 7 and 28 between cheese types with different superscript are significantly different (*P* < 0.05).

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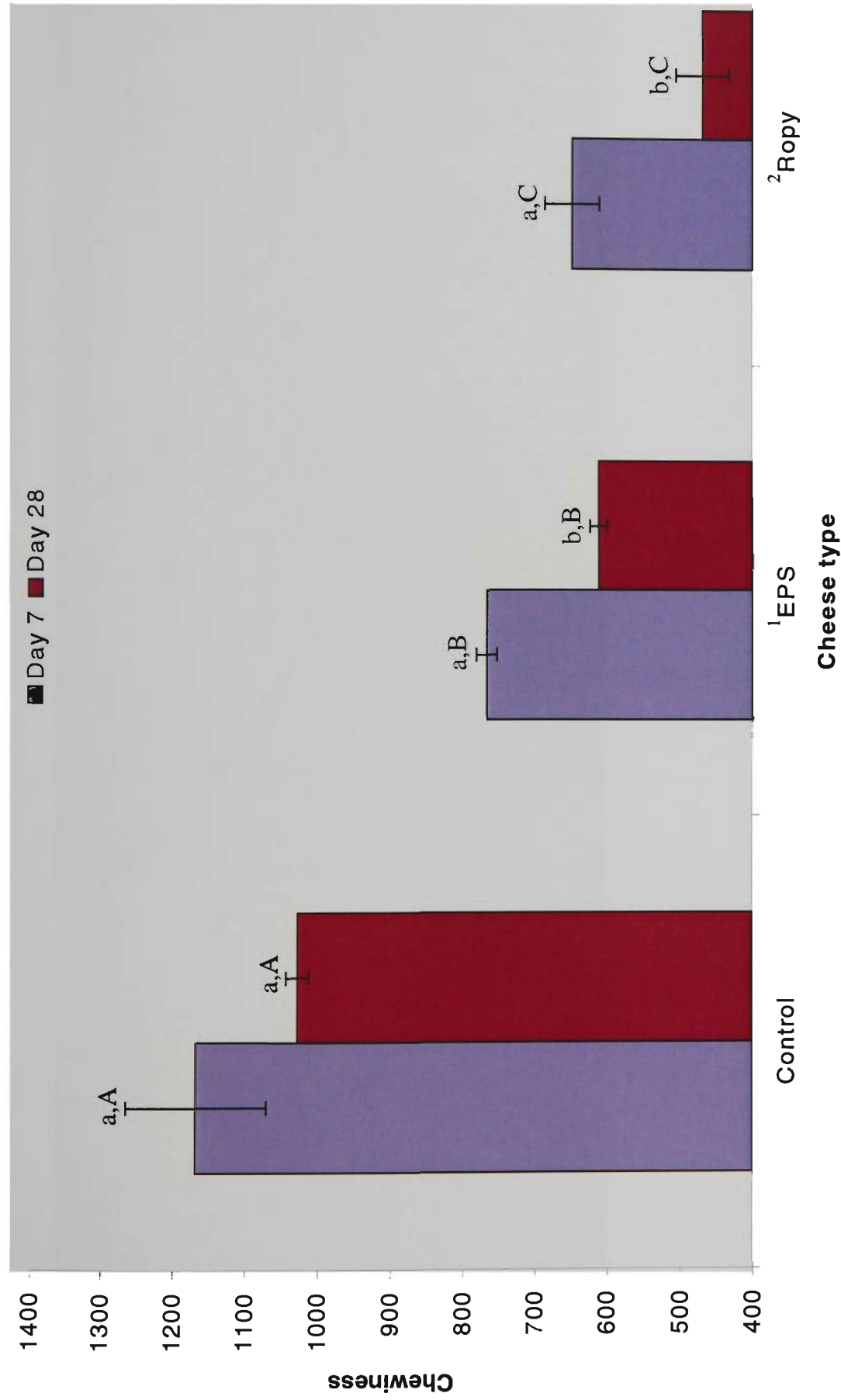
Figure 5.9. Chewiness of control, [1]EPS (cheeses made with capsular EPS producing *S. thermophilus* 285) and [2]ropy (cheeses made with ropy EPS producing *S. thermophilus* 1275) cheeses over 28 d of storage at 4°C. [abc]One-way ANOVA of means (n = 6) between d 7 and d 28 within a cheese type and [ABC]one-way ANOVA of means at d 7 and 28 between cheese types with different superscript are significantly different ($P < 0.05$).

Figure 5.10. Pizza bake characteristics of control (A), EPS (cheeses made with capsular EPS producing *S. thermophilus* 285) (B) and ropy (cheeses made with ropy EPS producing *S. thermophilus* 1275) (C) cheeses baked at 232°C for 7 min after 28 d of storage.

CHAPTER 6.0

Texture and Functionality of Low Fat Mozzarella Cheeses as Influenced by Pre-acidification with Citric Acid and use of Capsular and Ropy EPS Starter

*A version of this chapter has been submitted for publication in the International Dairy Journal.

6.1. INTRODUCTION

The calcium content in low fat Mozzarella cheeses is higher than the full fat variety and exists in the form of bound calcium phosphate with casein in a colloidal phase. Calcium contributes to the texture and functionality of cheeses by cross linking with proteins leading to hardness of the product (Geurts *et al.*, 1972; Metzger *et al.*, 2000b; Solarza and Bell, 1995; Yun *et al.*, 1994). By destabilising and removing some calcium from the casein micelles by pre-acidification of the cheese milk with a food grade acid prior to the addition of a coagulant, the amount of cross-linking between the casein polymers is reduced, thus allowing the manufacture of a softer cheese with greater flow and stretch properties. Pre-acidification has also been reported to increase the susceptibility of casein to primary proteolysis by interacting with residual coagulant (Feeney *et al.*, 2002; Joshi *et al.*, 2003). The combined action of proteolysis and reduction in calcium content has been shown to improve meltability of Mozzarella cheeses (Fife *et al.*, 1996).

Unbound moisture increases the ability of cheese particles to flow when melted and influences the overall texture and functionality of cheeses. The role of moisture on functionality of low fat Mozzarella cheeses has been investigated in various studies (Fife *et al.*, 1996; Low *et al.*, 1998; McMahon *et al.*, 1996; McMahon and Oberg, 1998; Petersen *et al.*, 2000). Low fat cheeses with improved meltability as a result of an increase in the moisture to protein ratio that was equal to or greater than that observed in the full fat variety was reported. Exopolysaccharide (EPS) producing starter bacteria have been used to promote an increase in the moisture content of low fat Mozzarella cheeses (Low *et al.*, 1998; Petersen *et al.*, 2000).

Many food-grade strains of *S. thermophilus* have the ability to synthesize EPS, which entraps moisture and retards whey expulsion. EPS has been successfully implemented to raise the moisture content of low fat Mozzarella cheeses (Low *et al.*, 1998). EPS plays a key role in the rheological behaviour and texture of fermented products without the need of additives, and

has been used to improve the functional properties of rennet set curds (Hassan and Frank, 1997; Hassan *et al.*, 1996; Perry *et al.*, 1997; Perry *et al.*, 1998; Petersen *et al.*, 2000). Several capsular strains have the ability to produce ropy EPS in addition to capsular EPS (Broadbent *et al.*, 2003; Cerning, 1990a; Duguid, 1951), including *S. thermophilus* 1275 reported earlier (Zisu and Shah, 2003). Capsular EPS is of particular importance as this form of the metabolite has little effect on whey viscosity (Low *et al.*, 1998; Perry *et al.*, 1997; Petersen *et al.*, 2000).

The amount of either form of EPS synthesised is highly variable and is strongly influenced by the culture used and growth conditions. Furthermore, production of EPS is growth associated, and dependent on temperature, pH and composition of the medium (De Vuyst *et al.*, 1998; Gamar-Nourani *et al.*, 1998; Gorret *et al.*, 2001; Grobben *et al.*, 2000; Zisu and Shah, 2003). In our earlier work, the optimum pH and temperature for EPS production for *S. thermophilus* 1275 in milk medium was found to be 5.5 and 40°C (Zisu and Shah, 2003). These conditions are reproducible during cheeses making.

The combined effects of pre-acidification and use of capsular and ropy EPS starter cultures on the texture and functionality of low fat Mozzarella cheese are not known. Similarly, little is known in regards to the synthesis of EPS in cheeses or during the cheese making process. The aims of this study were to investigate the combined effects of pre-acidification and use of capsular and ropy EPS producing strains of *S. thermophilus* on EPS production, moisture retention and textural and functional characteristics of low fat Mozzarella cheeses containing 6% fat.

6.2 MATERIALS AND METHODS

6.2.1. Starter Cultures

S. thermophilus strains 1275 (a mixed capsular/ropy EPS producer), 285 (a capsular EPS producer) and the non-EPS 1303 and *Lactobacillus delbrueckii* ssp. *bulgaricus* strain 1368

(described in Chapter 3.0) were used in this study. Working cultures were prepared as described in Chapter 3.0, section 3.2.1.

6.2.2. Cheese Making

Each batch of cheese was made with a total of 20 L of milk in 30 L capacity custom made, temperature controlled cheese vats. Fresh whole milk was obtained from Mamma Lucia Cheese (Fresh Cheese Co. Pty. Ltd., Brunswick, VIC, Australia) and separated to skim milk (0.005% fat) and cream (45% fat) using a batch 107 AE type cream separator (Alfa Laval; APV Australia, Clayton, VIC, Australia) at 50°C. The skim milk and cream were pasteurised at 72°C for 15 sec using an Alfa Laval P20 HRB pasteuriser (Alfa Laval). These served as the ingredients to standardise cheese milk at 0.5% fat that was used to make cheeses with a final fat content of 6%. The various treatments used are as indicated below:

Batch 1: _S. thermophilus_ 1303 (non-EPS) + _L. delbrueckii_ ssp. _bulgaricus_ 1368 (non-EPS) (Control),

Batch 2: Pre-acidification + _S. thermophilus_ 1303 + _L. delbrueckii_ ssp. _bulgaricus_ 1368,

Batch 3: Pre-acidification + _S. thermophilus_ 285 (capsular EPS) + _L. delbrueckii_ ssp. _bulgaricus_ 1368,

Batch 4: Pre-acidification + _S. thermophilus_ 1275 (ropy EPS) + _L. delbrueckii_ ssp. _bulgaricus_ 1368.

Starter bacteria (2.4%) were inoculated with 1.6% of _S. thermophilus_ and 0.8% of _L. delbrueckii_ ssp. _bulgaricus_ and the milk was tempered at 35°C for half an hour. An aliquot of 100 mL of milk was collected immediately after inoculation and frozen at −20°C for later analysis. Single strength chymosin (Chymax; Chr. Hansen, Pty. Ltd., Bayswater, VIC, Australia) was added at the rate of 8 mL per 20 L of milk. Milk of the relevant batches (batch 2, 3, 4) was pre-acidified to pH 6.1 using 50% (wt/vol) citric acid. A setting time of 25 min was

used except for those batches that were pre-acidified for which a setting time of 15 min was applied. The increase in milk acidity resulting from the addition of citric acid reduced coagulation time, hence, cutting of the curd occurred after only 15 min. The curd was cut using a cheese knife (1 cm^2 wire grid) based on setting time and subjective assessment of the coagulum with a knife. After cutting, the curd was cooked for 20 min where the temperature was gradually increased to 40°C. Whey was drained at pH 6.2 (pH 6.1 for pre-acidified cheeses) and stored at –20°C for later analysis. The curd was cheddered into slabs before milling at pH 5.2. Salt was added to the curd at 1.5% (wt/wt) and held for 30 min. Curd (40 g) was collected for later analysis and frozen at –20°C. The remaining salted curd was stretched for 7 min in hot water maintained at 75°C. The volume of stretching water was 2.5 times the weight of the curd containing 3% salt (wt/wt).

Cheese blocks were immediately placed into barrier bags and vacuum sealed using a Multivac A300/16 machine (Multivac Sepp Haggenmuller KG, Wolfertschwenden, Germany) before storing at 4°C in a fan forced cold room. An appropriate aliquot of each cheese was removed on the day following production and frozen at –20°C for later quantification of EPS and calcium determination.

6.2.3. Cheese Analysis

An appropriate sample size was collected from cheese blocks on the day following production and each cheese was analysed for fat (Babcock method), moisture (atmospheric oven method) and protein (Kjeldahl method) contents in accordance with AOAC methods (AOAC, 1999). Textural characteristics, stretch and meltability of each cheese were performed at d 7, 14, 28, 45 and 90. A simulated pizza bake test was carried out a d 28, 45 and 90.

6.2.4. Isolation and Quantification of Exopolysaccharides

A 100 mL aliquot of each of the frozen milk, whey and stretching water was thawed overnight at 4°C. To prepare cheese and curd for analysis, 100 mL of distilled water was mixed with 15 g of finely chopped sample and homogenised completely at 24,000 rpm for 2 min using a ultra turrax homogeniser (T25, Janke & Kunkel GmbH & CoKG, 1KA Labortechnik, Staufen, Germany). The homogenous mixture was sonicated for 90 min using a FX 14PH sonication bath (Unisonics Pty Ltd, Sydney, Australia). EPS was extracted and quantified from each sample according to the procedure described in Chapter 3.0, section 3.2.5.

6.2.5. Determination of Calcium

Determination of the calcium content in cheese, curd, milk, whey and stretching water was undertaken as described by Metzger _et al._ (2000a). Sample sizes and dilution volumes were slightly modified to allow for variations in mineral content. An aliquot of sample (0.75 g of milk and whey; 1.5 g of cheese and curd; 5 g of stretching water) was mixed with a volume of 12% trichloro-acetic acid (TCA) (wt/vol) (Sigma-Aldrich Co., St. Louis, MO, USA) (29.25 g for milk and whey samples; 48 g for cheese and curd samples; 25 g for the stretching water sample). The mixture was homogenised at 24,000 rpm for 2 min and sonicated for 90 min using the ultra turrax homogeniser and the sonication bath describes in section 6.2.4. The homogenous mixture was filtered using Whatman 541 filter paper (Whatman International Ltd., Maidstone, England). An aliquot of the filtered sample (15 g of milk and stretching water; 25 g of whey; 2 g of cheese and curd) was blended with a solution of 49.2% distilled water, 48.7% of 12% (wt/vol) trichloro-acetic acid and 2.1% of 5% (wt/vol) lanthanum oxide (75 g for milk, whey and stretching water samples; 78 g for cheese and curd samples). A calcium atomic spectroscopy standard (containing 993 μg/mL calcium) (Sigma-Aldrich) was used to prepare

working standards (in the range of 1 to 5 mg/L) in the mixture of distilled water, 12% TCA and 5% lanthanum oxide. A standard curve for the determination of calcium is shown in Figure 6.1. The distilled water, 12% TCA and 5% lanthanum oxide mixture was run as a blank and the calcium content of samples was determined using an atomic absorption spectrophotometer (Spectra AA400, Varian ustralia Pty. Ltd., Mulgrave, Victoria, Australia) with an air (13.5 L/min)/acetylene (2.0 L/min) flame.

6.2.6. Texture Profile Analysis

Textural characteristics of cheeses including hardness, adhesiveness, cohesiveness, springiness and chewiness were analysed by means of an Instron Universal Testing Machine (UTM) (5564, Instron Ltd., London, England) at room temperature (~22°C) according to the procedure described in Chapter 5.0, section 5.2.7. The details of the selected parameters have been described by (Pons and Fiszman, 1996).

6.2.7. Cheese Stretch

Stretch distance was recorded by elongation using the UTM by a modified version of the method described by (Bhaskaracharya and Shah, 2002) as used in Chapter 5.0, section 5.2.6. A cross bar spindle was placed at the bottom of a beaker containing 50 g of melted sample and the stretch limit was increased to 450 mm at a speed of 450 mm/min.

6.2.8. Pizza Bake

Shredded Mozzarella cheese (300 g) was prepared for pizza baking and baked as described in Chapter 5.0, section 5.2.8. The appearance of the cheese on the pizza was visually evaluated for shred fusion and melt, colour and the extent of blistering. The Hunter L,a,b system was used to quantify L-values (whiteness), a-values (redness) and b-values (yellowness) of cheeses before cooking, immediately after baking and after half-an-hour of cooling (to reach ~22°C) with a Minolta chroma-meter (CR-300, Minolta Corporation, Ramsey, New Jersey, USA). Multiple L,a,b values were measured (9 to 12 readings) across the entire surface of the pizza in the section covered with cheese to obtain a reliable result. Pizza baking was performed at d 28, d 45 and d 90.

6.2.9. Cheese Melt

The meltability of Mozzarella cheeses was determined in 250 mm long glass tubes with a diameter of 24 mm and thickness of 3 mm (R.B. Instruments, Mt. Eliza, VIC, Australia), using 10 g of cheese and heating at 110°C for 100 min as described in Chapter 5, section 5.2.5.

6.2.10. Statistical Analysis

Each batch of cheese was made in triplicate and results are presented as means ± standard error of replicates. Moisture, protein, hardness, adhesiveness, cohesiveness, springiness and chewiness data are an average of 9 analyses, while the results of fat, EPS, melt and stretch are representative of 6 analyses. Calcium results are an average of 6 to 12 analyses. The pizza bake experiment was replicated three times. Hunter L,a,b values obtained from the pizza bake are representative of 9 to 12 readings. To find significant differences between data, the means were

analyzed using one-way analysis of variance (ANOVA) with a 95% confidence interval using Microsoft® Excel StatPro™ (Palaside Corporation, Newfield, NY). ANOVA data with a $P <$ 0.05 was classified as statistically significant.

6.3 RESULTS AND DISCUSSION

6.3.1. Composition of Cheese

Table 6.1 shows the average composition of low fat Mozzarella cheeses. Control cheeses made with non-EPS starter and without pre-acidification had the lowest moisture content of 54.84% on a wet basis and 58.13% as moisture in non-fat substance (MNFS), exhibiting a moisture to protein (M:P) ration of 1.61. Cheeses made with pre-acidified milk and non-EPS starter exhibited a moisture content of 55.28% and 58.62% MNFS with a M:P ratio of 1.67. Although the resulting moisture was higher, the increase was not statistically significant ($P >$ 0.05). With capsular EPS synthesised by *S. thermophilus* 285 and ropy EPS produced by *S. thermophilus* 1275, there was a significant increase in the moisture content in pre-acidified cheeses to 56.67% (60.10% as MNFS) and 56.21% (59.64% as MNFS), respectively ($P <$ 0.05). The M:P ratio also increased to 1.72 and 1.79 in cheeses made with capsular EPS synthesised by *S. thermophilus* 285 and ropy EPS produced by *S. thermophilus* 1275, respectively. No significant difference in the moisture content was, however, detected between the EPS containing cheeses ($P >$ 0.05).

Table 6.2 shows EPS contents in cheeses, curds, whey, stretch water and milk. The increase in moisture retention was attributed to the water binding properties of microbial EPS. EPS was absent in control cheeses, while cheeses containing *S. thermophilus* 285 and *S. thermophilus* 1275 exhibited 30.42 mg/g and 30.55 mg/g of EPS, respectively. The EPS concentrations isolated form cheeses containing capsular and ropy EPS were similar and this was reflected in comparable moisture contents ($P >$ 0.05). It was anticipated that the amount of

ropy EPS released by _S. thermophilus_ 1275 would be greater than that produced by the capsular form which remained attached to the cells of _S. thermophilus_ 285 and is dependent on the cell numbers (Chapter 4.0; Zisu & Shah, 2003a). In an earlier study, ropy EPS was isolated in substantially greater amounts in comparison to that of the capsular type when cells were grown independently in culture media, from curds made without pre-acidification (Chapter 3.0, section 3.3.4), and from results obtained during fermentation of milk with _S. thermophilus_ 1275 (Chapter 4.0; Zisu & Shah, 2003a). In the present study, although EPS concentrations were lower than expected, they remained high enough to increase the moisture content by approximately 1%. Pre-acidification of the cheese milk could have hindered the ability of starter bacteria to produce EPS, particularly that of the ropy type due to EPS synthesis being growth associated. Pre-acidification involves rapid lowering of the pH of milk. Under regular cheese making practices, it takes a considerable amount of time to achieve the desired pH as the starter bacteria gradually release lactic acid as they multiply. Under regular cheese making conditions, the starter cultures would multiply during this stage, however, when milk is pre-acidified and processing time is shortened, cell numbers remained low affecting EPS concentrations. Furthermore, some EPS was lost (between 6 and 10 mg/mL of EPS) through the whey.

The concentrations of EPS in curds were similar to those found in cheeses made with the capsular form of EPS ($P > 0.05$). The amount of EPS was, however, significantly higher in the curd made with ropy EPS ($P < 0.05$). This was related to higher content of unbound moisture in the curd before stretching. As mentioned earlier, a considerable amount of EPS was retrieved from whey samples (6 to 10 mg/mL) as large numbers of starter culture cells were removed through whey. Despite the loss, EPS concentrations remained significantly higher in cheeses ($P < 0.05$). EPS in stretch water was minute and was not detected by the method adopted for the extraction of EPS from samples. This suggests that the majority of EPS remained within the cheese protein matrix after whey draining and stretching of the curd. The culture introduced

into cheese milk did not increase the concentration of EPS, as it was not detected in milk before processing. The build up of EPS occurred mainly during the cheese making process.

The fat content on a wet basis and as a fat in dry matter (Table 6.1) was identical between cheese types ($P > 0.05$) and was not expected to influence texture and functionality of cheeses. The protein content was influenced by the moisture increase in EPS cheeses. As the moisture content increased, the protein content became lower and the M:P ratio increased and vice versa. The calcium content was similar in cheeses made with pre-acidification ($P > 0.05$) (Table 6.3), but was lower than the calcium content in control cheeses made without an acid pre-treatment ($P < 0.05$). Variations in texture and functionality between the control and pre-acidified cheeses made with non-EPS strains of _S. thermophilus_ were attributed to lower calcium concentrations. The association between acid treatment, its influence on calcium and the related functionality of low fat Mozzarella cheeses has been demonstrated by other researchers (Guinee _et al._, 2002; Joshi _et al._, 2003; Paulson _et al._, 1998; Shakeel-Ur-Rehman _et al._, 2003).

6.3.2. Cheese Melt

Meltability of low fat Mozzarella cheeses is shown in Figure 6.2. All types of cheeses exhibited a significant increase in melt over 90 d of storage at 4°C ($P < 0.05$). An increase in melt distance occurred due to proteolysis, and hydration of unbound moisture. By d 90 of maturation, all types of cheeses exhibited a similar flow distance ($P > 0.05$). The pre-treatment of cheese milk with citric acid was applied to increase meltability further by accelerating the rate of proteolysis as demonstrated by other researchers (Joshi _et al._, 2003).

Control cheeses made without EPS and pre-acidification showed a significant increase in melt distance between d 7 (of 53 mm) and d 90 (of 72 mm) ($P < 0.05$). Although, melt distance progressively increased with storage time, a large standard error was recorded at d 7 prior to the

hydration of the cheese system. This was further emphasised by results between d 14 (of 61 mm) and d 28 (of 62 mm), which showed a significant difference to those at d 90 ($P < 0.05$).

The melt distance of control pre-acidified cheeses notably increased between d 14, d 28 and d 90 reaching 53 mm, 69 mm and 80 mm, respectively ($P < 0.05$). These cheeses displayed a short melt distance that was similar to that of the control at d 7 and d 14 ($P > 0.05$). At d 28, the melt distance was significantly greater than the control cheeses ($P < 0.05$). The accelerated rate of aging suggests that proteolysis was influenced by pre-acidification. This was supported by previous studies (Fife _et al._, 1996; Zisu and Shah, 2004).

Cheeses containing EPS made with pre-acidified milk had a significant increase in flow distance between d 7 (of 60 mm), d 14 (of 65 mm), d 45 (of 73 mm) and d 90 (of 77 mm) ($P < 0.05$). Pre-acidification was shown to increase enzyme activity and increase the rate of proteolysis in chapter 8, section 8.3.3, thereby assisting meltability. These cheeses also had a large flow distance at d 7, d 14 and d 28 and showed the greatest flow distance at d 45 ($P < 0.05$). Perry _et al._ (1997, 1998) and Petersen _et al._ (2000) reported an improvement in melt distance in low fat Mozzarella cheeses made from EPS cultures due to an increase in the moisture content.

Acid treated cheeses made with ropy EPS producing _S. thermophilus_ 1275 had an increase in flow distance between d 7 (of 54 mm), d 14 (of 63 mm) and d 90 (of 75 mm) ($P < 0.05$). Although these cheeses generally showed a good flow distance that was comparable to EPS cheese, meltability was slightly lower at d 45 ($P < 0.05$). In a separate experiment, when cheeses were made without an acid treatment, the melt distance achieved by the ropy type was superior (Chapter 5.0, section 5.3.2). Moisture seepage from ropy type cheeses was evident when melted at d 7 and d 14 and to a lesser degree at d 28. By d 45, moisture no longer exuded when heated due to the hydration of the protein matrix. Although this did not interfere with meltability results, it presented problems when attempting to stretch.

6.3.3. Texture Profile Analysis

6.3.3.1. Cheese hardness

Figure 6.3 shows the changes in hardness over 90 d of storage at 4°C. Hardness decreased between d 7 and d 90 in all cheese types ($P < 0.05$). In like manner to the factors contributing to meltability, the softening of cheeses was related to the hydration of the protein matrix and the effects of proteolysis (Chapter 8.0). Control cheeses were the hardest at d 7 ($P < 0.05$). Between d 14 and d 90, the control and non-EPS pre-acidified cheeses had a similar hardness value ($P > 0.05$). Hardness was reduced to favourable levels by d 28. Control cheeses showed a significant softening between d 7, d 14 and d 28, whereas non-EPS pre-acidified cheeses showed softening between d 28 and d 45 ($P < 0.05$).

Pre-acidification of cheese milk and use of EPS cultures significantly reduced the overall hardness ($P < 0.05$). (Hassan and Frank, 1997) found that capsular EPS reduced the tension and firmness of non-fat rennet curd to similar levels detected in curd standardised to 40 g fat per litre. Similar to non-EPS pre-acidified cheeses, those containing EPS showed significant softening after 28 d of storage ($P < 0.05$). Cheeses made with capsular and ropy EPS exhibited similar hardness ($P > 0.05$) and showed a similar pattern of softening. In our earlier work, cheeses made with ropy EPS were found to be softer than those made with capsular EPS in the absence of an acid treatment (Chapter 5.0, section 5.3.4.1). The most desirable softness was present in cheeses made with capsular EPS. Ropy EPS created a slime covering on the surface of cheeses and liquid seepage was evident when samples were compressed with the UTM up to 28 d of storage. The slime became less apparent with storage time and was not detected after 28 d.

6.3.3.2. Cheese adhesiveness

Adhesive forces measured over 90 d of storage at 4°C are given in Figure 6.4. The work necessary to overcome the forces of adhesion was low in all cheese types and increased with storage of the product. By d 90, all cheeses had similar adhesiveness values ($P < 0.05$). Control cheeses had a consistent and low adhesion over 28 d ($P > 0.05$), before increasing significantly at d 45 and d 90 ($P < 0.05$). These cheeses also showed changes in hardness and springiness at a similar time period. Adhesiveness was absent in cheeses made with pre-acidified milk and non-EPS starter at d 7 and slightly increased at d 14 and again at d 90 ($P < 0.05$). The cheeses without EPS recorded similar adhesiveness at all time points except at d 45 ($P < 0.05$) where adhesion was greater in the control batch. Adhesion was primarily monitored to investigate the effects implemented by the action of EPS and the higher moisture content associated with these cheeses. Ropy EPS in particular raised our interest as it was believed that seepage of moisture and the sticky coating of slime detected on the surface of the cheese may interfere and influence the forces of adhesion. Cheeses made with the two forms of EPS and pre-acidified milk required a greater force to overcome adhesion over the control cheeses at d 7, d 14 and d 28 in general, but not at d 45 and d 90. Capsular and ropy EPS cheeses exhibited similar adhesion between the two types at all time points ($P < 0.05$), indicating that the slime coating did not contribute to the adhesion of cheeses. Cheeses made with capsular EPS produced by *S. thermophilus* 285 showed a significant increase in adhesion between d 7 and d 14 ($P < 0.05$) and remained unchanged up to d 90 ($P > 0.05$). Hardness of these cheeses was also found to vary slightly between d 7 and d 45. Cheeses made with the ropy type of EPS produced by *S. thermophilus* 1275 showed similar adhesiveness and remained unchanged up to d 45 ($P > 0.05$) before increasing at d 90 ($P < 0.05$). In our earlier work with cheeses made without pre-acidification, adhesion was also similar in cheeses made with ropy EPS (Chapter 5.0, section 5.3.4.2).

6.3.3.3. Cheese cohesiveness

Figure 6.5 shows the cohesiveness of cheeses over 90 d stored at 4°C. The cohesiveness of cheeses was found to be similar for each variation ($P > 0.05$) with the exception of d 7 where cohesiveness was lower than other time points. The similarity in cohesiveness is related to the moisture content which varied within 2% (MNFS) between batches. This was contrary to EPS cheese made without pre-acidification which had greater moisture retention resulting in more cohesiveness (Chapter 5.0, section 5.3.4.3).

6.3.3.4 Cheese springiness

Springiness recorded over 90 d of storage at 4°C is presented in Figure 6.6. A compression of the sample to 50% of its original size was used to prevent the destruction of the specimen. In the absence of EPS cultures, cheeses made with or without pre-acidification had similar springiness ($P > 0.05$), except at d 28. They also had the greatest overall springiness, but this only became conclusively apparent beyond d 45 ($P < 0.05$). Springiness appeared to become lower with time, particularly after 90 d of storage, but the reduction was not significant ($P > 0.05$). Non-EPS cheeses resisted deformation forces and maintained their integrity throughout storage. These were also hardest, had low moisture content and showed least adhesiveness. EPS pre-acidified cheeses had lower springiness than those made with non-EPS starters, but were statistically similar up to 28 d of storage ($P > 0.05$). Capsular and ropy EPS cheeses generally showed similar springiness values ($P < 0.05$). At d 45 and at d 90, respectively, capsular and ropy EPS cheeses showed permanent structural deformation, leading to a drop in springiness ($P < 0.05$).

6.3.3.5. Cheese chewiness

Figure 6.7 shows the chewiness of cheeses measured over 90 d of storage at 4°C. As a secondary parameter corresponding to the primary parameters, chewiness was calculated as the

product of hardness × cohesiveness × springiness. Cheeses made with or without pre-acidified milk and non-EPS starter had similar chewiness ($P > 0.05$), but was greater than those made with EPS and acid treated milk ($P < 0.05$). EPS cheeses also showed similar chewiness ($P > 0.05$). Chewiness values appear to reduce with time in all cheese types, however, this parameter does not become significant until d 90 ($P < 0.05$). Results from previous work without pre-acidification also showed that chewiness was lowest in ropy type cheeses and there was a reduction in chewiness between d 7 and d 28 in cheeses made with both types of EPS (Chapter 5.0, section 5.3.4.5).

6.3.4. Cheese Stretch

Figure 6.8 represents the stretch performance of cheeses over 90 d of storage at 4°C. The lowest stretch distance was recorded at d 7 in all cheese types ($P < 0.05$). By d 90 all cheeses exhibited greatest stretch and showed significant improvement from the initial measurements at d 7 ($P < 0.05$). Unlike results from d 7, there was no significant difference identified between cheese varieties ($P > 0.05$).

At d 7, control cheeses had the lowest stretch ($P < 0.05$), reaching 295 mm. By d 14, control cheeses increased stretch significantly to 384 mm. The stretch of these cheeses also became similar to all other variables ($P < 0.05$). No substantial increases were noted at d 28 (of 395 mm) and d 45 (of 347 mm) ($P > 0.05$) until reaching 437 mm at d 90 ($P < 0.05$). At d 45, the stretch distance of control cheeses was again lower than that observed in the EPS cheeses ($P < 0.05$), although, it was comparable to that of the control pre-acidified cheeses ($P > 0.05$). Non-EPS pre-acidified cheeses showed a similar stretch at d 7 (of 382 mm) and d 28 (of 331 mm) ($P > 0.05$). At d 7, stretch was similar to the EPS cheeses (of 385 mm) ($P > 0.05$). The stretch distance significantly increased to 450 mm at d 90 ($P < 0.05$).

At d 7, capsular EPS cheeses made with pre-acidified milk showed a substantial stretch distance of 385 mm, and there was no significant improvement until d 90 where a stretch distance of 450 mm was recorded ($P < 0.05$). Ropy type pre-acidified cheeses did not register any readings at d 7 and will be discussed in the section to follow. At d 14, a stretch distance of 391 mm was recorded. Between d 14 and d 28 (of 400 mm) there was no significant change in the stretch distance recorded ($P > 0.05$) until d 45 (of 434 mm) and d 90 (of 450 mm) ($P < 0.05$).

Several cheese samples were particularly tough and brittle at d 7 and analyses were lost. The cheese shreds did not completely fuse causing several samples to lift away from the bottom of the beaker as a mass when attempting to stretch (similar to Figure 6.9, D). Control cheeses and those made from pre-acidified milk performed acceptably at d 7 (Figure 6.9, A and B, respectively) and d 14. At d 28 and beyond, control cheeses began to deteriorate and displayed a thin strand formation when stretched creating an unappealing and inferior appearance (Figure 6.9, E). Control pre-acidified cheeses continued to show a higher quality stretch throughout the storage (Figure 6.9, F). Cheeses made with ropy EPS, however, exuded an excessive amount of unbound water when heated at d 7. The build-up of water in beakers caused samples to slip and lift away from the bottom of the beaker resulting in loss of data for all replicates of the ropy type cheeses (Figure 6.9, D). The surface of cheeses was also coated in an undesired layer of sticky slime. The seepage of moisture and slime observations continued at d 14 and to a lesser degree at d 28 (Figure 6.9, H), however, cheeses became more pliable with time and a smoother stretch was achieved. After d 45 and d 90 of storage, unbound water was absorbed into the protein matrix and slime formation had dissipated. Unbound water also seeped out of EPS cheeses at d 7 (Figure 6.9, C), however, this was not evident at d 14 of storage and beyond following the subsequent hydration of the casein protein matrix. EPS cheeses exhibited a fuller, smoother and more fibrous stretch throughout the storage and were best overall (Figure 6.9, G). Our observations suggest that the water holding capacity of EPS, particularly the loose, ropy type, is low on heating and unbound moisture within cheese is easily lost prior to hydration of the casein

proteins. Although a similar concentration of capsular and ropy EPS was isolated from cheeses, the uniqueness of each type of EPS may have caused it to behave differently in the final product. The ropy form is less acceptable in cheese making not only due to its interference during whey processing, but also due to the problems created in cheeses.

6.3.5 Pizza Bake

Results from the pizza bake tests performed at d 28 and d 90 are shown in Figure 6.10, A to H. The browning behaviour, melt and flow of shredded cheese were evaluated subjectively after baking at 232°C in a similar fashion to that described by other researchers (Metzger _et al._, 2000a; Rudan and Barbano, 1998a; Rudan and Barbano, 1998b). When baked at d 28, all cheese types performed poorly, exhibiting a high degree of scorching due to high moisture loss from the cheese shreds, leading to incomplete shred fusion and inadequate melt (Figure 6.10, A to D). By d 90, a substantial improvement in melt, flow, shred fusion and a reduction in surface blistering was evident for all type of cheeses (Figure 6.10, E to H). Control and non-EPS pre-acidified cheeses (Figure 6.10, E and F, respectively) continued to show a degree of inadequate shred fusion. EPS and ropy type cheeses made with acid treated milk (Figure 6.10, G and H, respectively) showed an acceptable appearance after baking. Although the pizza bake images at d 45 are not shown, there was a substantial improvement in the pizza bake characteristics compared to those observed at d 28. At d 45, cheeses appeared similar to those observed at d 90.

6.3.5.1. L,a,b values: before cooking

Table 6.4 shows L-values, representing cheese whiteness before cooking, immediately after baking and after cooling for half-an-hour at d 28, d 45 and d 90 of storage. Due to the similarity in cheese composition, no significant difference in L-values was observed between

cheeses prior to cooking ($P > 0.05$), with the exception of the EPS cheeses, which showed greater L-values at d 45 ($P < 0.05$).

Table 6.5 shows a-values, representing redness before cooking, immediately after baking and after cooling for half an hour at d 28, d 45 and d 90 of storage. The a-values were similar between cheeses before cooking at d 28, however, EPS based cheeses appear to have lower a-values after storage, particularly at d 45 ($P < 0.05$). Furthermore, the redness of cheeses increased during storage between d 28 and d 90.

Table 6.6 shows b-values, representing yellowness before cooking, immediately after baking and after cooling for half-an-hour at d 28, d 45 and d 90 of storage. Before cooking, cheeses exhibited similar b-values that did not change over time between d 28 and d 90.

6.3.5.2. L,a,b values: warm

Whiteness increased significantly immediately after baking ($P < 0.05$). L-values were often greater in EPS containing cheeses due to a higher moisture content, but were similar between cheeses made with the two types of EPS. Highest degree of scorching was measured in control cheeses at all time points. Pre-acidified cheeses, although similar to the control ($P > 0.05$) performed better and L-values were comparable to those exhibited by the EPS batches after baking. All cheese types showed an increase in L-values between d 28 and d 45 ($P < 0.05$). No such improvement occurred between d 45 and d 90, suggesting that aging low fat Mozzarella cheese in excess of 45 d was not required to achieve good pizza bake performance.

After baking, a-values increased due to browning of the cheese surface ($P < 0.05$). When cheeses were warm, a-values were lower in EPS cheeses due to higher moisture content signifying a reduction in scorching. After maturation for 45 d and 90 d, surface scorching was reduced (Figure 6.10, E to H) and a-values became similar between cheeses ($P > 0.05$). Cheeses made with ropy _S. thermophilus_ 1275, however, had lower a-values (_ie._ less scorching) than control cheeses. Pre-acidification did not influence a-values and they were not different

between non-EPS cheeses. The b-values of cheeses also increased after baking ($P < 0.05$) but showed a similar degree of yellowness between cheese types.

6.3.5.3 L,a,b values: cool

When cooled, all cheeses became translucent and shiny in appearance. L-values became lower and were similar to those recorded before cooking (section 6.3.5.1). Cheeses containing EPS exhibited higher L-values. This was also the pattern observed when cheeses were warm. The cheeses made with non-EPS starter were similar, however, the pre-acidification treatment did improve baking characteristics and showed less burning, _ie._ greater L-values over the control cheeses at d 45 ($P < 0.05$). At d 28 and d 45, ropy cheeses showed the highest readings followed by the EPS cheeses and the control cheeses ($P < 0.05$). At d 45, the two EPS cheeses were similar in L-values ($P > 0.05$).

After cooling the pizzas, a-values were similar between cheese, except ropy EPS based cheeses which were lower than controls. The b-values of cheeses were also similar but remained greater to those before cooking (section 6.3.5.1).

6.4 CONCLUSIONS

The control cheeses showed lowest moisture content. Pre-acidification of the cheese milk did not influence moisture retention unless made with EPS producing cultures. Pre-acidification improved texture and functionality during the early stages of storage. Stretch distance, although similar to that recorded in the control cheeses, improved overall upon pre-acidification. EPS concentrations were similar in pre-acidified cheeses made with capsular EPS producing _S. thermophilus_ 285 and ropy EPS producing _S. thermophilus_ 1275. The moisture retention was similar between the two batches of cheeses. Ropy EPS showed poor water holding capacity with excessive of moisture seepage when cheeses were heated during the first 28 d of storage. Furthermore, the cheese surface was coated with a sticky layer of slime. Upon

hydration with unbound moisture, ropy EPS cheeses showed good characteristics and were indistinguishable from those made with capsular EPS. EPS improved the overall textural and functional characteristics of cheeses. Upon baking, EPS cheeses generally exhibited good characteristics after 45 d of storage and maturation to 90 d was not required to achieve acceptable functionality. Given that capsular and ropy EPS have similar beneficial effects on low fat Mozzarella cheeses when milk is pre-acidified, the selective use of the capsular EPS producing strains over the ropy type is rational when considering the implications associated with slime formation and utilisation of whey.

Table 6.1. Composition of low fat Mozzarella cheeses (mean ± standard error).

Cheese	Moisture (%) (n = 9)	Fat (%) (n = 6)	[1]MNFS (%) (n = 6)	[2]FDM (%) (n = 6)	Protein (%) (n = 9)	[3]M:P
Control	54.84 ± 0.17[c]	5.66 ± 0.07[a]	58.13 ± 0.12[c]	12.53 ± 0.12[a]	34.01 ± 0.33[a]	1.61
Control [4]pre-acid	55.28 ± 0.26[bc]	5.69 ± 0.06[a]	58.62 ± 0.16[bc]	12.72 ± 0.15[a]	33.10 ± 0.48[a]	1.67
[5]EPS pre-acid	56.67 ± 0.44[a]	5.71 ± 0.09[a]	60.10 ± 0.21[a]	13.18 ± 0.34[a]	32.34 ± 1.10[ab]	1.72
[6]Ropy pre-acid	56.21 ± 0.23[a]	5.75 ± 0.05[a]	59.64 ± 0.14[a]	13.13 ± 0.14[a]	31.39 ± 0.42[b]	1.79

[abc]One-way ANOVA of means in a column with different superscript are significantly different ($P < 0.05$).

[1]MNFS = Moisture in non-fat substance.

[2]FDM = Fat in dry matter.

[3]M:P = Moisture to protein ratio based on final (average) moisture and protein contents.

[4]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

[5]EPS = Capsular exopolysaccharides (EPS) produced by *S. thermophilus* 285.

[6]Ropy = Ropy EPS produced by *S. thermophilus* 1275.

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Table 6.2. EPS extracted from cheese, curd, whey, stretch water and milk (n = 6 ± standard error).

Sample	Control	Control¹ pre-acid	²EPS pre-acid	³Ropy pre-acid
Cheese (mg/g)	^{4}ND^{c,A}	ND^{bc,A}	30.42 ± 1.71^{a,A}	30.55 ± 3.16^{a,A}
Curd (mg/g)	ND^{c,A}	ND^{bc,A}	36.353 ± 1.24^{a,A}	43.40 ± 5.30^{a,B}
Whey (mg/mL)	ND^{c,A}	ND^{bc,A}	6.45 ± 3.33^{a,B}	10.77 ± 4.98^{a,C}
Stretch water (mg/mL)	ND^{a,A}	ND^{a,A}	ND^{a,CD}	ND^{a,DE}
Milk (mg/mL)	ND^{a,A}	ND^{a,A}	ND^{a,D}	ND^{a,E}

^{abc}One-way ANOVA of means across a row and ^{ABC}one-way ANOVA of means in a column with different superscript are significantly different (P <0.05).

¹Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

²EPS = Capsular exopolysaccharides (EPS) produced by S. thermophilus 285.

³Ropy = Ropy EPS produced by S. thermophilus 1275.

⁴ND = Not detected.

Table 6.3. Calcium content in cheese, curd, whey, stretch water and milk (n = 6 to 12 ± standard error).

Sample (mg/100g)	Control	Control[1] pre-acid	[2]EPS pre-acid	[3]Ropy pre-acid
Cheese	1234.23 ± 21.93[a,A]	1181.72 ± 7.60[bcd,A]	1143.43 ± 18.51[cd,A]	1142.99 ± 18.45[d,A]
Cheese (mg/g protein)	16.39 ± 0.28[a,D]	15.96 ± 0.32[bcd,D]	15.32 ± 0.38[d,D]	15.95 ± 0.22[cd,D]
Curd	1251.83 ± 19.93[a,A]	1147.11 ± 15.09[d,A]	1176.80 ± 21.37[cd,A]	1187.17 ± 19.42[bcd,A]
Whey	51.62 ± 2.24[a,C]	55.95 ± 1.92[a,C]	52.95 ± 0.88[a,C]	52.41 ± 1.29[a,C]
Stretch water	9.54 ± 0.43[a,E]	10.42 ± 0.65[a,E]	9.31 ± 0.85[a,E]	9.22 ± 0.23[a,E]
Milk	116.37 ± 2.02[a,B]	121.78 ± 3.29[a,B]	119.99 ± 0.35,B[a]	107.52 ± 4.91[a,B]

[abc]One-way ANOVA of means across a row and [ABC]one-way ANOVA of means in a column with different superscript are significantly different (P < 0.05).
[1]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.
[2]EPS = Capsular exopolysaccharides (EPS) produced by S. thermophilus 285.
[3]Ropy = Ropy EPS produced by S. thermophilus 1275.

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Table 6.4. Mean Hunter L-values (n = 9 to 12 ± SE) for whiteness measurements of low fat Mozzarella cheeses for given variables, treatments and processing stages.

	Control	Control ^{1}pre-acid	^{2}EPS pre-acid	^{3}Ropy pre-acid
Before cook$_{28}$	63.04 ± 0.75^{a,EFGH}	63.43 ± 0.73^{a,GH}	63.37 ± 0.49^{a,H}	63.43 ± 0.94^{a,H}
Before cook$_{45}$	64.62 ± 0.45^{bc,DE}	63.89 ± 0.95^{c,EFGH}	66.56 ± 0.44^{a,EF}	65.77 ± 0.51^{abc,FG}
Before cook$_{90}$	65.82 ± 0.92^{a,CDE}	65.38 ± 0.51^{a,CDEF}	65.95 ± 0.52^{a,FG}	64.30 ± 0.62^{a,GH}
Warm$_{28}$	68.29 ± 1.68^{b,BC}	70.51 ± 0.86^{ab,B}	72.56 ± 0.47^{a,B}	71.80 ± 0.64^{ab,BCD}
Warm$_{45}$	73.57 ± 0.82^{d,A}	74.96 ± 0.64^{cd,A}	75.26 ± 0.72^{bcd,A}	77.74 ± 0.49^{a,A}
Warm$_{90}$	73.26 ± 0.86^{b,A}	75.35 ± 0.87^{ab,A}	76.85 ± 0.48^{a,A}	76.73 ± 10.41^{a,A}
Cool$_{28}$	61.52 ± 1.61^{d,H}	63.66 ± 0.80^{cd,FGH}	67.53 ± 10.8^{b,DEF}	70.33 ± 0.50^{a,D}
Cool$_{45}$	61.69 ± 0.75^{c,GH}	64.79 ± 0.85^{b,DEFG}	68.78 ± 0.77^{a,CDE}	70.42 ± 0.67^{a,CD}
Cool$_{90}$	61.73 ± 0.96^{cd,FGH}	60.42 ± 0.64^{d,H}	64.43 ± 0.59^{b,GH}	67.79 ± 0.52^{a,E}

^{abc}One-way ANOVA of means across a row and ^{ABC}one-way ANOVA of means in a column with different superscript are significantly different (P < 0.05).

^{1}Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

^{2}EPS = Capsular exopolysaccharides (EPS) produced by *S. thermophilus* 285.

^{3}Ropy = Ropy EPS produced *S. thermophilus* 1275.

$_{28,45,90}$Pizza bake performed after 28, 45 and 90 d of storage at 4°C.

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Table 6.5. Mean Hunter a-values (n = 9 to 12 ± SE) for redness measurements of low fat Mozzarella cheeses for given variables, treatments and processing stages.

	Control	Control[1] pre-acid	[2]EPS pre-acid	[3]Ropy pre-acid
Before cook$_{28}$	$-2.15 \pm 0.15^{a,D}$	$-2.20 \pm 0.14^{a,G}$	$-2.22 \pm 0.18^{a,E}$	$-2.21 \pm 0.22^{a,FG}$
Before cook$_{45}$	$-1.85 \pm 0.08^{a,CD}$	$-1.73 \pm 0.21^{a,FG}$	$-2.15 \pm 0.11^{bc,DE}$	$-2.30 \pm 0.12^{c,G}$
Before cook$_{90}$	$-1.50 \pm 0.16^{abc,BC}$	$-1.32 \pm 0.11^{a,EFG}$	$-1.72 \pm 0.13^{c,C}$	$-1.66 \pm 0.11^{bc,CDE}$
Warm$_{28}$	$1.86 \pm 1.15^{a,A}$	$0.86 \pm 0.54^{a,AB}$	$-1.05 \pm 0.36^{c,BC}$	$-0.90 \pm 0.58^{bc,ABCD}$
Warm$_{45}$	$0.03 \pm 0.60^{a,AB}$	$-0.75 \pm 0.40^{a,CDE}$	$-0.85 \pm 0.43^{a,ABC}$	$-2.16 \pm 0.26^{b,EFG}$
Warm$_{90}$	$0.45 \pm 0.29^{a,A}$	$-0.23 \pm 0.38^{ab,BCD}$	$-0.68 \pm 0.40^{ab,AB}$	$-0.98 \pm 0.29^{b,BCD}$
Cool$_{28}$	$0.73 \pm 0.48^{a,A}$	$0.96 \pm 0.50^{a,AB}$	$-0.02 \pm 0.29^{ab,A}$	$-0.93 \pm 0.40^{b,ABCD}$
Cool$_{45}$	$0.26 \pm 0.34^{a,A}$	$-0.83 \pm 0.38^{b,DE}$	$-0.53 \pm 0.53^{ab,ABC}$	$-2.03 \pm 0.41^{c,DEFG}$
Cool$_{90}$	$0.85 \pm 0.46^{ab,A}$	$1.53 \pm 0.53^{a,A}$	$0.13 \pm 0.32^{b,A}$	$0.21 \pm 0.46^{ab,A}$

[abc]One-way ANOVA of means across a row and [ABC]one-way ANOVA of means in a column with different superscript are significantly different (P <0.05).

[1]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

[2]EPS = Capsular exopolysaccharides (EPS) produced by *S. thermophilus* 285.

[3]Ropy = Ropy EPS produced *S. thermophilus* 1275.

[28,45,90]Pizza bake performed after 28, 45 and 90 d of storage at 4°C.

Table 6.6. Mean Hunter b-values (n = 9 to 12 ± SE) for yellowness measurements of low fat Mozzarella cheeses for given variables, treatments and processing stages.

	Control	Control [1]pre-acid	[2]EPS pre-acid	[3]Ropy pre-acid
Before cook[28]	19.25 ± 0.27[a,DE]	18.41 ± 0.49[abc,E]	18.34 ± 0.26[bc,H]	17.76 ± 0.58[c,EF]
Before cook[45]	17.61 ± 0.21[a,E]	16.74 ± 0.53[a,F]	16.99 ± 0.36[a,I]	16.85 ± 0.36[a,F]
Before cook[90]	19.56 ± 0.59[a,CDE]	19.08 ± 0.36[ab,DE]	19.09 ± 0.30[a,GH]	18.03 ± 0.40[b,DE]
Warm[28]	28.71 ± 1.02[a,A]	27.64 ± 0.72[a,AB]	27.03 ± 0.33[a,CDE]	26.97 ± 0.87[a,ABC]
Warm[45]	26.93 ± 0.60[a,A]	26.05 ± 0.42[ab,B]	26.42 ± 0.61[ab,E]	25.20 ± 0.56[b,C]
Warm[90]	26.50 ± 0.97[a,A]	26.48 ± 0.74[a,AB]	26.56 ± 0.64[a,DE]	25.84 ± 0.64[a,ABC]
Cool[28]	28.42 ± 0.62[a,A]	26.66 ± 0.64[a,AB]	27.50 ± 0.34[a,BCDE]	27.05 ± 0.56[a,AB]
Cool[45]	27.36 ± 0.48[ab,A]	27.95 ± 0.56[ab,A]	29.17 ± 0.62[a,A]	27.17 ± 0.44[b,A]
Cool[90]	23.69 ± 0.37[ab,B]	22.14 ± 0.68[b,C]	23.84 ± 0.49[ab,F]	25.64 ± 0.41[a,BC]

[abc]One-way ANOVA of means across a row and [ABC]one-way ANOVA of means in a column with different superscript are significantly different ($P < 0.05$).

[1]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.
[2]EPS = Capsular exopolysaccharides (EPS) produced by *S. thermophilus* 285.
[3]Ropy = Ropy EPS produced *S. thermophilus* 1275.
[28,45,90]Pizza bake performed after 28, 45 and 90 d of storage at 4°C.

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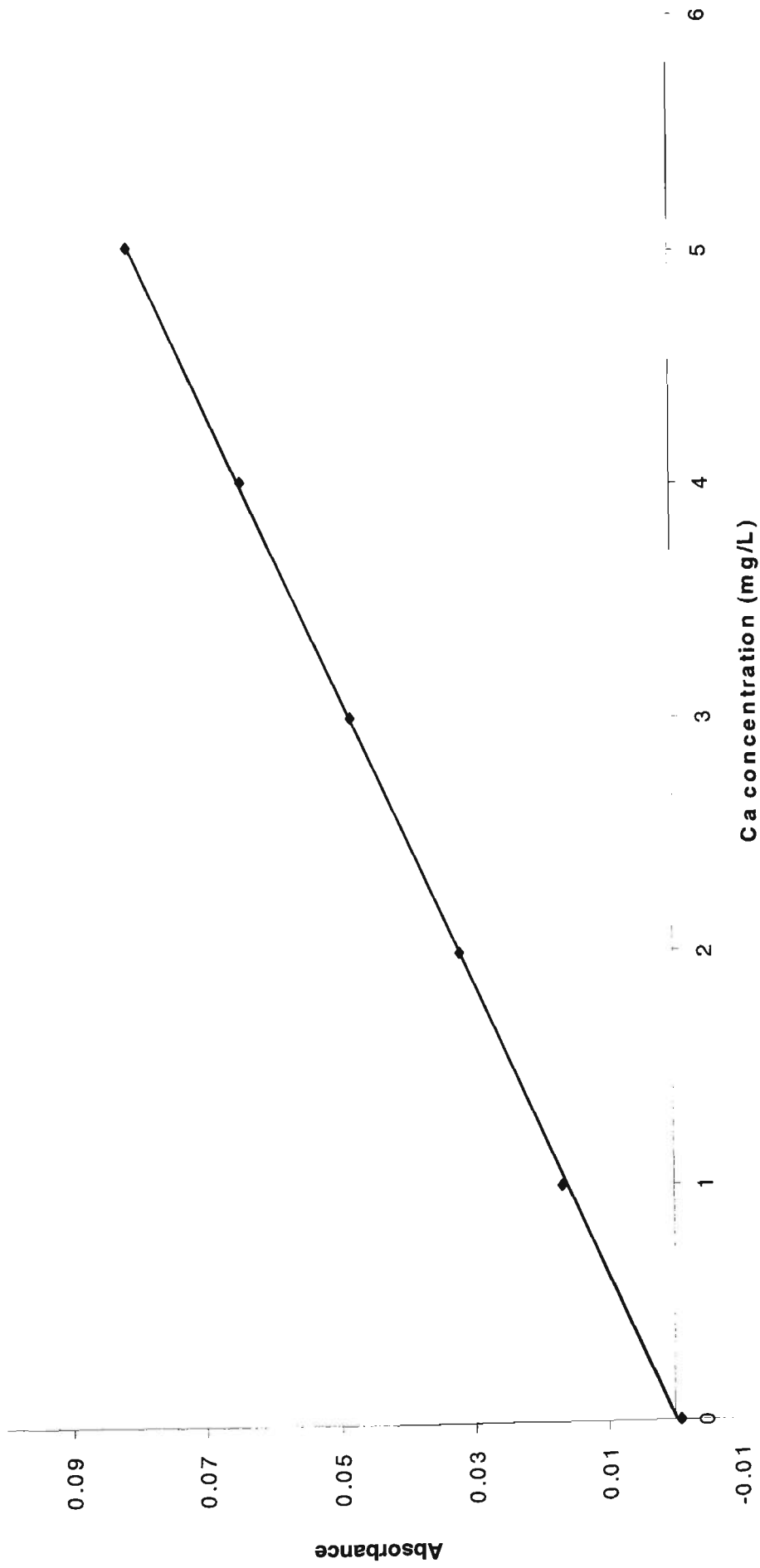
Figure 6.1. Standard curve for the determination of calcium in cheese, curd, milk, whey and stretching water by atomic absorption spectroscopy.
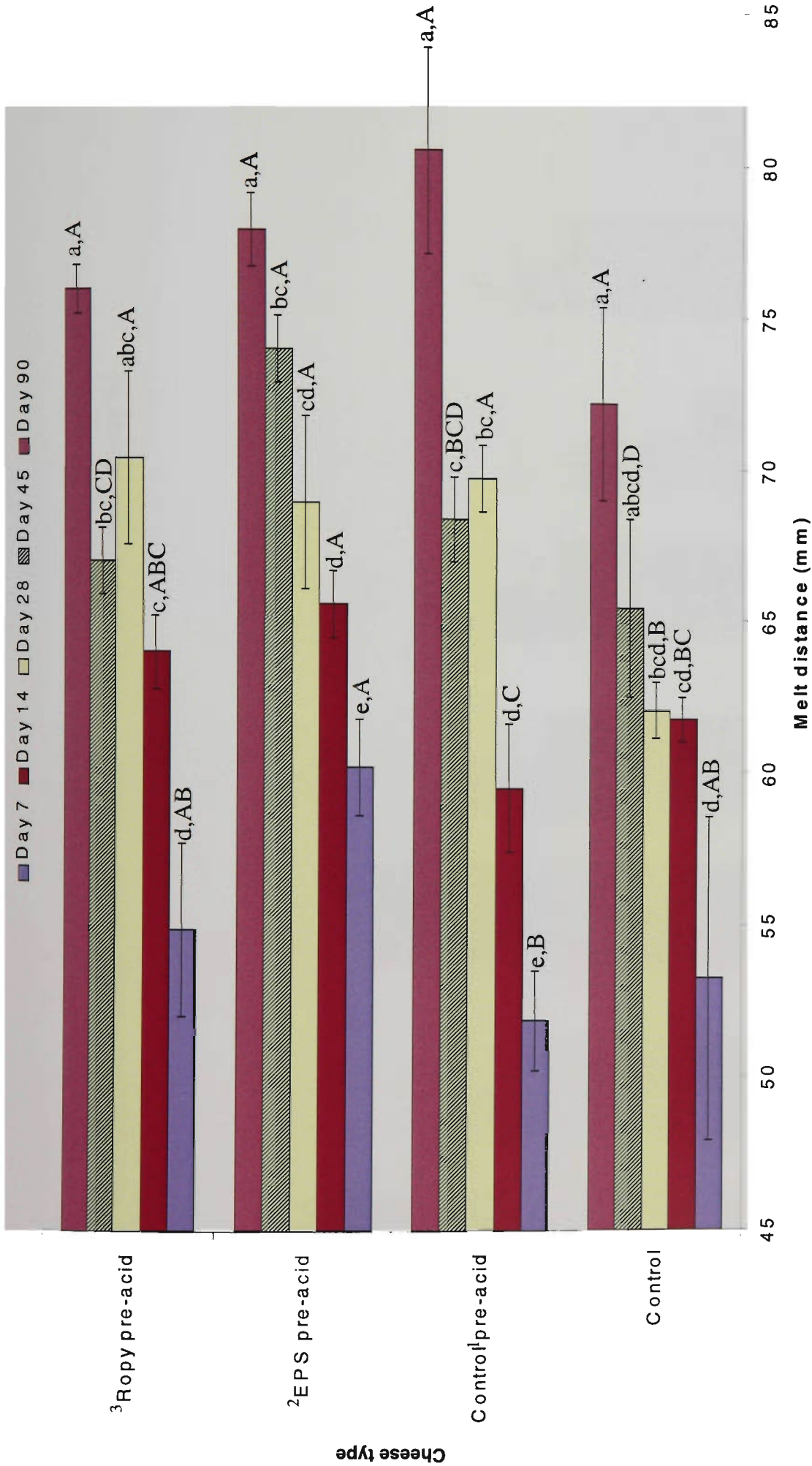
Figure 6.2. Cheese melt of control, control [1] pre-acid (cheese milk pre-acidified to pH 6.1), [2]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285) pre-acid, and [3]ropy (ropy EPS produced *S. thermophilus* 1275) pre-acid cheeses over 90 d of storage at 4°C. [abc]One-way ANOVA of means ($n = 6$) between d 7 and d 90 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28, 45 and 90 between cheese types with different superscript are significantly different ($P < 0.05$).

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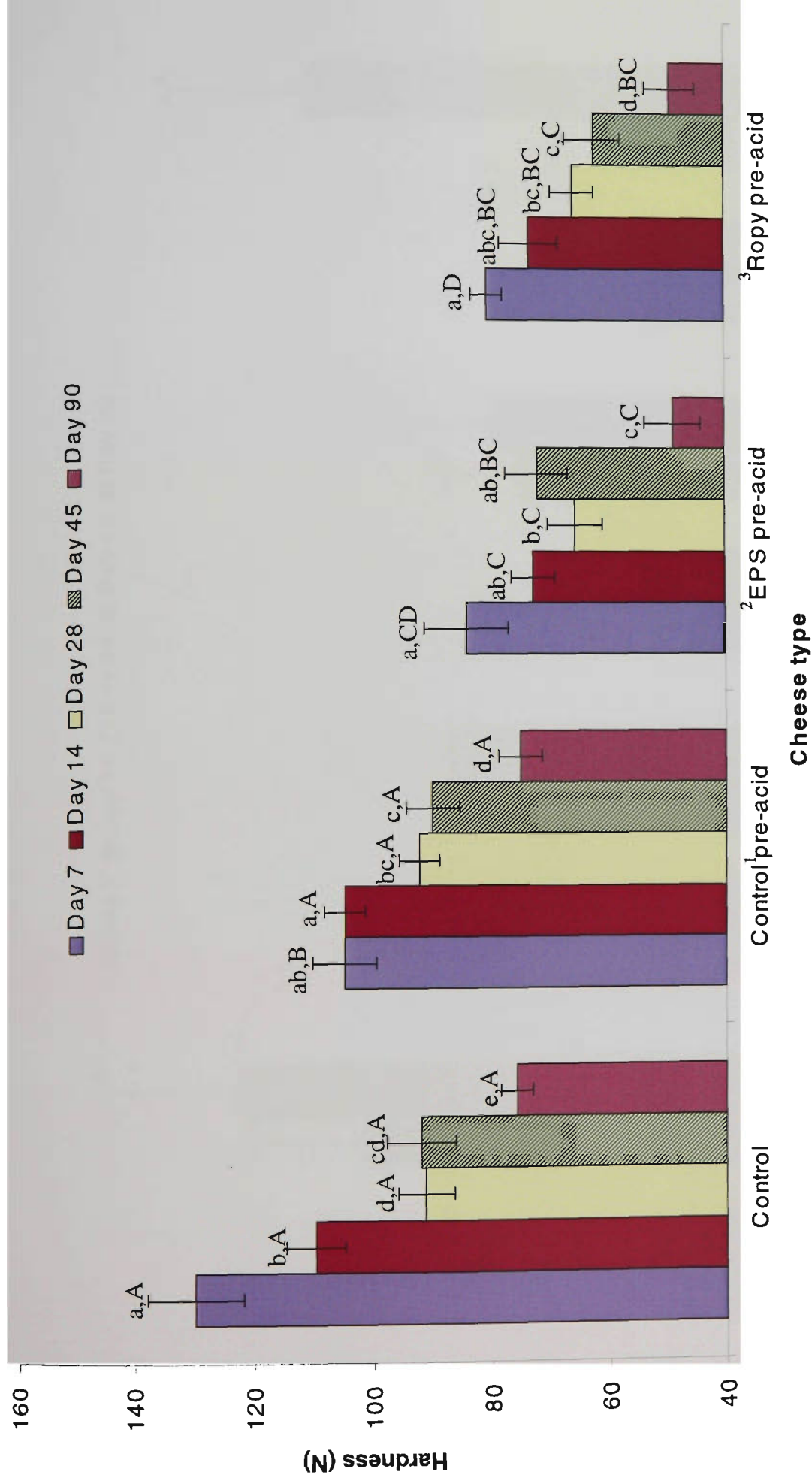
Figure 6.3. Hardness of control, control [1] pre-acid (cheese milk pre-acidified to pH 6.1), [2]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285) pre-acid, and [3]ropy (ropy EPS produced *S. thermophilus* 1275) pre-acid cheeses over 90 d of storage at 4°C. [abc]One-way ANOVA of means (n = 9) between d 7 and d 90 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28, 45 and 90 between cheese types with different superscript are significantly different (*P* < 0.05).

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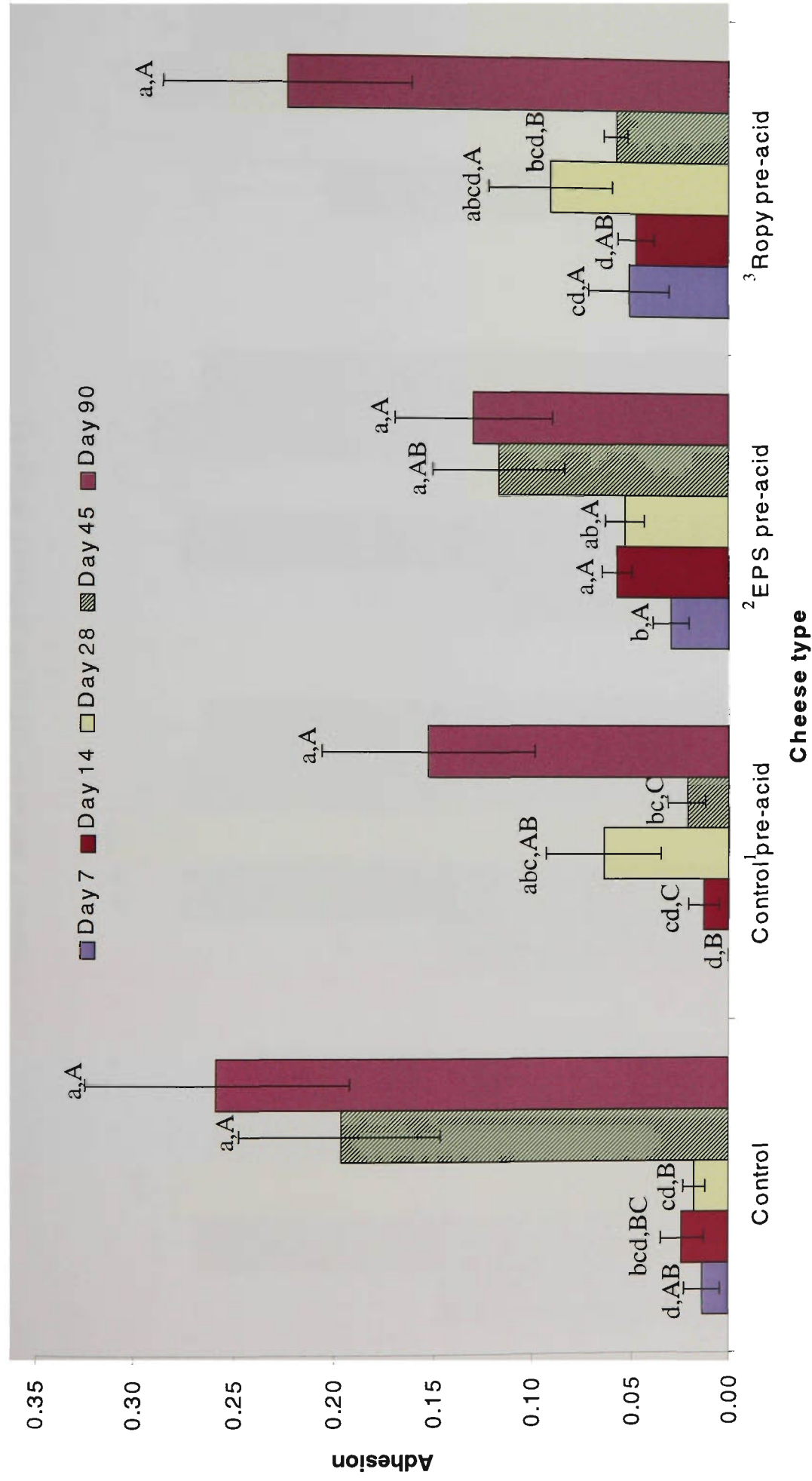
Figure 6.4. Adhesiveness of control, control [1] pre-acid (cheese milk pre-acidified to pH 6.1), [2]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285) pre-acid, and [3]ropy (ropy EPS produced *S. thermophilus* 1275) pre-acid cheeses over 90 d of storage at 4°C. [abc]One-way ANOVA of means (n = 9) between d 7 and d 90 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28, 45 and 90 between cheese types with different superscript are significantly different (*P* < 0.05).

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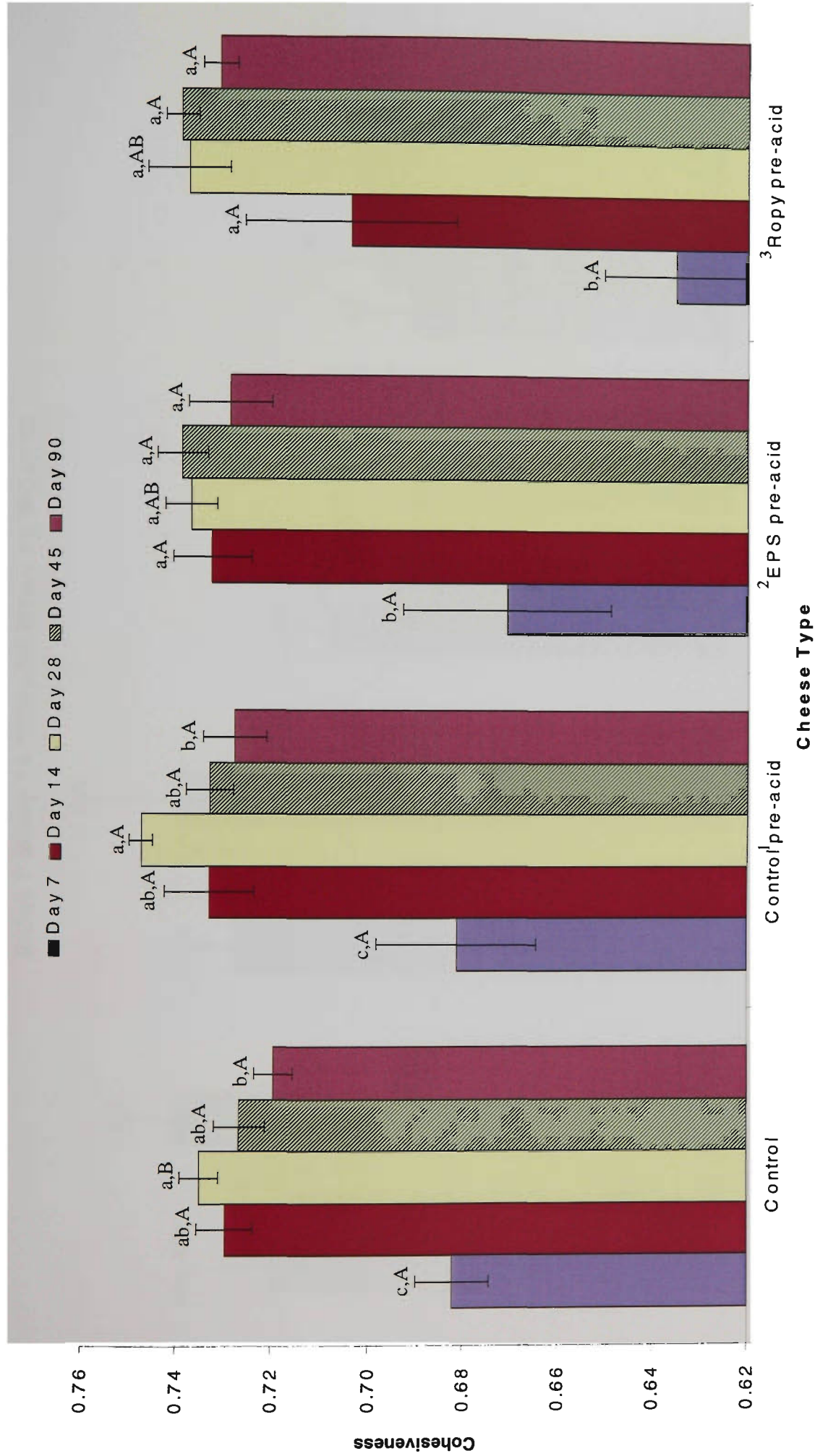
Figure 6.5. Cohesiveness of control, control [1] pre-acid (cheese milk pre-acidified to pH 6.1), [2]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285) pre-acid, and [3]ropy (ropy EPS produced *S. thermophilus* 1275) pre-acid cheeses over 90 d of storage at 4°C. [abc]One-way ANOVA of means (n = 9) between d 7 and d 90 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28, 45 and 90 between cheese types with different superscript are significantly different ($P < 0.05$).

154
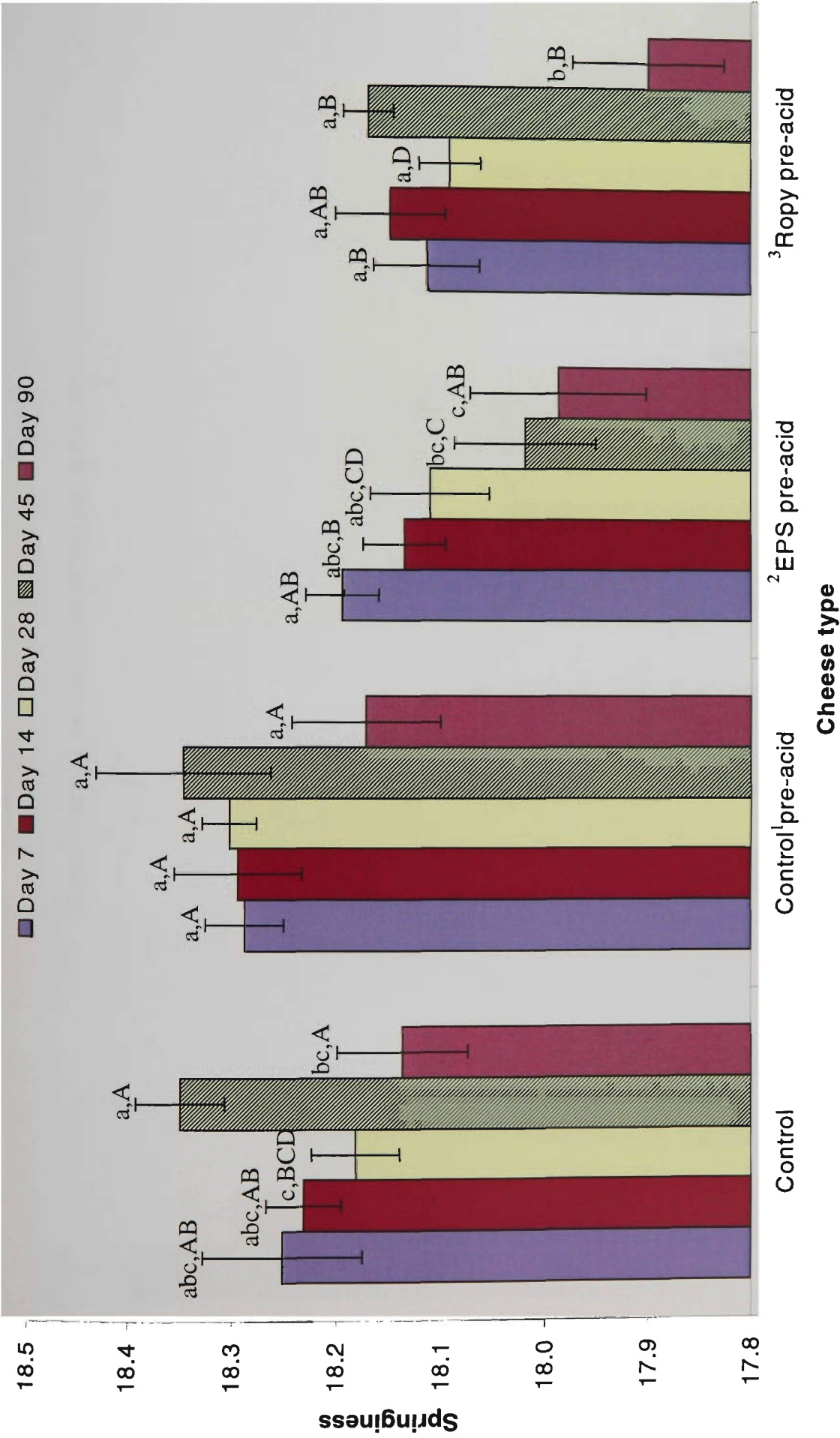
Figure 6.6. Springiness of control, control [1] pre-acid (cheese milk pre-acidified to pH 6.1), [2]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285) pre-acid, and [3]ropy (ropy EPS produced *S. thermophilus* 1275) pre-acid cheeses over 90 d of storage at 4°C. [abc]One-way ANOVA of means (n = 9) between d 7 and d 90 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28, 45 and 90 between cheese types with different superscript are significantly different (*P* < 0.05).

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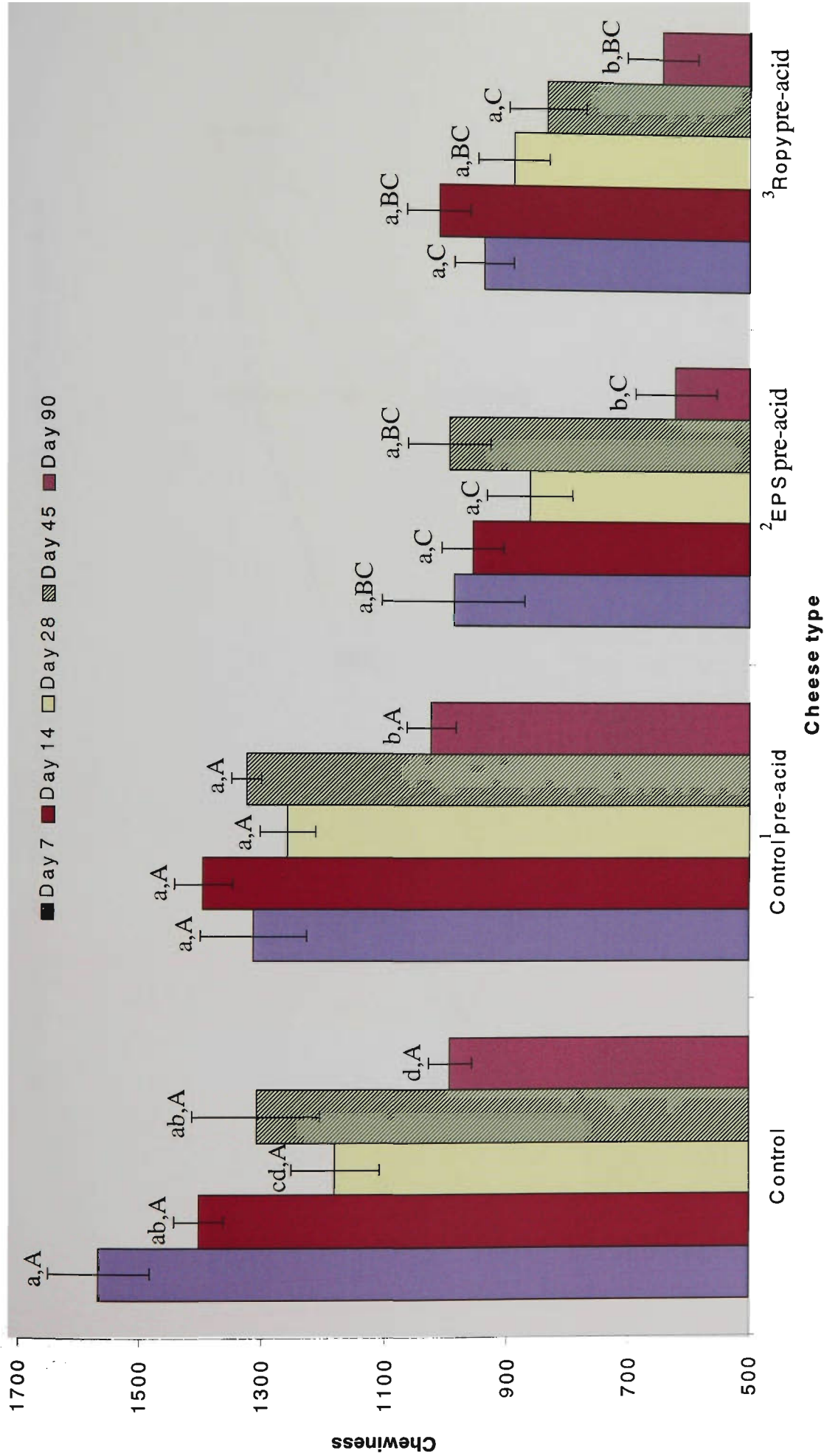
Figure 6.7. Chewiness of control, control [1] pre-acid (cheese milk pre-acidified to pH 6.1), [2]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285) pre-acid, and [3]ropy (ropy EPS produced *S. thermophilus* 1275) pre-acid cheeses over 90 d of storage at 4°C. [abc]One-way ANOVA of means (n = 9) between d 7 and d 90 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28, 45 and 90 between cheese types with different superscript are significantly different (*P* < 0.05).

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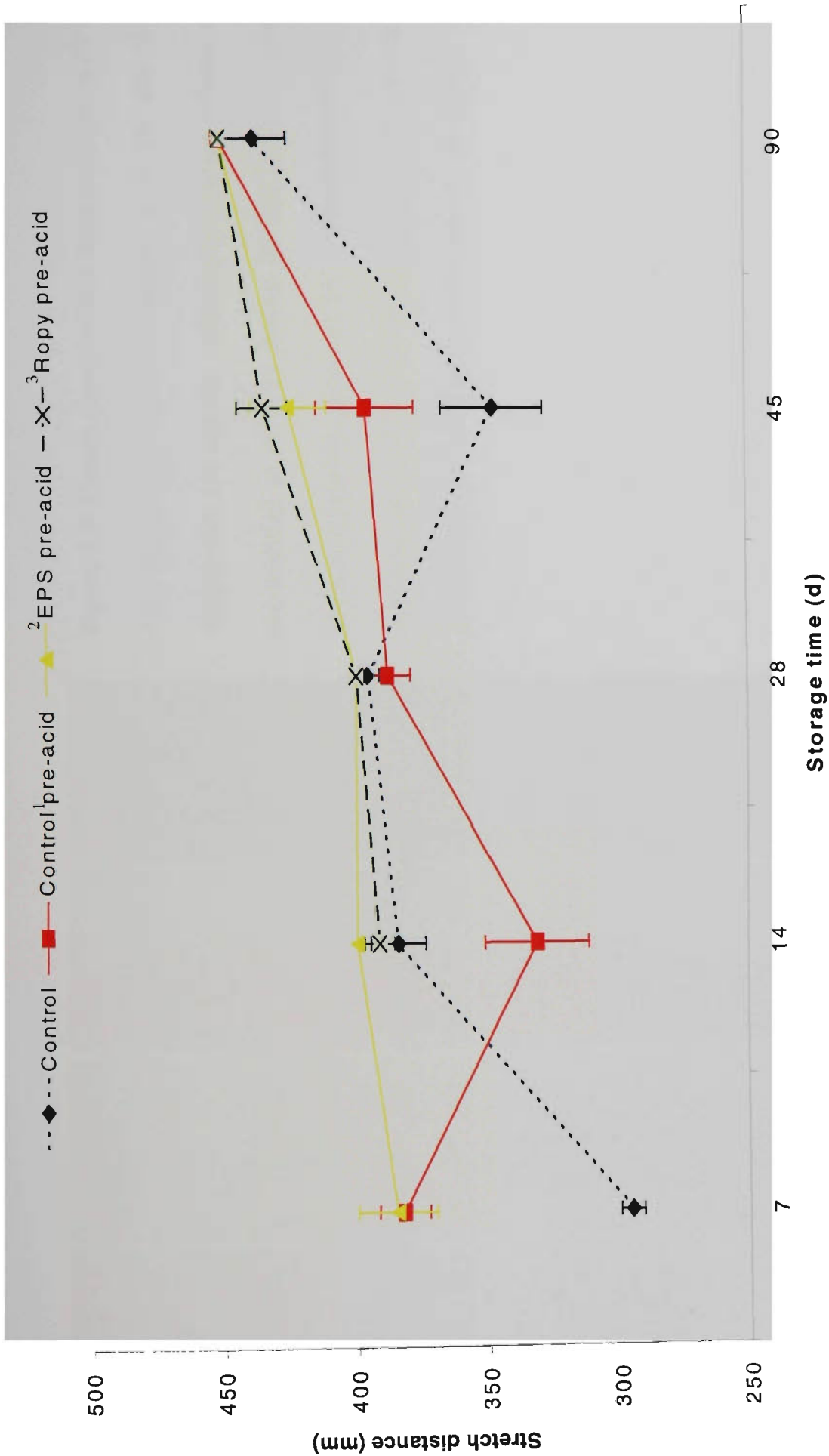
Figure 6.8. Stretch characteristics of control, control [1] pre-acid (cheese milk pre-acidified to pH 6.1), [2]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285) pre-acid, and [3]ropy (ropy EPS produced *S. thermophilus* 1275) pre-acid cheeses over 90 d of storage at 4°C (n = 6).

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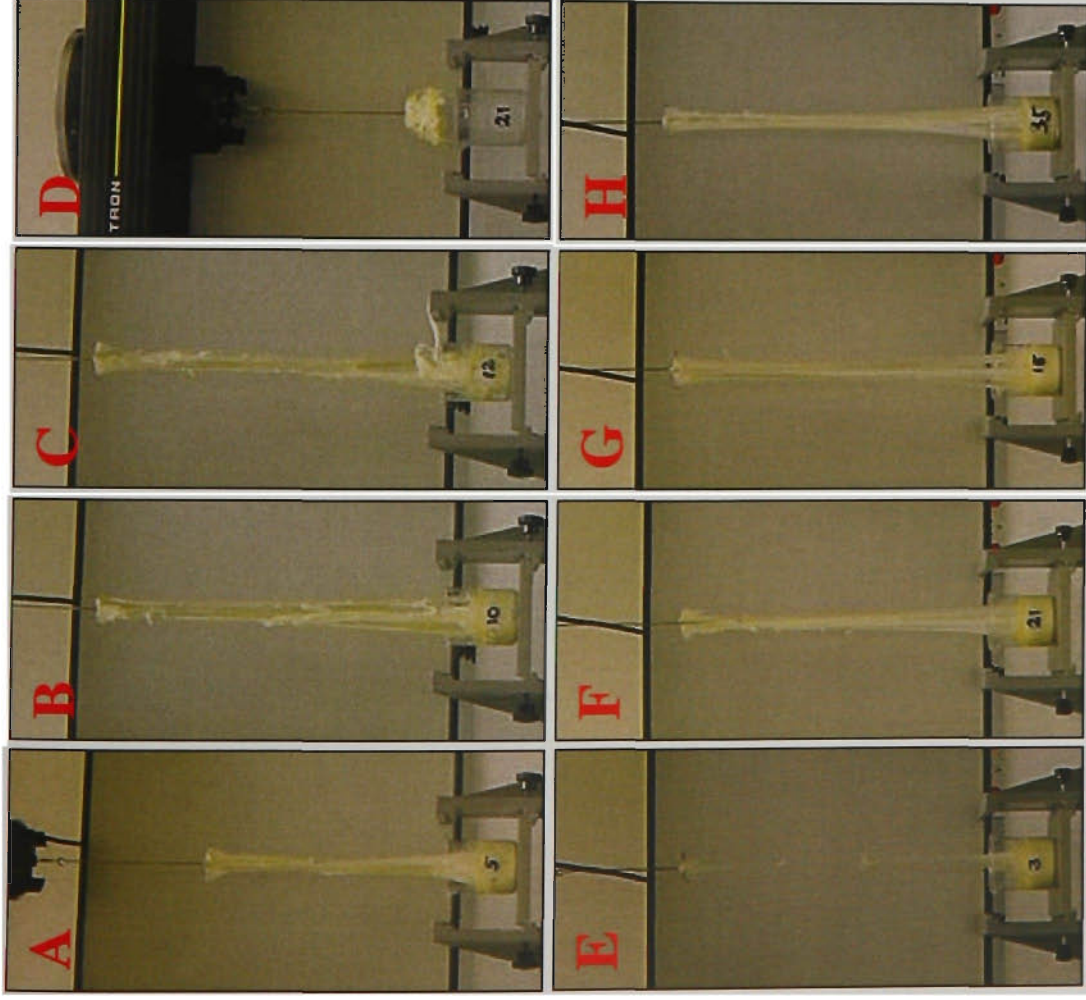
Figure 6.9. Cheese characteristics when stretched at 62°C to 450 mm/min for control cheeses at d 28 and d 90, respectively (A and E), control pre-acidified (cheese milk pre-acidified to pH 6.1) cheeses (B and F), EPS (capsular exopolysaccharides produced by *S. thermophilus* 285) pre-acidified cheeses (C and G) and ropy (ropy EPS produced by *S. thermophilus* 1275) pre-acidified cheeses (D and H).
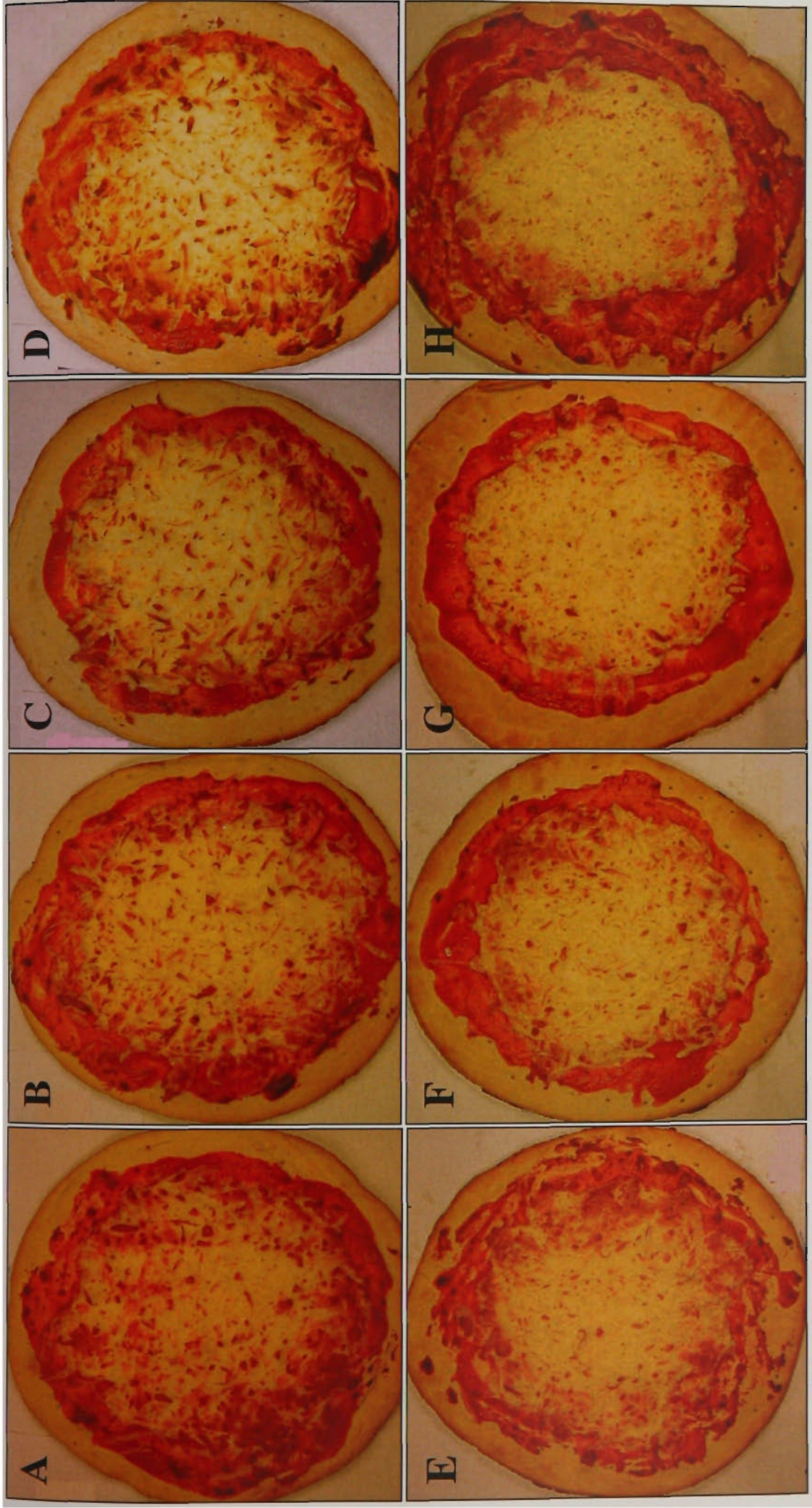
Figure 6.10. Pizza bake characteristics at 232°C / 7 min for control cheeses at d 28 and d 90 respectively (A and E), control pre-acidified (cheese milk pre-acidified to pH 6.1) cheeses (B and F), EPS (capsular exopolysaccharides produced by *S. thermophilus* 285) pre-acidified cheeses (C and G) and ropy (ropy EPS produced by *S. thermophilus* 1275) pre-acidified cheeses (D and H).

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CHAPTER 7.0

Texture and Functionality of Pre-acidified, Low Fat Mozzarella as Influenced by EPS Starter and Supplementation with WPC

7.1 INTRODUCTION

In our earlier study (Chapter 4.0; Zisu and Shah, 2003a), we found that co-culturing an EPS producing _Streptococcus thermophilus_ strain with a non-EPS _S. thermophilus_ greatly increased EPS production. The increase in EPS synthesis was due to the complementary relationship between the two strains of _S. thermophilus_ to produce a constant supply of essential nutrients.

We also reported that EPS production increased when the medium was supplemented with whey protein concentrate (WPC) (Chapter 4.0; Zisu and Shah, 2003a). In addition to its influence on cell growth and EPS synthesis, incorporation of WPC may influence changes in texture and functionality of Mozzarella cheese. Such changes, however, may depend on the solubility of commercially available WPC that is known to be highly variable.

Our findings (Zisu and Shah, 2005), supported by others (Feeney _et al._, 2002; Joshi _et al._, 2003) suggest that pre-acidification also increases the rate of primary proteolysis by the action of residual coagulant in low fat Mozzarella cheeses. As a combined result of proteolysis and degradation of the milk protein matrix, the meltability of Mozzarella cheese has been shown to improve (Fife _et al._, 1996; Zisu and Shah, 2005).

The baking performance of low fat Mozzarella cheeses, although improved in Chapters 4 and 5 remained inadequate as cheeses showed excessive surface scorching and incomplete shred fusion. To prevent this, the concept introduced by Rudan and Barbano (1998b) was adopted. These researchers showed that low fat Mozzarella cheeses could be baked to exhibit similar melting and browning characteristics to those of the full fat variety by coating cheese shreds with a hydrophobic material prior to baking to prevent surface dehydration and subsequent skin formation.

The effects of combining the technique of pre-acidification, the use EPS starter cultures and incorporation of WPC on the functionality of low fat Mozzarella cheese are not known.

Similarly, little is known about EPS synthesis in cheeses, in general, as influenced by co-culturing and use of WPC. The aims of this study were to investigate the effects of combining pre-acidification and use of EPS starter, alone and as a co-culture with a non-EPS producer, and WPC on moisture retention, EPS production, and texture and functionality of low fat Mozzarella cheeses containing 6% fat. The impact of coating such cheeses with a layer of canola oil and examining their behaviour after baking was also investigated.

7.2 MATERIALS AND METHODS

7.2.1 Starter Cultures

S. thermophilus strains 285 (capsular EPS producer) and 1303 (non-EPS) and _Lactobacillus delbrueckii_ ssp. _bulgaricus_ strain 1368 (described in Chapter 3.0) were used in this study. Working cultures were prepared as described in Chapter 3.0, section 3.2.1.

7.2.2 Cheese Making

Each batch of cheese was made with 25 L of whole, raw milk obtained from Mamma Lucia Cheese (Fresh Cheese Co. Pty. Ltd., Brunswick, VIC, Australia). The cream was separated using a batch 107 AE type cream separator (Alfa Laval; APV Australia, Clayton, VIC, Australia). Skim milk (0.005% fat) and whole milk (3.4% fat) were pasteurized at 72°C for 15 sec using an Alfa Laval P20 HRB pasteurizer (Alfa Laval). The pasteurized skim milk and whole milk were standardized to 0.5% fat, which was used to make four batches of cheese with a final fat content of 6% as indicated below:

Batch 1: Pre-acidification + _S. thermophilus_ 1303 (non-EPS) + _L. delbrueckii_ ssp. _bulgaricus_ 1368 (non-EPS) (Control),

Batch 2: Pre-acidification + _S. thermophilus_ 285 (EPS) + _L. delbrueckii_ ssp. _bulgaricus_ 1368 (non-EPS),

Batch 3: Pre-acidification + _S. thermophilus_ 285 (EPS) (75%) + _S. thermophilus_ 1303 (non-EPS) (25%) + _L. delbrueckii_ ssp. _bulgaricus_ 1368 (non-EPS),

Batch 4: Pre-acidification + _S. thermophilus_ 285 (EPS) + _L. delbrueckii_ ssp. _bulgaricus_ 1368 (non-EPS) + WPC 392 (0.125% wt/vol) (80.4% protein; New Zealand Milk Products (Australia) Pty. Ltd., Rowville, VIC, Australia).

Starter bacteria were inoculated at a concentration of 1.6% of _S. thermophilus_ and 0.8% of _L. delbrueckii_ ssp. _bulgaricus_. Cheese milk of batch 3 was inoculated with a co-culture of EPS producing _S. thermophilus_ 285 (0.75%) and non-EPS _S. thermophilus_ 1303 (0.25%) of the inoculum size of 1.6%. WPC 392 was added to milk at 0.125% (wt/wt) for batch 4.

Cheese milk was tempered to 35°C for half an hour and pre-acidified to pH 6.1 using 50% (wt/vol) citric acid. A 100 mL aliquot of milk was collected and stored at –20°C for analysis. Single strength chymosin (Chymax; Chr. Hansen, Pty. Ltd., Bayswater, VIC, Australia) was added at the rate of 10 mL per 25 L of milk. A setting time of 15 min was applied. The curd was cut using a cheese knife (1 cm^2 wire grid) based on setting time and subjective assessment of the coagulum with a knife. After cutting, curds were cooked for 20 min, gradually increasing the temperature to 40°C. Whey was drained at pH 6.1 and stored at –20°C for analysis. The curd was cheddered into slabs before milling at pH 5.2. Salt was added to the curd at 1.5% (wt/wt) and held for 30 min. Curd (40 g) was collected for later analysis and stored at –20°C. The remaining salted curd was then stretched for 7 min in hot water maintained at 75°C containing 3% salt (wt/wt) at a volume 2.5 times the weight of the curd. Stretch water was frozen at –20°C for later analysis.

Cheese blocks were immediately placed into barrier bags and vacuum sealed using a Multivac A300/16 machine (Multivac Sepp Haggenmuller KG, Wolfertschwenden, Germany) followed by storing at 4°C in a fan forced cold room. Appropriate aliquots from each cheese

block were removed on the day following production and frozen at –20°C for quantification of EPS and determination of calcium.

7.2.3 Cheese Analysis

An appropriate sample size was collected from cheese blocks on the day following production and each cheese was analyzed for fat (Babcock method), moisture (atmospheric oven method) and protein (Kjeldahl method) contents in accordance with AOAC methods (AOAC, 1999). Textural characteristics, stretch and meltability of each cheese were performed at d 7, 14, 28, and 45. A pizza bake test was carried out a d 28 and 45.

7.2.4 Isolation and Quantification of Exopolysaccharides

The preparation of samples for quantification of microbial EPS from milk, whey and stretching water was performed according to the procedure described in Chapter 6.0, section 6.2.4. EPS was isolated and quantified according to the procedure described in Chapter 3.0, section 3.2.5.

7.2.5 Determination of Calcium

An atomic absorption spectrophotometer was used to determine the calcium content in cheese, curd, milk, whey and stretch water as described in Chapter 6.0, section 6.2.5. A standard curve for the determination of calcium is shown in Figure 7.1.

7.2.6 Cheese Melt

The meltability of Mozzarella cheeses was determined in 250 mm long glass tubes with a diameter of 24 mm and thickness of 3 mm (R.B. Instruments, Mt. Eliza, VIC, Australia), using 10 g of cheese and heating at 110°C for 100 min as described in Chapter 5, section 5.2.5.

7.2.7 Texture Profile Analysis

Textural characteristics of cheeses including hardness, adhesiveness, cohesiveness, springiness and chewiness were analysed by means of an Instron Universal Testing Machine (UTM) (5564, Instron Ltd., London, England) at room temperature (~22°C) according to the procedure described in Chapter 5.0, section 5.2.7. The details of the selected parameters have been described by Pons and Fiszman (1996).

7.2.8 Cheese Stretch

Stretch distance was recorded by elongation to 450 mm using the UTM by a modified version of the method described by Bhaskaracharya and Shah (2002) as used in Chapter 5.0, section 5.2.6. A cross bar spindle was placed at the bottom of a beaker containing 50 g of melted sample and the stretch limit was increased to 450 mm/min.

7.2.9 Pizza Bake

A 300 g aliquot of shredded Mozzarella cheese was used as a topping placed on a pizza base (Don Emilio's, Freshwell Foods, Coolaroo, VIC, Australia) covered with a thin layer of tomato paste (Leggos, Simplot Australia Pty. Ltd., Cheltenham, VIC, Australia). One half of

each pizza was topped with untreated cheese shreds (150 g) and the remaining half was covered with cheese shreds coated with 1% (wt/wt) canola oil (Woolworths Home Brand, Yennora, NSW, Australia). Canola oil was sprayed uniformly in a fine mist over cheese shreds with a spray bottle. Coated cheese shreds were mixed to aid oil dispersion and to ensure the entire surface of the shreds was covered in oil. Cheeses were coated in oil to provide a hydrophobic barrier to reduce moisture loss from the shred surface and to reduce subsequent skin formation to improve melting and browning. Pizzas were baked at 262°C for 7 min using a Lincoln Impinger 1304-4 conventional pizza oven (Lincoln Foodservice Products Inc., Fort Wayne, IN, USA) to imitate the conditions used by pizza restaurants (Rudan and Barbano, 1998b) at d 28 and 45. The appearance of the cheese on the pizza was evaluated visually for shred fusion and melt, color and extent of blistering. The Hunter L,a,b system was used to measure L- (whiteness), a- (redness) and b-values (yellowness) of cheese before cooking, immediately after baking and after cooling (half-an-hour after baking) with a Minolta Chroma-meter (CR-300, Minolta Corporation, Ramsey, NJ, USA).

7.2.10 Statistical Analysis

Each batch of cheese was made in triplicate and results are presented as a mean ± standard error of replicates. Moisture, protein, hardness, adhesiveness, cohesiveness, springiness and chewiness results are an average of nine analyses, while the results of fat, EPS, melt, and stretch are representative of six analyses. Calcium analysis was recorded as a mean of six to twelve replicates. Pizza bakes were replicated three times. Hunter L,a,b values obtained from the pizza bake are representative of nine readings. To find significant differences between analyses, the means were analyzed using one-way analysis of variance (ANOVA) with a 95% confidence interval using Microsoft® Excel StatPro™ (Palaside Corporation, Newfield, NY, USA). ANOVA data with a $P < 0.05$ was considered as statistically significant.

7.3 RESULTS AND DISCUSSION

All cheeses were made from milk pre-acidified with citric acid to maintain the uniformity of calcium levels. Any variation between cheeses in terms of texture and functionality would therefore be attributed to the effects of EPS and WPC. Our previous findings (Chapter 6.0) revealed that pre-acidification of the cheese milk had positive effects on textural and functional characteristics of cheeses over those that were not made with pre-acidified milk. Pre-acidification did not, however, improve moisture retention. The relationship between acid treatment and its influence on calcium content, proteolysis and functionality of low fat Mozzarella cheeses has been demonstrated by other researchers (Guinee _et al._, 2002; Joshi _et al._, 2003; Paulson _et al._, 1998; Shakeel-Ur-Rehman _et al._, 2003).

7.3.1 Composition of Cheeses

The role of incorporating WPC as well as using the co-culture combination of 75% of EPS producing _S. thermophilus_ and 25% of non-EPS _S. thermophilus_ on EPS synthesis was described in our earlier work (Chapter 4.0; Zisu and Shah, 2003a). It was found that EPS production was substantially increased by the use of WPC as well as by co-culturing with non-EPS producing _S. thermophilus_ strains. In the presented study, we examined the effects of these in cheese systems for increasing EPS synthesis in order to influence moisture retention. The average composition of various low fat Mozzarella cheeses is shown in Table 7.1. Control pre-acidified cheeses without EPS had the lowest moisture content of 53.75% (57.26% as MNFS). All cheeses containing EPS made with pre-acidified milk had significantly higher moisture contents as compared to control cheeses ($P < 0.05$). No significant difference in the moisture content was, however, found between the EPS containing cheeses ($P > 0.05$). Cheeses made with 100% EPS producing _S. thermophilus_ 285 had a moisture content of 55.08% (58.70% as MNFS), whereas when co-cultured with 75% of EPS producing _S. thermophilus_ 285 and 25% of

non-EPS *S. thermophilus* 1303, the cheeses contained 54.79% moisture (58.37% as MNFS). WPC and EPS containing cheeses had a moisture content of 55.82% (59.49% as MNFS).

Table 7.2 shows the amount of EPS isolated from cheeses and curds, whey, stretch water and milk collected during cheese making. EPS was absent in control cheeses made with EPS negative strains. Cheeses made with EPS contained 41.18 mg of EPS per g of cheese and those containing WPC had 44.23 mg of EPS per g of cheese. The concentration of EPS was, however, significantly lower in cheeses made by co-culturing at 28.61 mg/g ($P < 0.05$). Although the amount of EPS in the co-cultured cheeses was lower, it was sufficient to influence the moisture content. In the work by Zisu and Shah (2003a) (Chapter 4.0), a ropy EPS producing strain was used. We anticipated that EPS concentrations would be lower if co-cultured with capsule producing strains. The amount of capsular EPS synthesized is growth associated and is related to the number of bacterial cells present as the capsules remain cell bound. Due to lower inoculum volume, EPS levels were affected. It must be noted that the fermentation time was also shorter with pre-acidification of the milk than that during regular cheese making. WPC also failed to increase the amount of EPS beyond levels recorded in EPS only cheeses ($P > 0.05$) and this reflected in the moisture content. Moisture retention did not increase further. This was possibly attributed to the pre-treatment with citric acid that limited the starter bacteria to multiply and the solubility of WPC. As previously stated, the addition of WPC to cheese milk was expected to increase the synthesis of EPS and together with the moisture retention properties of the WPC were expected to result in an improved moisture content in cheeses. As shown in Table 7.1, the protein content in cheeses made with WPC was not significantly higher than other cheeses ($P > 0.05$). This is possibly due to metabolism of part of WPC by starter bacteria (Zisu and Shah, 2003a) and losses of the insoluble WPC in the whey. To achieve increased EPS synthesis in cheeses, the supplementation concentration of WPC should be increased or solubilisation should be improved.

EPS concentration in curd was similar to that found in cheeses ($P > 0.05$). The EPS level in stretch water was minute and was not detected by the EPS extraction method adopted, suggesting that the majority of EPS remained within the cheese protein matrix after draining of the whey. A considerable amount of EPS was, however, retrieved from whey samples (18 to 28 mg/mL) as large numbers of starter culture cells were drained in the whey. Despite the loss in whey, the EPS concentration remained significantly higher in cheeses ($P < 0.05$). No EPS was detected in milk, hence the build up in the EPS concentration occurred during the cheese making process.

Although the moisture content of EPS cheeses was significantly greater, the protein content remained similar between cheeses ($P > 0.05$) (Table 7.1). The moisture to protein ratio increased from 1.60 in control pre-acidified cheeses to 1.71 in WPC pre-acidified cheeses. The fat and calcium (Table 7.3) contents were similar between cheeses and curds ($P > 0.05$). The influence of fat and calcium on moisture retention, texture and functionality of cheeses were expected to be negligible. WPC caused some variation in the calcium content of milk, as this mineral was present in the product. The calcium content in WPC was small, resulting in a slight increase in calcium content in cheese milk. However, the difference in calcium concentration upon addition of WPC was insignificant ($P > 0.05$). There was a minor but insignificant difference in the concentration of calcium in stretching water and whey.

7.3.2 Cheese Melt

Figure 7.2 presents the changes in meltability of low fat Mozzarella cheeses over 45 d of storage at 4°C. All cheeses exhibited a significant increase in melt between d 7 and 45 ($P < 0.05$) as a result of proteolysis and the hydration of the protein network. The control cheeses made with pre-acidified milk and non-EPS cultures displayed the lowest melt ($P < 0.05$) at d 7, at 30.10 mm. Melt distance increased by 23.07 mm to 53.18 mm at d 45 ($P < 0.05$). This

represented a higher melt than WPC pre-acidified cheeses (48.86 mm) ($P < 0.05$), although there was a similar melt to that of the EPS pre-acidified cheeses (55.78 mm) ($P > 0.05$) at d 45. The meltability of co-cultured cheeses made with acid treated milk remained higher after 45 d of maturation (60.54 mm) ($P < 0.05$). It was also observed that the control cheeses maintained a gradual increase in melt at each time point, whereas cheeses containing EPS showed a significant increase in melt distance between d 14 and d 28 only ($P < 0.05$).

Melt of pre-acidified cheeses containing EPS was 42.03 mm at d 7. Meltability was greater than the control cheeses that were also pre-acidified at d 7, 14, and 28 ($P < 0.05$). By d 45, melt distance increased by 13.75 mm to 55.78 mm, however, this was similar to that of the control cheeses. In a separate study, no significant difference was recorded in the melt distance between cheeses made with non-EPS and EPS cultures in the absence of an acid treatment (Chapter 5.0, section 5.3.2).

The melt of pre-acidified cheeses made with co-culturing of EPS and non-EPS producing strains of _S. thermophilus_ was similar to that of EPS cheeses during the first 14 d of storage ($P > 0.05$). At d 7, a melt of 47.13 mm was recorded. At d 28 and d 45, the melt was greatest in co-cultured cheeses ($P < 0.05$), reaching 62.04 mm and 60.54 mm, respectively. A substantial increase in the melt distance (an increase by 13.41 mm) of co-cultured cheeses was likely to be due to greater secondary proteolysis.

Pre-acidified cheeses containing WPC had a melt distance of 41.61 mm at d 7. This was greater than that of the control cheeses ($P < 0.05$). No significant difference existed between the two type of cheeses at d 14, 28 and 45 ($P > 0.05$). WPC containing cheeses were expected to show greater melt resulting from the combined effects of increased moisture retention of EPS and due to pre-acidification that reduced the calcium/casein interactions and stimulated primary proteolysis. This did not, however, occur. Meltability increased by only 7.25 mm over 45 d of storage to 48.86 mm. The poor performance of cheeses containing WPC over time indicates that WPC may have interfered with the physical ability of the cheese to flow when melted.

7.3.3 Texture Profile Analysis

7.3.3.1 Cheese hardness

Figure 7.3 illustrates the changes in hardness in cheeses over 45 d of storage at 4°C. Hardness decreased between d 7 and 45 in all cheese types except in those made with WPC. A reduction in hardness is due to the hydration and proteolysis of the protein matrix. Control pre-acidified cheeses were hardest in general. The overall hardness was reduced by pre-acidification of milk and introducing EPS producing starter bacteria alone, and as a co-culture. At 45 d of maturation, the EPS and EPS co-cultured cheeses were softer than the control ($P < 0.05$). The two variables, however, showed similar hardness ($P > 0.05$). The most desirable softness was present in cheeses made with EPS and by co-culturing with non-EPS starter.

At d 7, WPC and EPS cheeses were softest due to the presence of EPS and WPC particles possibly disrupting the protein matrix, allowing the protein network to become less compact. Between d 7 and 45, cheese hardness remained similar ($P > 0.05$), while the cheeses made with EPS and EPS and co-culture became comparably softer. WPC cheeses remained softer than the control cheeses at all time points. As the hardness of other cheeses reduced, the WPC within the protein matrix of WPC cheeses was likely to have contributed to sustaining similar hardness that did not reduce during storage.

7.3.3.2 Cheese adhesiveness

The adhesiveness of cheeses over 45 d of storage at 4°C is shown in Figure 7.4. Adhesiveness was not significant between cheeses in general and was negligible. At d 7, however, adhesiveness was absent in control pre-acidified cheeses which had the lowest moisture content and were hardest, but was detected in all other cheese types. There was

significantly higher adhesiveness between control pre-acidified- and WPC pre-acidified cheeses at d 7 ($P < 0.05$). At d 14 and beyond, adhesiveness was present in control pre-acidified cheeses.

7.3.3.3 Cheese cohesiveness

Figure 7.5 shows cohesiveness of cheeses over 45 d of storage at 4°C. Cohesiveness was similar between pre-acidified cheeses made with EPS producing starter bacteria, and there was no change within a cheese type over 45 d of storage ($P > 0.05$). The composition between each type of cheese varied little, particularly in the protein and moisture contents, hence the similarity in cohesiveness. The overall cohesiveness was lower in control pre-acidified cheeses without EPS and reduced moisture content (Table 7.1). Later findings suggest that the strength of the internal bonds of the para-casein fibers that represent cohesiveness reduced over lengthy storage in cheeses with higher moisture content and pre-acidification (Chapter 8.0, section 8.3.5.2; Zisu and Shah, 2005).

7.3.3.4 Cheese springiness

Figure 7.6 represents springiness in pre-acidified cheeses over 45 d of storage as the height recovered after deformation. In general, springiness was greatest in control cheeses. These were also the hardest cheeses with the lowest moisture and cohesiveness. Cheeses maintained their structural integrity over 45 d to resist permanent deformation and springiness remained unchanged throughout storage ($P > 0.05$).

7.3.3.5 Cheese chewiness

The chewiness of cheeses is a measure of the primary textural parameters and is closely related to the hardness of the product. Figure 7.7 represents the chewiness of cheeses calculated as hardness × cohesiveness × springiness. Control cheeses made with pre-acidified milk having the lowest moisture content and highest springiness generally exhibited more chewiness than those made with EPS producing _S. thermophilus_ and pre-acidification. Although control cheeses

appeared to have greater chewiness than EPS only cheeses over 45 d and co-cultured cheeses at d 14 and 28, they were statistically similar ($P < 0.05$). EPS and co-cultured cheeses exhibited similar chewiness at all time points ($P > 0.05$). All cheeses with the exception of those containing WPC tended to show a decrease in chewiness over time. The reduction was not significant until d 45 ($P < 0.05$). Chewiness appeared to be lower in acid treated cheeses made with WPC, however, chewiness was statistically similar to those made with EPS at d 14, 28 and 45 and co-culturing at d 28 and 45 ($P > 0.05$). Cheeses containing WPC showed no changes in chewiness over 45 d of storage ($P > 0.05$).

7.3.4 Cheese Stretch

The stretch pattern of cheeses over 45 d of storage at 4°C is shown in Figure 7.8. Stretch was lowest at d 7 in all batches of cheese ($P < 0.05$). All types of cheeses showed an improvement in stretch distance over time between d 7 and 28 ($P < 0.05$). Stretch was similar between cheese types at d 7, 28 and 45. At d 14, acid treated cheeses made with WPC and EPS and co-culture had a lower stretch than EPS and control cheeses. Control, pre-acidified cheeses showed a significant increase in stretch distance between d 7 (279 mm) and d 14 (396 mm) ($P < 0.05$). Between d 14 (396 mm) and d 45 (404 mm), there was no significant change in the stretch distance ($P > 0.05$).

Pre-acidified cheeses made with EPS, EPS co-culture and WPC did not show a substantial increase in the stretch distance between d 7 (310 mm, 286 mm and 319 mm, respectively) and d 14 (387 mm, 318 mm and 323 mm, respectively) ($P > 0.05$). Stretch increased significantly between d 14 and d 28 (423 mm, 410 mm and 410 mm, respectively) ($P < 0.05$), and remained similar at d 45 (402 mm, 417 mm and 378 mm, respectively).

Based on our observation, control and WPC based cheeses were exceptionally tough at d 7, causing several test samples to lift away from the bottom of the sample beaker when stretched (similar to Figure 6.8, D). The performance of control cheeses was acceptable at d 14 and

beyond. WPC cheeses also showed good stretch at d 14, however, they became excessively soft and pasty when melted after 28 d, creating an inferior appearance. Cheeses made with EPS and EPS co-culture exhibited good stretch characteristic throughout the storage. Our work with EPS and fat replacers suggest that a loss in the ability to stretch with time due to the increase in protein breakdown associated with maintaining the structure was expected beyond d 45 (Chapter 8.0, section 8.3.6; Zisu and Shah, 2005).

7.3.5 Pizza Bake

Results from the simulated pizza bake tests at d 28 and d 45 are shown in Figure 7.9, A to H. The browning behaviour, melt and flow of cheese shreds when baked were evaluated as per other researchers (Metzger _et al._, 2000a; Rudan and Barbano, 1998a; Rudan and Barbano, 1998b). The hydrophobic barrier concept applied to low fat cheese shreds was introduced by Rudan and Barbano (1998a,b), as it was found to prevent surface moisture loss when baked and improved the overall pizza bake attributes. At d 28, all four batches of cheeses performed poorly in the absence of an external coating of canola oil. Cheeses showed scorching, and lacked shred fusion and adequate melt. An application of canola oil to coat the surface of the cheese shreds before baking resulted in improved cheese fusion and melt and reduced scorching by lowering moisture loss from the shred surface. EPS and pre-acidified cheeses showed the best bake characteristics with larger white, non-scorched portions towards the middle of the pizza (Figure 7.9, B). Excessive burning continued to occur around edges of the pizza where more evaporation took place. Control and co-cultured cheeses made with pre-acidified milk (Figure 7.9, A and C, respectively) were similar in appearance, while WPC containing cheeses (Figure 7.9, D) exhibited most scorching, creating a blister like appearance. When heated, WPC appeared to have low heat stability and WPC cheeses behaved poorly. The inferior behaviour of

WPC in cheeses when heated was also identified and discussed earlier in the sections related to cheese melt and stretch.

By d 45, a substantial improvement in melt, flow, shred fusion and reduced blistering was evident for all 4 types of cheeses. Control, EPS and co-culture cheeses (Figure 7.9, E, F and G, respectively) appeared acceptable when coated with oil. WPC cheeses (Figure 7.9, H) continued to show more surface browning than other cheeses.

7.3.5.1 L,a,b values: before cooking

Table 7.4 shows the L-values as a measure of cheese whiteness before cooking, after cooking when warm and after cooling of cheeses baked at d 28 and d 45 of storage. Due to the similarity in cheese composition, no significant difference in L-values were observed among cheese types prior to cooking ($P > 0.05$).

Table 7.5 shows the a-values as a measure of cheese redness and Table 7.6 shows the b-values as a measure of cheese yellowness before cooking, after cooking when warm and after cooling of cheeses baked at d 28 and d 45 of storage. As was observed with L-values, the a- and b-values were similar between cheese types and between oil coated and non-coated cheese shreds before cooking. The a-values did, however, increase with storage time between d 28 and d 45 ($P < 0.05$).

7.3.5.2 L,a,b values: warm

Whiteness increased significantly immediately after baking ($P < 0.05$) (Table 7.4). When cheese shreds were coated with oil, L-values were greater at d 28 and d 45 in control and EPS cheeses. At d 28, cheeses made with co-culturing at a ratio of 75:25 of EPS to non-EPS _S. thermophilus_ and those made with WPC showed similar readings between oil-coated and non-coated shreds. A high degree of scorching was evident in these cheeses at this time. At d 45 as the baking performance improved, L-values became greater for oil-coated cheeses.

Baking performance was most improved in EPS cheeses. Between d 28 and d 45, L-values were significantly higher for both the oil-coated and non-coated cheeses ($P < 0.05$). The control cheeses and those made using co-culturing recorded a substantial improvement when coated with oil ($P < 0.05$), but not in the untreated batch ($P > 0.05$). WPC cheeses, although showing improved melt and flow, did not show greater whiteness ($P > 0.05$). The lactose content of WPC 392 is typically 6.7%. Lactose was a likely contributing factor for blistering and reduced whiteness. As a reducing sugar, lactose and its constituents will react in the presence of amino acids leading to non-enzymatic Maillard browning. Due to excessive moisture loss in all non-coated cheeses, shred fusion remained a problem. Surface scorching was severe, L-values remained low and cheeses were not acceptable after baking. An external oil coating remained necessary for all cheese types.

An increase in a-values (Table 7.5) occurred during baking of the pizzas due to surface browning. This was more evident for untreated cheeses lacking the protective hydrophobic oil barrier to prevent evaporation of surface moisture. Although this was apparent, it was only significant for EPS pre-acidified cheeses at d 28 and WPC pre-acidified cheeses at d 45 ($P < 0.05$). Hunter b-values (Table 7.6) also increased after baking corresponding to the increase in L-values.

7.3.5.3 L,a,b values: cool

L-values were generally lower after cooling and all cheeses became translucent and shiny in appearance. Cheeses containing WPC, however, showed similar L-values after cooling as the warm counterpart. The oil coated cheeses continued to show significantly higher L-values at d 28 and d 45 to those lacking an oil coating ($P < 0.05$). The a- and b-values remained higher to those before cooking (section 7.3.5.1).

7.4 CONCLUSIONS

The moisture content of control (pre-acidified) low fat Mozzarella cheeses was lowest. Moisture retention in pre-acidified cheeses increased with the use of capsular EPS producing *S. thermophilus* 285 when used as a starter culture singly, as a co-culture with non-EPS *S. thermophilus* or in the presence of WPC. EPS also improved the textural and functional characteristics of cheeses by increasing meltability, reducing hardness, springiness, chewiness and improving the visual appeal of cheese strands when stretched. Pizza bake characteristics were relatively inferior in all cheeses (exhibiting scorching and incomplete shred fusion) unless cheese shreds were coated with oil. Co-culturing an EPS producing *S. thermophilus* with a non-EPS producing strain and the use of WPC did not increase EPS synthesis during cheese manufacture. A significant amount of EPS was lost through the whey. *S. thermophilus* 285 used as a co-culture limited the amount of EPS produced in cheese, however, co-culturing led to improved melt and texture. On the contrary, the use of the WPC in cheeses interfered with the melt, texture and pizza bake performance.

Table 7.1. Average composition of low fat Mozzarella cheeses (mean ± standard error).

Cheese	Moisture (%) (n = 9)	Fat (%) (n = 6)	MNFS[2] (%) (n = 6)	FDM[1] (%) (n = 6)	Protein (%) (n = 9)	M:P[3]
Control [4] pre-acid	53.75 ± 0.14[b]	6.13 ± 0.06[a]	57.26 ± 0.11[b]	13.25 ± 0.10[a]	32.75 ± 0.34[a]	1.60
EPS[5] pre-acid	55.08 ± 0.29[a]	6.17 ± 0.05[a]	58.70 ± 0.18[a]	13.74 ± 0.17[a]	33.28 ± 0.24[a]	1.66
75:25[6] pre-acid	54.79 ± 0.43[a]	6.13 ± 0.06[a]	58.37 ± 0.26[a]	13.56 ± 0.24[a]	32.70 ± 1.42[a]	1.68
WPC[7] pre-acid	55.82 ± 0.43[a]	6.17 ± 0.05[a]	59.49 ± 0.27[a]	13.89 ± 0.28[a]	32.62 ± 0.27[a]	1.71

[abc]One-way ANOVA of means in a column with different superscript are significantly different ($P < 0.05$).

[1]FDM = Fat in dry matter.

[2]MNFS = Moisture in non-fat substance.

[3]M:P = Moisture to protein ratio based on final (average) moisture and protein contents.

[4]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

[5]EPS = Capsular exopolysaccharides (EPS) produced by S. thermophilus 285.

[6]75:25 = Mixture of 75% EPS producing S. thermophilus 285 and 25% non-EPS S. thermophilus 285.

[7]WPC = Cheeses made with whey protein concentrate and EPS – S. thermophilus 285.

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Table 7.2. EPS isolated from cheese, curd, whey, stretch water and milk (n = 6 ± standard error).

Sample	Control [1] pre-acid	EPS [2] pre-acid	75:25 [3] pre-acid	WPC [4] pre-acid
Cheese (mg/g)	ND[5] [c,A]	41.18 ± 1.21 [a,A]	28.61 ± 1.71 [b,A]	44.23 ± 3.16 [a,A]
Curd (mg/g)	ND [c,A]	38.14 ± 2.21 [a,A]	28.16 ± 1.24 [b,A]	42.73 ± 5.30 [a,AB]
Whey (mg/mL)	ND [b,A]	19.80 ± 2.33 [a,B]	18.13 ± 3.33 [a,B]	28.36 ± 4.98 [a,B]
Stretch water (mg/mL)	ND [a,A]	ND [a,CD]	ND [a,CD]	ND [a,CD]
Milk (mg/mL)	ND [a,A]	ND [a,D]	ND [a,D]	ND [a,D]

[a,b,c]One-way ANOVA of means across a row and [A,B,C]one-way ANOVA of means in a column with different superscript are significantly different ($P < 0.05$).
[1]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.
[2]EPS = Capsular exopolysaccharides (EPS) produced by S. thermophilus 285.
[3]75:25 = Mixture of 75% EPS producing S. thermophilus 285 and 25% non-EPS S. thermophilus 1303.
[4]WPC = Cheeses made with whey protein concentrate and EPS – S. thermophilus 285.
[5]ND = Not detected.

Table 7.3. Calcium content in cheese, curd, whey, stretch water and milk (n = 6 to 12 ± standard error).

Sample (mg/100g)	Control[1] pre-acid	[2]EPS pre-acid	[3]75:25 pre-acid	[4]WPC pre-acid
Cheese	1041.37 ± 11.52^a	1041.34 ± 9.28^a	1075.77 ± 14.94^a	1044.50 ± 12.61^a
Cheese (mg/g protein)	14.71 ± 0.15^a	14.10 ± 0.12^a	14.87 ± 0.25^a	14.10 ± 0.21^a
Curd	924.88 ± 24.76^a	926.97 ± 25.82^a	991.46 ± 25.59^a	950.84 ± 19.39^a
Whey	32.81 ± 0.45^d	34.60 ± 0.37^c	36.47 ± 0.36^b	38.84 ± 0.42^a
Stretch water	14.61 ± 0.42^a	11.32 ± 0.20^{cd}	10.96 ± 0.31^d	11.80 ± 0.07^b
Milk	95.10 ± 0.87^a	93.29 ± 2.26^a	92.96 ± 2.67^a	99.54 ± 2.57^a

[abc]One-way ANOVA of means across a row with different superscript are significantly different ($P < 0.05$).
[1]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.
[2]EPS = Capsular exopolysaccharides (EPS) produced by *S. thermophilus* 285.
[3]75:25 = Mixture of 75% EPS producing *S. thermophilus* 285 and 25% non-EPS *S. thermophilus* 1303.
[4]WPC = Cheeses made with whey protein concentrate and EPS – *S. thermophilus* 285.

Table 7.4. Mean Hunter L-values (n = 9 ± SE) for whiteness measurements of low fat Mozzarella cheeses for given variables, treatments and processing stages.

	Control[1] pre-acid		[2]EPS pre-acid		[3]75:25 pre-acid		[4]WPC pre-acid	
	No Oil	Oil	No Oil	Oil	No Oil	Oil	No Oil	Oil
Before cook$_{28}$	65.25 ± 0.92[ac,A]	62.19 ± 1.33[c,F]	64.60 ± 0.85[abc,BC]	64.29 ± 0.73[abc,EF]	65.36 ± 0.87[a,A]	62.77 ± 0.71[bc,EF]	66.30 ± 1.19[a,A]	63.95 ± 1.21[abc,CD]
Before cook$_{45}$	65.28 ± 0.63[ab,A]	63.45 ± 0.82[bcdef,DEF]	65.73 ± 0.49[a,ABC]	62.48 ± 1.24[ef,F]	63.02 ± 0.49[cdef,BC]	62.11 ± 0.91[f,F]	65.21 ± 0.89[abcde,AB]	62.96 ± 0.89[de,D]
Warm$_{28}$	61.09 ± 2.11[d,A]	66.71 ± 1.22[ab,BC]	63.19 ± 1.11[cd,C]	68.82 ± 0.67[a,C]	64.72 ± 2.07[abcd,ABC]	67.40 ± 0.53[a,CD]	64.69 ± 0.74[bcd,AB]	66.81 ± 0.98[ab,ABC]
Warm$_{45}$	64.28 ± 0.89[fg,A]	71.02 ± 0.86[bc,A]	67.81 ± 0.93[de,A]	73.37 ± 0.51[a,A]	66.80 ± 0.94[ef,A]	72.56 ± 0.52[ab,A]	61.28 ± 1.90[g,BCD]	69.16 ± 1.17[cde,A]
Cool$_{28}$	52.37 ± 2.76[e,C]	63.04 ± 1.70[ab,EF]	52.83 ± 2.04[de,E]	66.06 ± 0.62[a,DE]	59.07 ± 1.45[bc,D]	65.24 ± 0.94[a,DE]	57.56 ± 2.00[de,D]	65.17 ± 0.80[a,BCD]
Cool$_{45}$	53.29 ± 1.71[f,B]	64.19 ± 1.88[bcd,CDEF]	57.33 ± 1.38[ef,DE]	69.01 ± 1.16[a,BC]	61.37 ± 0.95[cd,CD]	67.57 ± 0.71[ab,BCD]	60.11 ± 0.85[de,CD]	68.14 ± 0.69[ab,A]

[abc]One-way ANOVA of means across a row and [ABC]one-way ANOVA of means in a column with different superscript are significantly different (P < 0.05).

[28,45]Pizza bake performed after 28 and 45 d of storage at 4°C.
[1]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.
[2]EPS = Capsular exopolysaccharides (EPS) produced by S. thermophilus 285.
[3]75:25 = Mixture of 75% EPS producing S. thermophilus 285 and 25% non-EPS S. thermophilus 1303.
[4]WPC = Cheeses made with whey protein concentrate and EPS – S. thermophilus 285.

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Table 7.5. Mean Hunter a-values (n = 9 ± SE) for redness measurements of low fat Mozzarella cheeses for given variables, treatments and processing stages.

	Control pre-acid[1]		[2]EPS pre-acid		[3]75:25 pre-acid		[4]WPC pre-acid	
	No Oil	Oil	No Oil	Oil	No Oil	Oil	No Oil	Oil
Before cook[28]	-3.79 ± 0.09^{aC}	-3.45 ± 0.23^{aC}	-3.62 ± 0.10^{aE}	-3.83 ± 0.07^{aF}	-3.63 ± 0.12^{aC}	-3.80 ± 0.08^{aC}	-3.56 ± 0.17^{aD}	-3.61 ± 0.18^{aD}
Before cook[45]	-3.39 ± 0.07^{cB}	$-3.22 \pm 0.12^{bc,BC}$	$-3.00 \pm 0.06^{ab,D}$	$-2.91 \pm 0.21^{ab,E}$	-2.72 ± 0.10^{aB}	$-3.04 \pm 0.17^{abc,B}$	$-3.09 \pm 0.16^{abc,CD}$	$-2.85 \pm 0.23^{ab,C}$
Warm[28]	$4.70 \pm 1.01^{abc,A}$	$1.69 \pm 1.08^{cde,A}$	$5.16 \pm 0.71^{a,BC}$	$0.67 \pm 0.86^{e,D}$	$3.84 \pm 1.18^{abcd,A}$	$1.42 \pm 0.71^{de,A}$	$3.93 \pm 0.53^{abc,B}$	$2.43 \pm 0.93^{bcde,AB}$
Warm[45]	$3.87 \pm 1.04^{abcde,A}$	$1.94 \pm 0.39^{e,A}$	$3.71 \pm 1.04^{abcde,C}$	$2.72 \pm 0.36^{bcde,A}$	$3.69 \pm 0.85^{abcde,A}$	$2.22 \pm 0.26^{de,A}$	$6.15 \pm 1.29^{a,AB}$	$2.69 \pm 0.59^{cde,A}$
Cool[28]	$6.15 \pm 0.47^{bc,A}$	$2.22 \pm 1.12^{fg,A}$	$7.91 \pm 0.65^{a,A}$	$1.34 \pm 0.50^{g,CD}$	$5.41 \pm 0.85^{c,A}$	$2.89 \pm 0.83^{efg,A}$	$6.65 \pm 0.32^{abc,A}$	$3.23 \pm 1.10^{def,A}$
Cool[45]	$4.68 \pm 0.73^{ab,A}$	$3.17 \pm 0.91^{bcde,A}$	$5.96 \pm 0.81^{a,ABC}$	$1.55 \pm 0.34^{cde,BCD}$	$3.74 \pm 0.86^{ab,A}$	$1.18 \pm 0.26^{de,A}$	$6.36 \pm 0.55^{a,AB}$	$1.06 \pm 0.24^{e,B}$

[abc]One-way ANOVA of means across a row and [ABC]one-way ANOVA of means in a column with different superscript are significantly different ($P < 0.05$).

[28,45]Pizza bake performed after 28 and 45 d of storage at 4°C.

[1]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

[2]EPS = Capsular exopolysaccharides (EPS) produced by S. thermophilus 285.

[3]75:25 = Mixture of 75% EPS producing S. thermophilus 285 and 25% non-EPS S. thermophilus 1303.

[4]WPC = Cheeses made with whey protein concentrate and EPS – S. thermophilus 285.

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Table 7.6. Mean Hunter b-values (n = 9 ± SE) for yellowness measurements of low fat Mozzarella cheeses for given variables, treatments and processing stages.

	Control [1] pre-acid		[2]EPS pre-acid		[3]75:25 pre-acid		[4]WPC pre-acid	
	No Oil	Oil	No Oil	Oil	No Oil	Oil	No Oil	Oil
Before cook$_{28}$	19.67 ± 0.28[ab,BC]	18.28 ± 0.24[c,B]	19.58 ± 0.22[ab,D]	19.25 ± 0.19[bcd,D]	20.19 ± 0.54[a,C]	19.60 ± 0.60[abc,D]	20.16 ± 0.31[a,C]	19.40 ± 0.52[ab,C]
Before cook$_{45}$	18.79 ± 0.35[a,C]	17.04 ± 0.39[f,C]	18.03 ± 0.27[abcdef,E]	17.15 ± 0.42[ef,F]	17.48 ± 0.38[bcdef,A]	17.44 ± 0.37[cde,E]	18.79 ± 0.47[ab,D]	17.23 ± 0.31[de,D]
Warm$_{28}$	26.76 ± 1.90[c,A]	29.30 ± 1.11[abc,A]	29.94 ± 0.99[abc,A]	31.47 ± 0.98[a,A]	28.22 ± 1.01[bc,AB]	32.16 ± 1.16[a,A]	28.87 ± 1.14[abc,A]	30.53 ± 0.69[abc,A]
Warm$_{45}$	25.54 ± 0.88[cd,A]	27.65 ± 0.60[abcd,A]	28.05 ± 1.27[abcd,ABC]	25.97 ± 0.77[bcd,C]	29.39 ± 0.74[a,A]	25.46 ± 1.12[d,C]	26.66 ± 1.39[abcd,AB]	28.99 ± 1.05[a,AB]
Cool$_{28}$	23.48 ± 2.00[f,A]	27.25 ± 0.72[cdef,A]	25.67 ± 1.42[ef,C]	30.52 ± 0.56[a,A]	28.25 ± 0.84[bcde,AB]	31.32 ± 0.72[a,A]	26.98 ± 1.41[def,AB]	30.44 ± 0.66[ab,A]
Cool$_{45}$	23.12 ± 1.12[f,A]	27.01 ± 0.75[abcde,A]	25.70 ± 0.65[def,BC]	28.65 ± 0.62[a,B]	25.81 ± 0.93[cdef,B]	27.47 ± 0.77[abc,BC]	24.38 ± 1.11[ef,B]	26.05 ± 0.58[bcde,B]

[abc]One-way ANOVA of means across a row and [ABC]one-way ANOVA of means in a column with different superscript are significantly different (P < 0.05).

[28,45]Pizza bake performed after 28 and 45 d of storage at 4°C.

[1]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

[2]EPS = Capsular exopolysaccharides (EPS) produced by S. thermophilus 285.

[3]75:25 = Mixture of 75% EPS producing S. thermophilus 285 and 25% non-EPS S. thermophilus 1303.

[4]WPC = Cheeses made with whey protein concentrate and EPS – S. thermophilus 285.

Figure 7.1. Standard curve for the determination of calcium in cheese, curd, milk, whey and stretching water by atomic absorption spectroscopy.
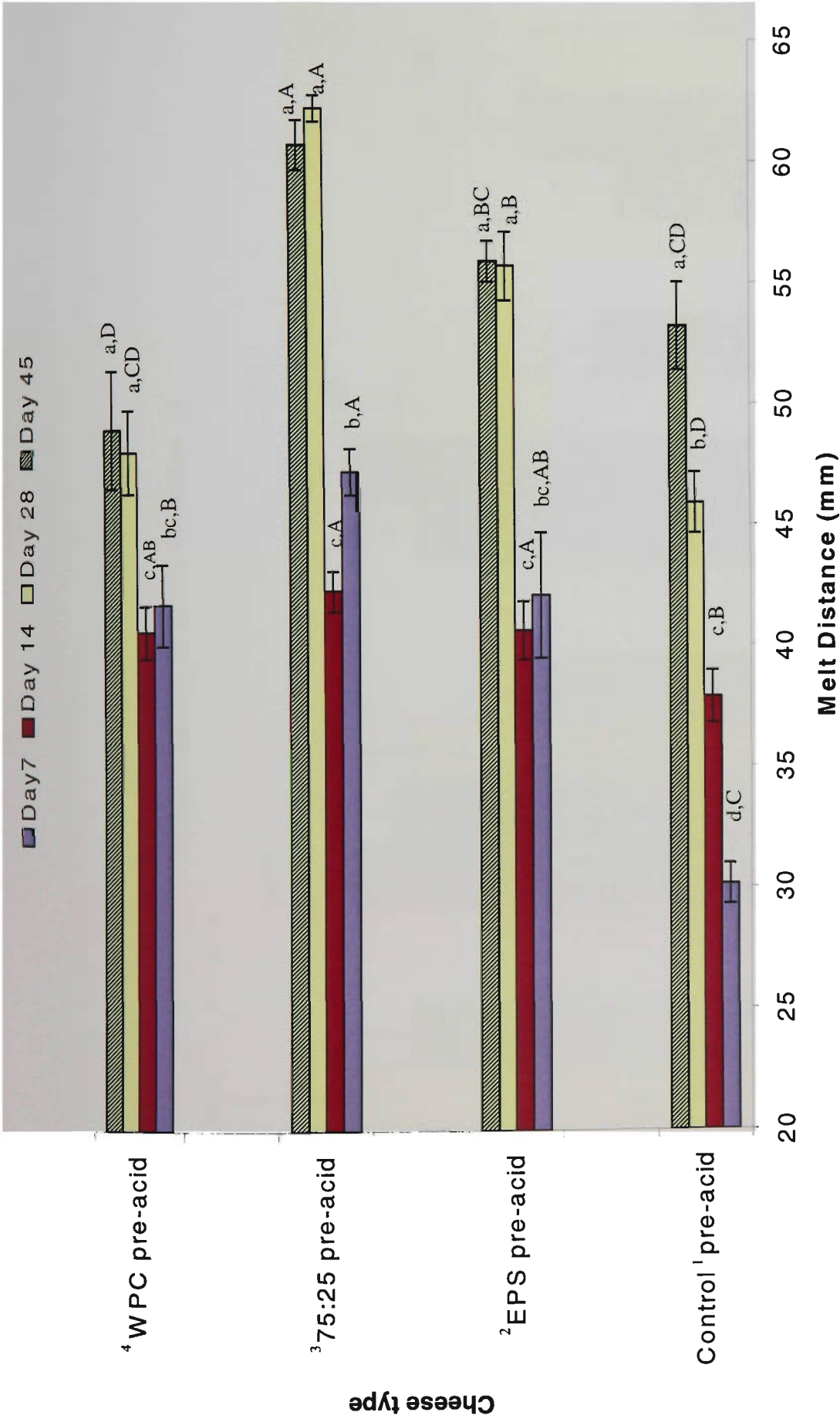
Figure 7.2. Cheese melt of control [1]pre-acid (cheese milk pre-acidified to pH 6.1), [2]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285) pre-acid, [3]75:25 (mixture of 75% EPS producing *S. thermophilus* 285 and 25% non-EPS *S. thermophilus* 1303) pre-acid and [4]WPC (whey protein concentrate and EPS – *S. thermophilus* 285) pre-acid cheeses over 45 d of storage at 4°C. [abc]One-way ANOVA of means (n = 6) between d 7 and d 45 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28 and 45 between cheese types with different superscript are significantly different (*P* < 0.05).

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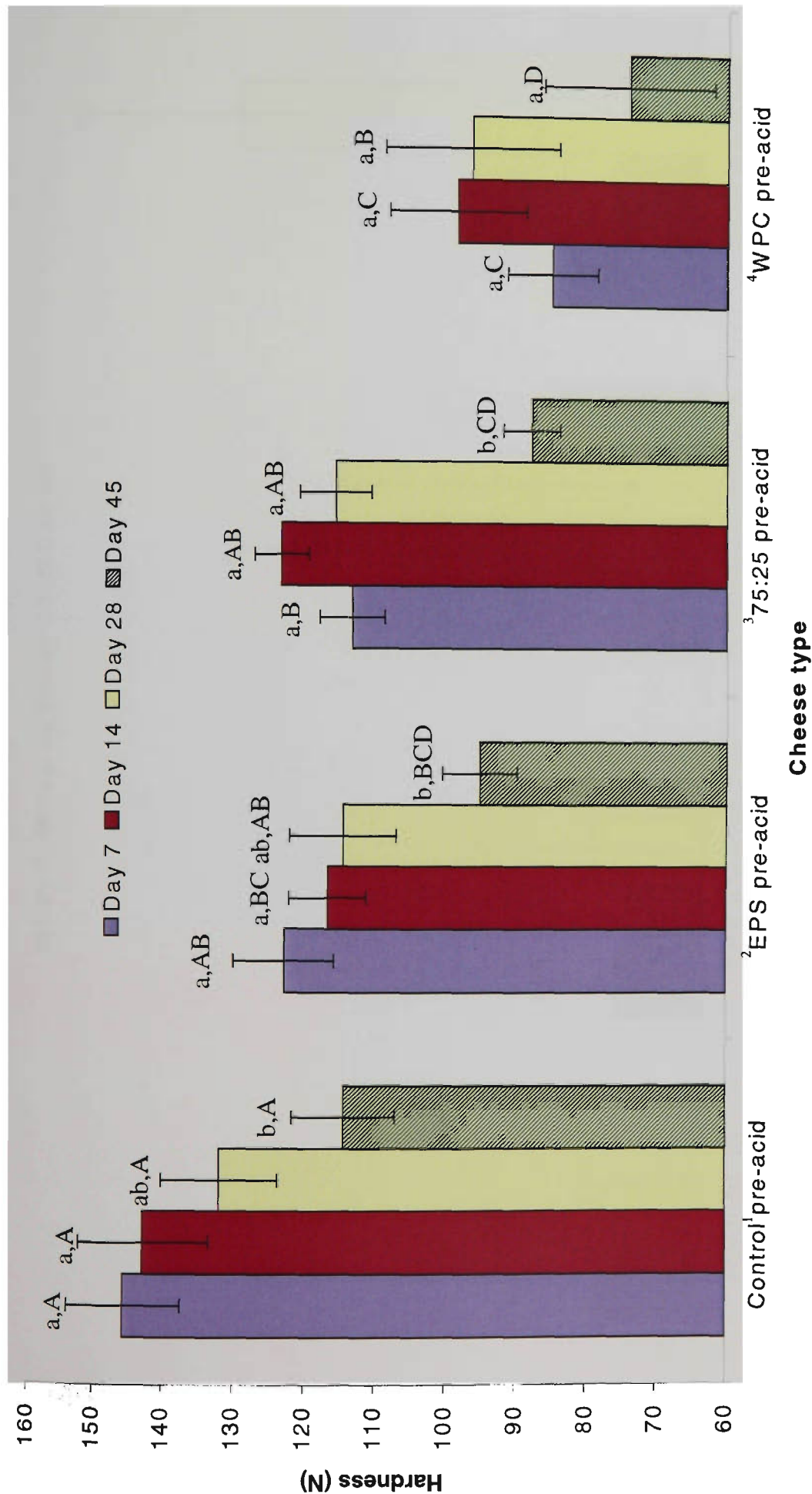
Figure 7.3. Hardness of control [1] pre-acid (cheese milk pre-acidified to pH 6.1), [2]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285) pre-acid, [3]75:25 (mixture of 75% EPS producing *S. thermophilus* 285 and 25% non-EPS *S. thermophilus* 1303) pre-acid and [4]WPC (whey protein concentrate and EPS – *S. thermophilus* 285) pre-acid cheeses over 45 d of storage at 4°C.
[abc]One-way ANOVA of means (n = 9) between d 7 and d 45 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28 and 45 between cheese types with different superscript are significantly different (*P* < 0.05).

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Figure 7.4. Adhesiveness of control[1] pre-acid (cheese milk pre-acidified to pH 6.1), [2]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285) pre-acid, [3]75:25 (mixture of 75% EPS producing *S. thermophilus* 285 and 25% non-EPS *S. thermophilus* 1303) pre-acid and [4]WPC (whey protein concentrate and EPS – *S. thermophilus* 285) pre-acid cheeses over 45 d of storage at 4°C. [abc]One-way ANOVA of means (n = 9) between d 7 and d 45 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28 and 45 between cheese types with different superscript are significantly different (*P* < 0.05).

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Figure 7.5. Cohesiveness of control [1] pre-acid (cheese milk pre-acidified to pH 6.1), [2]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285) pre-acid, [3]75:25 (mixture of 75% EPS producing *S. thermophilus* 285 and 25% non-EPS *S. thermophilus* 1303) pre-acid and [4]WPC (whey protein concentrate and EPS – *S. thermophilus* 285) pre-acid cheeses over 45 d of storage at 4°C. [abc]One-way ANOVA of means (n = 9) between d 7 and d 45 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28 and 45 between cheese types with different superscript are significantly different (*P* < 0.05).

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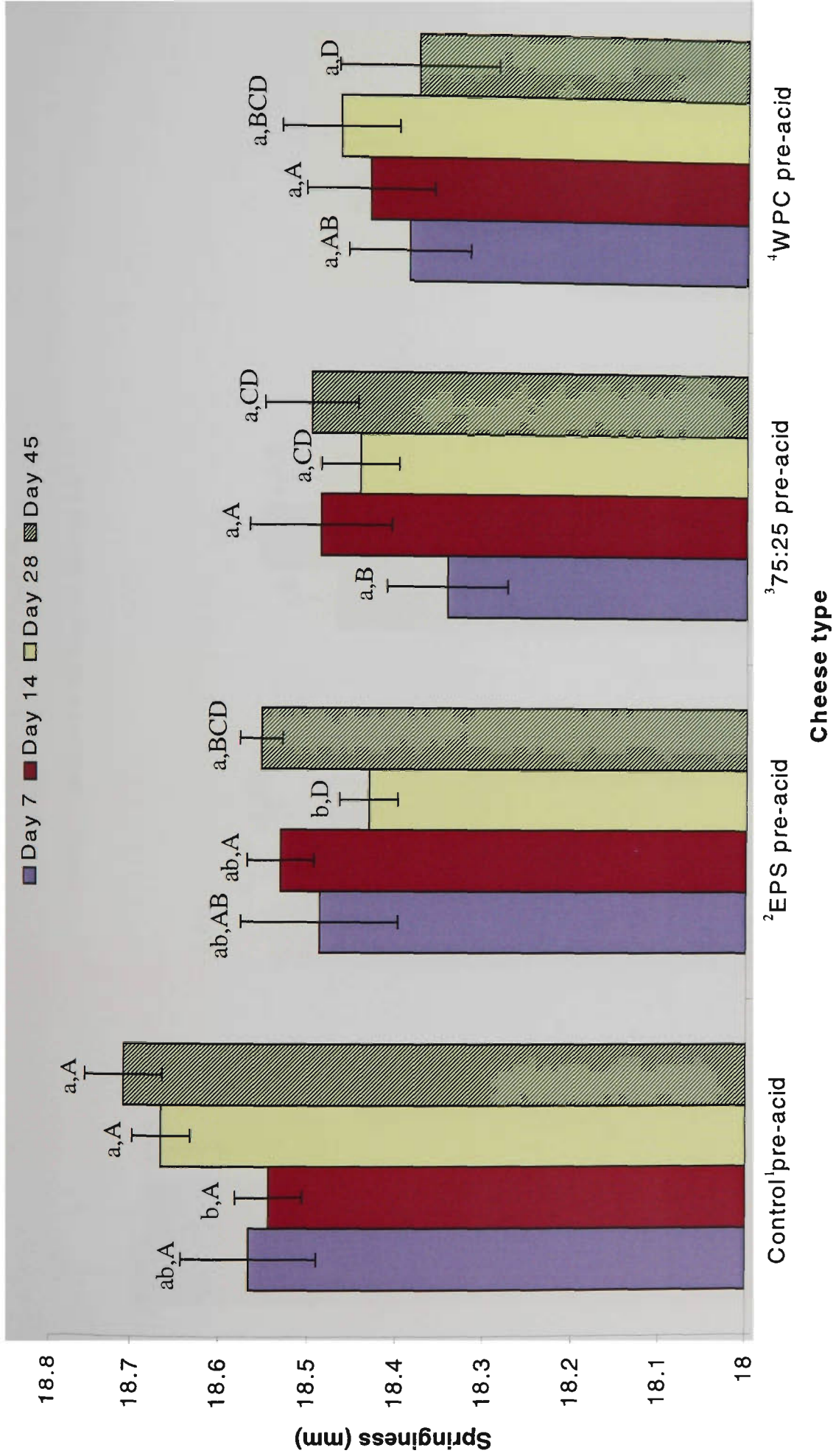
Figure 7.6. Springiness of control[1] pre-acid (cheese milk pre-acidified to pH 6.1), [2]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285) pre-acid, [3]75:25 (mixture of 75% EPS producing *S. thermophilus* 285 and 25% non-EPS *S. thermophilus* 1303) pre-acid and [4]WPC (whey protein concentrate and EPS – *S. thermophilus* 285) pre-acid cheeses over 45 d of storage at 4°C.
[abc]One-way ANOVA of means (n = 9) between d 7 and d 45 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28 and 45 between cheese types with different superscript are significantly different (*P* < 0.05).

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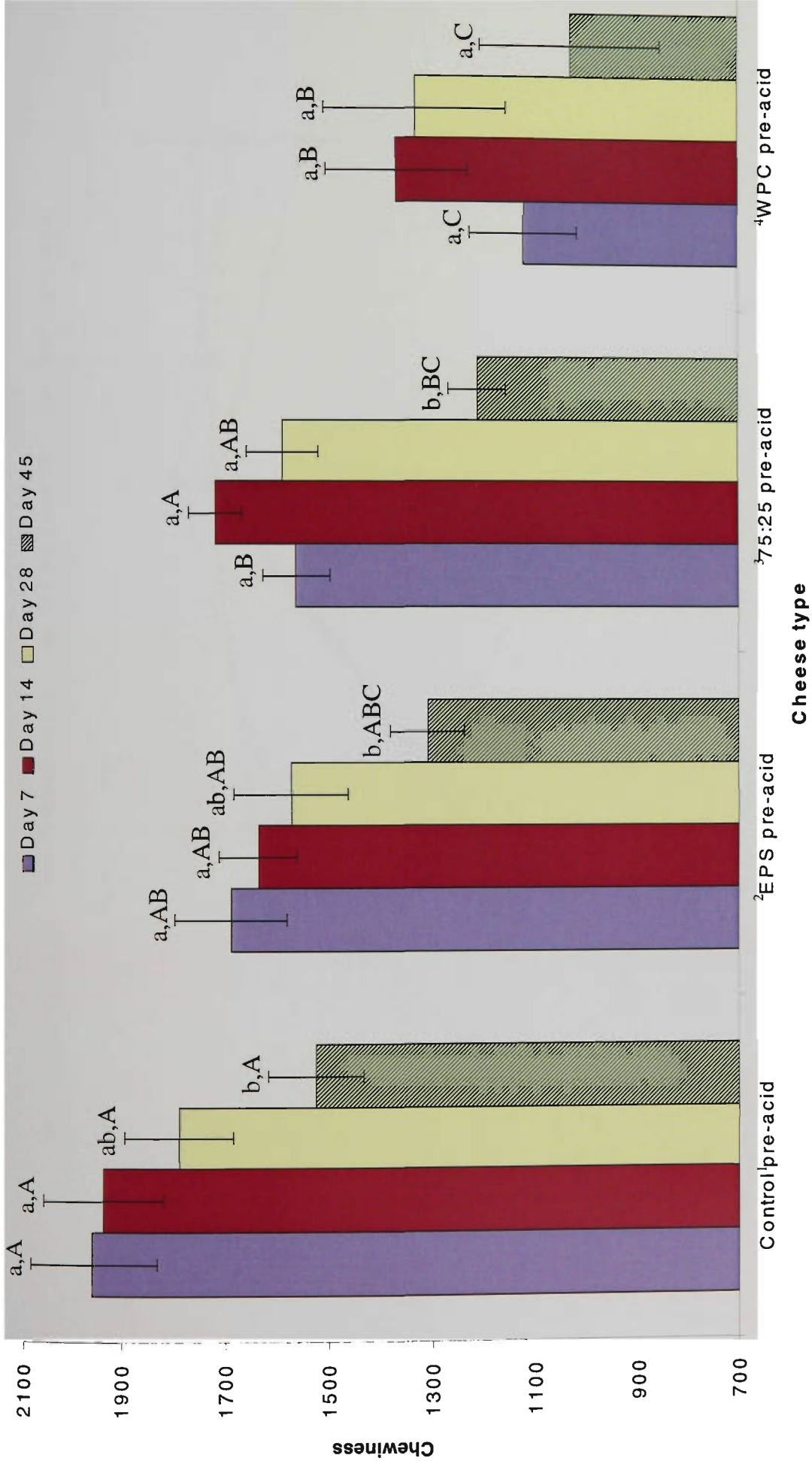
Figure 7.7. Chewiness of control [1] pre-acid (cheese milk pre-acidified to pH 6.1), [2]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285), [3]75:25 (mixture of 75% EPS producing *S. thermophilus* 285 and 25% non-EPS *S. thermophilus* 1303) pre-acid and [4]WPC (whey protein concentrate and EPS – *S. thermophilus* 285) pre-acid cheeses over 45 d of storage at 4°C.
[abc]One-way ANOVA of means (n = 9) between d 7 and d 45 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28 and 45 between cheese types with different superscript are significantly different (*P* < 0.05).

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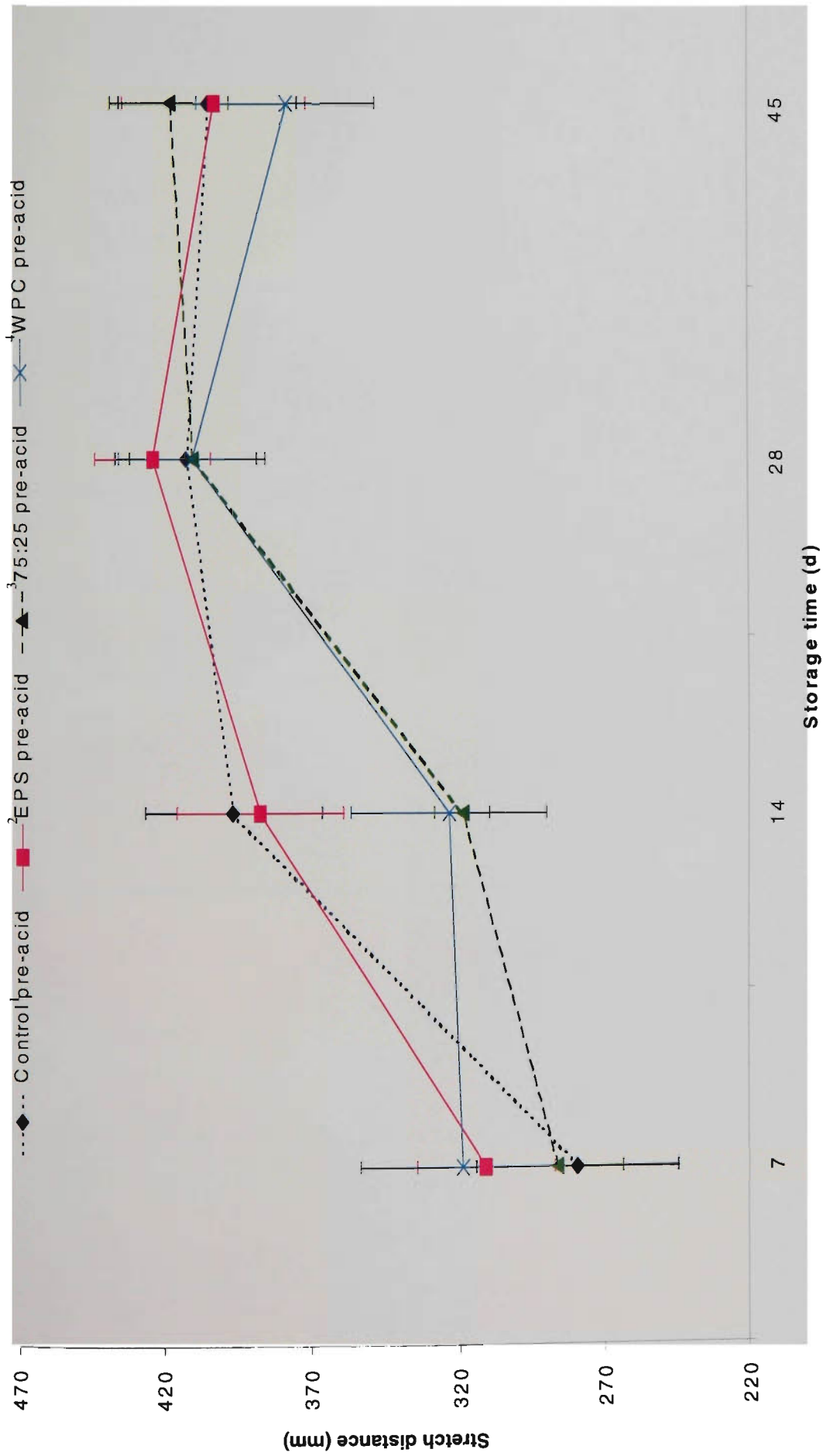
Figure 7.8. Stretch characteristics of control [1]pre-acid (cheese milk pre-acidified to pH 6.1), [2]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285) pre-acid , [3]75:25 (mixture of 75% EPS producing *S. thermophilus* 285 and 25% non-EPS *S. thermophilus* 1303) pre-acid and [4]WPC (whey protein concentrate and EPS – *S. thermophilus* 285) pre-acid cheeses over 45 d of storage at 4°C (n = 6).
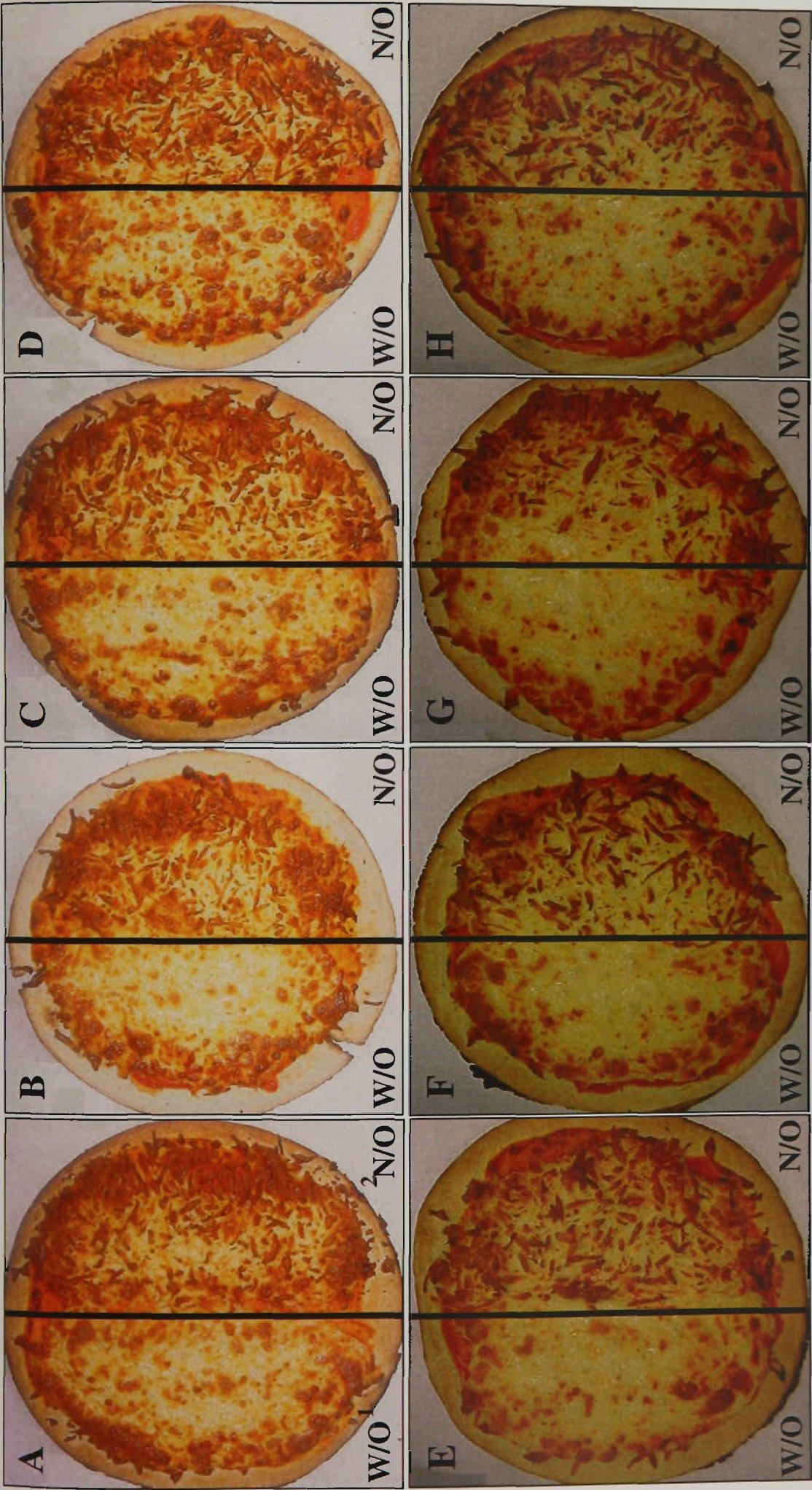
Figure 7.9. Pizza bake characteristics of control pre-acidified cheeses (cheese milk pre-acidified to pH 6.1) at d 28 and d 45 respectively (A and E), EPS (capsular exopolysaccharides produced by *S. thermophilus* 285) pre-acidified cheeses (B and F), 75 % (EPS *S. thermophilus*) : 25 % (non-EPS *S. thermophilus*) co-cultured and pre-acidified cheeses (C and G) and WPC (whey protein concentrate and EPS – *S. thermophilus* 285) pre-acidified cheeses (D and H).

[1]W/O = With oil.

[2]N/O = No oil.

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CHAPTER 8.0

Textural and Functional Changes in Low Fat Mozzarella Cheeses in Relation to Proteolysis and Micro-structure as Influenced by the use of Fat Replacers, Pre-acidification and EPS Starter

8.1 INTRODUCTION

Mozzarella cheese is often used as a functional ingredient on pizzas, and its ability to stretch when melted has made this cheese the choice for use as a pizza topping. Fat content is important in allowing moisture to be retained in cheeses as well as for providing a lubricating effect during heating. By reducing the fat content of Mozzarella cheeses below 10%, a reduction in the functionality demanded by the pizza industry occurs (McMahon _et al._, 1996). Specific changes occur in the overall composition and structure of cheeses including a lower ratio of moisture to protein.

According to McMahon and Oberg (1998a), the most important method for improving the functional properties of low fat cheese is to increase the moisture content and create a moisture to protein ratio equal to or greater than that observed in full fat Mozzarella cheese. The size of the serum channels between the protein strands can also be expanded by the use of fat replacers that act as 'fillers'.

Strains of encapsulated exopolysaccharide (EPS) producing lactic acid bacteria have been used successfully to manufacture low fat Mozzarella cheese with improved moisture retention, thereby enhancing cheese yield and improving its functional properties (Perry _et al._, 1997; Petersen _et al._, 2000).

The use of fat replacers has also been shown to increase the moisture content of low fat Mozzarella cheeses (McMahon _et al._, 1996; Rudan _et al._, 1998). The contribution of the fat replacer to improve the textural and functional properties of cheeses, however, varies. Such compounds can be derived from carbohydrate, protein, lipid and synthetic chemical groups. The use of carbohydrate based fat replacers in foods are of particular interest as they appear to closely resemble the desired effects lost after the removal of fat as a consequence of the bulking associated with moisture retention (Fennema, 1996).

Calcium also plays a significant role in Mozzarella cheese functionality and texture by cross linking protein (Metzger *et al.*, 2000b). By removing some of the bound calcium from casein micelles, it is possible to manufacture a low fat cheese with improved characteristics.

Proteolysis and degradation of the protein matrix has been shown to improve the meltability of Mozzarella cheese (Fife *et al.*, 1996) and to improve the texture by reducing hardness. Recent findings also suggest that a reduction in the calcium content increases the rate of proteolysis in low fat Mozzarella cheeses (Feeney *et al.*, 2002; Joshi *et al.*, 2003).

Confocal scanning laser microscopy may be used to study the microstructure of Mozzarella cheeses to monitor the changes of the protein matrix that occur with time. The advantage of this form of microscopy is that samples remain relatively unaffected during preparation, not requiring freezing, dehydration or the removal of fat and can be viewed close to their natural form. Individual components such as protein, fat and EPS may be visualised and comparisons of their distribution can be made. Microscopic observations are, however, limited by the resolution that can be achieved and therefore images can only be viewed at relatively low magnifications. Confocal microscopy has been used to study microstructure of dairy products by several authors (Auty *et al.*, 2001; Hassan *et al.*, 2002b; Hassan *et al.*, 1995).

The effects of combining the technique of pre-acidification and the use of fat replacers and EPS starter cultures on functionality of low fat Mozzarella cheese are not known. The aims of this study were to investigate the effects of combining pre-acidification, fat replacers and EPS starter cultures in low fat Mozzarella cheeses containing 6% fat and examine the influence on moisture retention and textural and functional attributes in relation to protelyitc and micro-structural changes.

8.2 MATERIALS AND METHODS

8.2.1 Starter Cultures

Capsular EPS producing _S. thermophilus_ 285 (described in Chapter 3.0) and _L. delbrueckii_ ssp. _bulgaricus_ strain 2515 obtained from the Victoria University Culture Collection (Melbourne, Vic., Australia) selected for its high protelytic activity as previously reported (Shihata and Shah, 2000) were used in this study. Working cultures were prepared according to Chapter 3.0, section 3.2.1.

8.2.2 Cheese Making

Each batch of cheese was made in triplicate using 25 L of milk in custom made cheese vats. Whole raw milk was obtained from Mamma Lucia Cheese (Fresh Cheese Co. Pty. Ltd., Brunswick, VIC, Australia) and cream was separated using a batch 107 AE type cream separator (Alfa Laval, APV Australia, Clayton, VIC, Australia). Skim milk (0.005% fat) and whole milk (3.6% fat) were pasteurised at 72°C with a holding time of 15 sec using an Alfa Laval P20 HRB pasteuriser (Alfa Laval). The pasteurised skim milk and whole milk were standardised to 0.5% fat and used to make cheeses at a final fat content of 6% as indicated below:

Batch 1: _S. thermophilus_ 285 (EPS) + _L. delbrueckii_ ssp. _bulgaricus_ 2515 (non-EPS) (Control),

Batch 2: _S. thermophilus_ 285 (EPS) + _L. delbrueckii_ ssp. _bulgaricus_ 2515 (non-EPS) + OptaMax® (referred to as FR1; Opta Food Ingredients, Inc., Bedford, MA, USA),

Batch 3: Pre-acidification + _S. thermophilus_ 285 (EPS) + _L. delbrueckii_ ssp. _bulgaricus_ 2515 (non-EPS) + FR1,

Batch 4: Pre-acidification + _S. thermophilus_ 285 (EPS) + _L. delbrueckii_ ssp. _bulgaricus_ 2515 (non-EPS) + Versagel® (referred to as FR2; Gelled Foods Australia, East Ringwood, VIC, Australia).

Starter bacteria (2.4%) were inoculated with a concentration of 1.6% _S. thermophilus_ 285 and 0.8% _L. delbrueckii_ ssp. _bulgaricus_ 2515 and the milk was tempered to 35°C for half-an-hour. Single strength chymosin (Chr. Hansen, Pty. Ltd., Bayswater, Australia) was added at the rate of 10 mL per 25 L of milk. The carbohydrate based fat replacer, FR1 and FR2 were heated to 95°C and held for 30 sec in a small volume of cheese milk to aid hydrolysis of starch granules and increase moisture retention before adding to appropriate vats (Batches 2, 3, and 4) at 0.25% (wt/wt). FR1 is a modified corn starch-based (high amylopectin content) product and behaves more like a hydrocolloid than a typical granular starch (as claimed by the manufacturers). FR2 is a blend of β-lactoglobulin, carageenan and xanthan gum. Milk of the relevant batches (Batches 3 and 4) was pre-acidified to pH 6.1 using 50% (wt/vol) citric acid while maintaining a temperature of 35°C. A setting time of 25 min was used except for those batches that were pre-acidified for which a setting time of 15 min was applied. The set curd was cut using a cheese knife (1 cm^2 wire grid) based on setting time and subjective assessment of the coagulum with a knife. After the cutting, the curd was cooked for 20 min and the temperature was gradually increased to 40°C. Whey was drained at pH 6.2 (pH 6.1 for pre-acidified cheeses) and the curd was cheddared into slabs before milling at pH 5.2. Salt was added to the curd at 1.5% (wt/wt) and held for 30 min. Salted curd was then hand stretched for 7 min in hot water maintained at 75°C. The volume of stretching water was 2.5 times the weight of the curd and contained 3% salt (wt/wt).

An appropriate sample size of cheese was removed from each cheese block and frozen at -20°C for analysis of proteolysis at d 0 (representing 100% intact casein). The remaining cheese blocks were immediately placed into barrier bags and vacuum sealed using a Multivac A300/16 machine (Multivac Sepp Haggenmuller KG, Wolfertschwenden, Germany) prior to cooling in a fan forced cold room and storage at 4°C.

8.2.3 Cheese Analysis

Cheese yield based on the weight of cheese manufactured from a known weight of milk was calculated and expressed as kilograms of cheese per 100 kilograms of milk as carried out by Hong *et al.* (1998) and Metzger *et al.* (2000a). Estimation of yield was carried out in triplicate.

An appropriate sample size of cheese was removed from the cheese blocks on the day following production (~30 h) and analysed for fat (using Babcock test) and moisture (using atmospheric oven method) contents. Cheese samples were also removed form cheese blocks and frozen at -20°C to determine the protein (using Kjeldahl method) content at a later date. Fat, moisture and protein contents were analysed in accordance with AOAC methods (AOAC, 1999). Moisture results are presented as an average of 9 analyses, while the results of fat and protein are representatives of 6 measurements.

Textural characteristics, stretch, meltability, and a simulated pizza bake test of each cheese were performed at day 7, 14, 28, 45, and 60.

8.2.4 Texture Profile Analysis

Textural characteristics of cheeses including hardness, cohesiveness, springiness and chewiness were analysed by means of an Instron Universal Testing Machine (UTM) (5564, Instron Ltd., London, England) at room temperature (~22°C) according to Chapter 5.0, section 5.2.7. The details of the selected parameters have been described by Pons and Fiszman (1996) and results of hardness, cohesiveness, springiness and chewiness are presented as an average of 9 analyses.

8.2.5 Cheese Stretch and Yield Point

Stretch distance was recorded by elongation to 450 mm using the UTM by a modified version of the method described by Bhaskaracharya and Shah (2002) as used in Chapter 5.0, section 5.2.6. A cross bar spindle was placed at the bottom of a beaker containing 50 g of melted sample and the stretch limit was increased to 450 mm/min. Along with stretch distance and resistance, the yield point (the sudden change is slope for a given sample in a force-distance curve) was also recorded. The stretch distance and yield point results are representatives of 6 measurements.

8.2.6 Pizza Bake

Pizza bake was carried out in triplicate according to Chapter 7.0, section 7.2.9 and Hunter L,a,b values are representative of 15 to 25 readings.

8.2.7 Cheese Melt

The meltability of Mozzarella cheeses was determined in 250 mm long glass tubes with a diameter of 24 mm and thickness of 3 mm (R.B. Instruments, Mt. Eliza, VIC, Australia), using 10 g of cheese and heating at 110°C for 100 min as described in Chapter 5, section 5.2.5. Results are representatives of 6 measurements.

8.2.8 Proteolysis

An aliquot of each cheese sample (0.25 g cheese) was used to prepare a stock solution by suspending in a mixture of 1 mL tris (10 mM) (Sigma-Aldrich Co., St. Louis, MO, USA)-EDTA

(1 mM) pH 8.0 buffer, 350 µL of 10% SDS (Sigma-Aldrich) and 50 µL of β–mercaptoethanol (Bio-Rad Laboratories, Ltd., Watford, UK) as described by Fife _et al._ (1996). The samples were boiled at 5 min intervals and vortexed until the cheese solids were completely dissolved. A 25 µL aliquot of stock solution was diluted with 100 µL of 2X treatment buffer (0.125 M Tris-chloride, 4% SDS, 20% (vol/vol) glycerol, 0.2 M dithiothreitol, 0.02% bromophenol blue (pH 6.8) (Sigma-Aldrich)).

Whole casein, and α-, β-, κ-caseins (Sigma-Aldrich) were used as standards. Whole casein was dissolved in water at pH 10.0 and α-, β-, κ-caseins were dissolved in water at neutral pH to a concentration of 2 mg/mL. A 20 µL aliquot of this mixture was diluted with 40 µL of 2X treatment buffer.

A working volume of 15 µL of each sample and 7.5 µL of each standard (α-, β-, κ- and whole caseins) were loaded into 12.5% running gels. A 20 µL volume of broad range, pre-stained SDS-PAGE standard (Bio-Rad) was used as a marker.

SDS-PAGE gels were run in a BIO-RAD Protean® II xi cell filled with tank buffer solution (0.025 M tris, 0.192 M glycine, 0.1% SDS (pH 8.3)), powered by a power pac 300 run at 50 mA. Gels were staining with a solution of 0.025% (wt/vol) Coomassie Brilliant blue (ICN Biochemicals Inc., Aurora, Ohio, USA) for 4 h. Proteolysis was performed in duplicate.

Gels were fixed in de-staining solution I (40% methanol, 7% acetic acid) for 30 min before staining with staining solution (0.025% Coomassie Brilliant blue (ICN Biochemicals Inc., Aurora, Ohio, USA), 40% methanol, 7% acetic acid) for 4 h. Gels were then de-stained in de-staining solution I for 1 h followed by de-staining in de-staining solution II (7% acetic acid, 5% methanol) until the background became clear.

Gel images were recorded using a Fuji Film Intelligent Dark Box II with Fuji Film LAS-1000 Lite V1.3 software and the intensity of bands was measured using Fuji Film Image Gauge V4.0 software (Fuji Photo Film Co., Ltd., Japan). Figure 8.1 shows the standard curve used to calculate the molecular weight of caseins in low fat Mozzarella cheeses.

8.2.9 Confocal Scanning Laser Microscopy (CSLM)

A sample from each cheese was removed using a trier and cut into thin circular strips of approximately 2 mm thickness with minimal distortion of the surface by using a surgical blade. For a fat/protein stain, cheese slices were soaked in a solution of Nile Red (100 mg/L) (Sigma-Aldrich) containing a minimal amount of ethanol for 5 min (for staining fat), washed with distilled water and soaked in a solution of Fast Green (100 mg/L) (Sigma-Aldrich) for a further 5 min (for staining protein) before a final wash with distilled water. For an EPS/protein stain, samples were soaked in Fast Green for 5 min, washed with distilled water and soaked in a solution of fluorescein isothiocyanate conjugated with wheat germ (WG) (Sigma-Aldrich) made up in liquid whey (1mg/mL) and diluted 20 × for 20 min at 4°C (for staining carbohydrates).

Samples were subjected to a Leica TCS-SP2 confocal scanning laser microscope powered by Ar/Kr and He/Ne lasers. The microstructure of samples was viewed with a Leica DMIRE2 inverted microscope (Leica Microsystems, Heilderberg, Germany) fitted with a HCX PL APO CS 100 × 1.4 oil immersion lens. Nile Red was excited at a wavelength of 534 nm, Fast Green at 633 nm and WG at 488 nm. Emission wavelengths were set at 552 to 617 nm for Nile Red, 668 to 708 nm for Fast Green and 495 to 559 nm for WG. Images were recorded at a depth of 12 to 15 μm from the surface of the sample. Leica software was used to acquire digital images of 16 average frames. CSLM was performed at d 7, 28 and 45.

8.2.10 Statistical Analysis

Results are presented as a mean ± standard error of replicated measurements. To find significant differences between analyses, the means were analysed using one-way analysis of variance (ANOVA) with a 95% confidence interval using Microsoft® Excel StatPro™ (Palaside

Corporation, Newfield, NY). ANOVA data with a $P < 0.05$ was classified as statistically significant.

8.3 RESULTS AND DISCUSSION

8.3.1 Yield and Composition

Control cheeses made with capsular EPS producing _S. thermophilus_ 285 resulted in the lowest yield at 7.71 kg of cheese per 100 kg of milk. These cheeses also had the lowest moisture at 52.84% (56.51% as MNFS) (Table 8.1). Although EPS only cheeses showed the lowest moisture content, our previous findings indicate that the EPS cheeses made with capsular EPS producing _S. thermophilus_ 285 contained a significantly higher moisture content than non-EPS cheeses (Chapter 5.0; Bhaskaracharya _et al.,_ 2004; Zisu _et al.,_ 2004; Zisu & Shah, 2002). Supplementation of FR1 and FR2 into cheese milk reduced curd syneresis during cheese making. Addition of FR1 to cheese milk significantly increased both yield and moisture contents of low fat Mozzarella cheeses to 8.72 kg of cheese per 100 kg of milk and 56.21% (59.80% as MNFS), respectively. Furthermore, by pre-acidifying the cheese milk containing FR1, a further substantial increase in yield and moisture was recorded, reaching 9.44 kg of cheese per 100 kg of milk and 60.25%, moisture (63.89% as MNFS). Such moisture content led to complications in sample handling and undesired textural and functional attributes during storage as also described by Olson and Johnson (1990). A much lower amount of FR1 is therefore recommended for use when using pre-acidified milk. Cheeses made with pre-acidified milk and FR2 also showed a substantial increase in yield to 9.11 kg per 100 kg of milk and a moisture content to 57.89% (61.59% as MNFS), although the latter moisture content was not statistically different from that of FR1 only cheeses ($P > 0.05$). However, due to absence of cheese containing FR2 (not pre-acidified) it must be noted that comparisons between cheeses made with FR1 (without pre-acidification) and those containing FR2 (pre-acidified) cannot be

made. The higher moisture retention of pre-acidified cheeses containing FR1 over those made with pre-acidification containing FR2 may be attributed to the unconventional interaction of starch molecules in FR1. According to the manufacturers, the starch granules form a gelled structure that is similar to hydrocolloids when cooled.

In our previous work, no increase in moisture content was recorded in cheeses made with pre-acidified milk using citric acid (Chapter 6.0; Zisu & Shah, 2004a). The higher moisture content associated with the pre-acidified cheeses containing FR1 was therefore likely to occur due to increased gelation of starch granules (heat treated to 95°C prior to addition into cheese milk) after treatment with citric acid. Adebowale & Lawal (2003) showed that the swelling power of native starches and various modified starches was dependent on pH. The swelling power of certain starches improved when subjected to increased acidity. Acid modification of starch granules also increases the hydration properties of starches (Fennema, 1996).

Accordingly, the protein content was shown to be proportional to the moisture content. The cheeses with the lowest moisture content exhibited the highest protein content and vice versa. The moisture to protein (M:P) ratio increased from 1.55 in EPS cheeses to 1.80 in FR1 based EPS cheeses. The M:P ration increased further to 2.11 and 1.87 in FR1 and FR2 based EPS cheeses that were pre-acidified, respectively. Because all cheeses are low in fat, the protein contents were higher than it would be expected in full fat Mozzarella cheeses and ranged from 28.59% to 34.03% (Table 8.1).

8.3.2 Micro-structure

Cheese microstructure was monitored during storage to study the protein matrix of various types of cheeses, the distribution of fat and EPS within the protein matrix, and the hydration of moisture from within the fat/serum channels. The protein and fat components are responsible for the resulting texture and functionality of cheeses. Two components were stained

at any one time to show the protein/fat and protein/EPS relationship. It was anticipated that all three constituents may be represented on one image, however, due the interactions between the fat and carbohydrate stains the two variables became indistinguishable.

Cheese microstructure and the relationship between its protein matrix and fat globules in all cheese variables at d 7 and d 45 is depicted in Figure 8.2, A to H. Fat was located within the protein matrix channels only and was observed to be a mixture of agglomerated masses of fat and individual globules of fat that have not coalesced. As the cheeses are low in fat, large pockets of the serum channels remained vacant at d 7 (Figure 8.2, A, C, E, G). As viewed in the microstructure at 28 d, the cheeses became more hydrated and expanded as the protein channels lengthened (images not shown). Microstructure at 45 d of storage (Figure 8.2, B, D, F, H), demonstrated that the protein matrix had expanded as influenced by the effects of proteolysis and hydration of the protein network to condense and fill in any of the smaller channels and to greatly reduce the size of the larger protein channels. The hydration theory is supported by the disappearance of whey as a decrease in expressible serum during aging (Auty _et al._, 2001).

At d 7, confocal microscopy revealed many large serum channels in EPS only cheeses (Figure 8.2 A). At the same point in time, FR1 based EPS cheeses (without pre-acidification) (Figure 8.2, C) appeared to have several larger and many smaller channels to have a more porous appearance, whereas FR1 pre-acidified EPS cheeses (Figure 8.2, E) showed a combination of many larger channels and smaller pores giving a very porous appearance to the overall protein network. Day 45 images, following the hydration and expansion of the protein network, show that EPS only cheeses (Figure 8.2, B) retained some of the larger protein channels that previously existed at d 7. EPS cheeses exhibited the greatest overall hardness and lowest moisture, whereas, high moisture FR1 only cheeses (Figure 8.2, D) became very dense and lacked this structure. FR1 only cheeses initially lacked the formation of larger channels. The protein channels of FR1 pre-acidified cheeses became elongated after hydration and there was an expansion of the protein matrix (Figure 8.2, F). Cheeses made using pre-acidified milk, EPS

starter and FR2 had the densest protein matrix of all the cheeses (Figure 8.2, G). At d 7, the few protein channels were observed to be thin and elongated in contrast to the rounded appearance observed in the previous cheeses. These protein channels that exhibited a linear arrangement showed a resemblance to those observed in Mozzarella cheese by Auty _et al._ (2001). At d 45, these channels almost completely coalesced (Figure 8.2, H) leaving only very tightly packed fat molecules embedded within the protein matrix and the few remaining serum channels.

The porosity of the protein matrix of FR1 pre-acidified cheeses may have partially contributed to a greater moisture retention over cheeses made with pre-acidified milk containing FR2 which exhibited a dense protein network. According to McMahon _et al._ (1996), fat replacers may interfere with shrinkage of the protein matrix and lower the force involved in expelling water from curd particles. These authors showed that a low fat Mozzarella cheese made with a particular type of fat replacer increased the openness of cheeses and led to an increase in moisture content.

Figure 8.3 shows the distribution of microbial EPS produced by _S. thermophilus_ 285 in the cheese protein network. EPS produced by this strain is of capsular type and generally remains attached to the cell surface, however, it is viewed as an agglomerated mass. Unlike the globules of fat which were seen in the serum channels, EPS appeared embedded within the dense protein matrix. The EPS capsules may still be attached to individual cells that appeared to be clustered into masses. The stains used do not distinguish between protein and individual bacterial cells, therefore the statement cannot be ruled out with any certainty. Exopolysaccharides were consistently viewed in fewer aggregated masses in the batches of cheese where pre-acidified milk was used (images not shown). Observations are in accordance with our previous work where EPS extracted from cheeses was quantified in lower amounts when pre-acidification was involved (Chapter 6.0). This is attributed to the reduced fermentation time between the priming of the milk and coagulation.

8.3.3 Proteolysis

Figure 8.4 shows a typical protein profile of low fat Mozzarella cheese and depicts the molecular weight of caseins. The major caseins α- and β- were detected at 25 and 24 kDa, respectively. Figure 8.5 demonstrates the proteolysis of α- and β-caseins that occurred in four types of cheeses between d 0 and d 60 of storage. A reduction by 38% of α-casein was recorded in cheeses made using pre-acidified milk and FR1, 37% for cheeses made using pre-acidified milk with FR2, 27.4% for cheeses made with FR1 and 25% for EPS only cheeses (Figure 8.6, A). Proteolysis of α-casein was gradual and statistically significant at all time points for all cheeses ($P < 0.05$) with the exception of EPS only cheeses between d 45 and d 60 where there was no statistical significance recorded in the rate of proteolysis ($P > 0.05$).

A reduction of β-casein by 31.3% in FR1 pre-acidified cheeses, 30.8% in FR2 pre-acidified cheeses, 24.7% in FR1 cheeses and 26.7% in EPS cheeses occurred (Figure 8.6, B). The proteolysis of β-casein was overall lower than that of α–casein (Feeney _et al._, 2002; Fox _et al._, 1996). Between d 14 and d 28, β–casein proteolysis remained stable in the cheeses made with fat replacers ($P > 0.05$), however, EPS only cheeses showed an increase from 9% to 14%. A high level of β–casein proteolysis also occurred between d 28 and d 45 before slowing between d 45 and d 60.

More proteolysis occurred in cheeses with higher moisture contents. A higher moisture content may have enhanced accessibility to proteolytic enzyme resulting in accelerated proteolysis. Furthermore, a greater amount of casein proteolysis was observed over time in cheeses made with pre-acidified milk and with lower calcium content, as previously recorded by other researchers (Feeney _et al._, 2002; Joshi _et al._, 2003). Joshi _et al._ (2003) correlated this to a higher chymosin activity due to the use of a low pH milk, as described by Holmes _et al._ (1977). Caseins demonstrate unique interactions with calcium ions. We believe that proteolysis is further affected by pre-acidifying milk and reducing the calcium levels in the cheeses. This

reduces the bonding of the protein network that has a particular influence on the calcium sensitive α_s- and β-caseins which have low solubilities in the presence of calcium ions, and increases the susceptibility to the overall proteolytic breakdown (Fennema, 1996).

8.3.4 Cheese Melt

Figure 8.7 shows the changes in meltability of low fat Mozzarella cheeses over 60 d of storage. The control, EPS only cheeses gradually increased melt distance over time as a result of proteolysis as the protein network became increasingly more hydrated. After 60 d of storage, melt distance increased by 12.3 mm to a maximum distance of 53.7 mm. Cheeses made with FR1 showed an excessive melt at d 7, particularly in those made from pre-acidified milk. At d 14, melt was reduced and gradually increased during storage thereafter. The excess melt observed at d 7 is likely to have occurred due to excess moisture that remained unbound within the serum channels of the protein matrix before stabilisation of the cheese system at d 14. As the microstructure revealed at d 7 (Figure 8.2, C and E), the protein matrix remained relatively unhydrated. The excess moisture escaping into the test tubes may have assisted cheeses to flow. By d 14, the moisture distribution in cheeses containing FR1 began to stabilise and these cheeses exhibited their actual melt properties. Cheeses made with pre-acidified milk containing FR1 displayed the greatest overall melt reaching 71.9 mm at d 60 and greater melt at all time points ($P < 0.05$). These results further highlight the association between acid treatment and its influence on functionality of low fat Mozzarella cheeses as indicated by other authors (Guinee _et al._, 2002; Joshi _et al._, 2003; Paulson _et al._, 1998; Shakeel-Ur-Rehman _et al._, 2003). In our earlier work, we found that pre-acidification of cheese milk reduces calcium contents in cheeses and such cheeses exhibited the greatest melt (Chapter 6.0; Zisu & Shah, 2004a). FR1 containing cheeses had a greater melt than EPS cheeses, however, after 60 d of maturation the melt distance achieved for the two variables was not significantly different ($P > 0.05$). FR2 cheeses made with pre-acidified milk showed the poorest melt of all the variables examined with no

improvement in melt distance over time. Cheese melt is an indication of the ability of the cheese particles to flow past one another when melted and this is an important characteristic for using these cheeses as a topping for making pizza. Although FR2 cheeses were pre-acidified to increase the interaction between the protein matrix and moisture, as well as having a high moisture content and increased proteolysis, they continued to show poor melt. Due to the nature of the fat replacer, the earlier attributes became secondary in importance and were small contributing factors when taking melt into consideration. The dense protein matrix observed (Figure 8.2, G and H) may have impeded slippage of proteins and slowing movement of cheese when melted. Furthermore, after melting and re-solidification of cheeses containing FR2, they were observed to exhibit a porous consistency from evaporated moisture droplets and may have physically disrupted flow.

8.3.5 Texture Profile Analysis

8.3.5.1 Cheese Hardness

Figure 8.8 illustrates the changes in hardness in cheeses over 60 d of storage. The hardness decreased over time for all cheese variables due to proteolytic changes and the hydration of the protein matrix. EPS cheeses were significantly hardest overall and at all time points ($P < 0.05$). These cheeses had the highest protein content, lowest moisture and were not pre-acidified to lower the calcium content and increase proteolysis of casein. FR1 containing cheeses and cheeses made with pre-acidified milk and FR2 showed similar hardness in the first 14 d of storage. After 28 d of storage, the softness was far greater in FR2 pre-acidified cheeses as influenced by increased proteolysis. Cheeses containing FR1 made with pre-acidified milk were softest and showed a great reduction in hardness due to a combination of high moisture and a high amount of proteolysis. These cheeses were excessively soft at d 7 and became unmanageably soft and sticky after 14 d of storage. The most desirable cheeses were the EPS

cheeses after 28 d of maturation. FR1 only and FR2 pre-acidified cheeses also showed desirable characteristics in the initial 14 d of storage, however, these cheeses became too soft afterwards.

8.3.5.2 Cheese Cohesiveness

Figure 8.9 represents the changes in cohesiveness in cheeses over 60 d of storage. Cohesiveness of EPS only cheeses was greatest and did not alter throughout storage ($P > 0.05$). FR1 only cheeses showed lower cohesiveness but remained unchanged throughout storage. The previous two cheese variables had the lowest moisture content and also exhibited the greatest hardness. Similarly, EPS control cheeses had the lower moisture content and greatest hardness of the two and consequently showed greater cohesiveness compared to those made with FR1 only. Cohesiveness values for pre-acidified FR1 cheeses were lower and reduced with time ($P < 0.05$). Cohesiveness was lowest in these cheeses at d 60. This was possibly due to the high moisture content and softness of these cheeses. After hydration of the protein matrix, cheeses may have saturated with moisture. The internal bonds were less able to maintain the structural integrity of cheeses and they became susceptible to irreversible deformation when subjected to the forces of the UTM. Values for FR2 pre-acidified cheeses were lowest initially and remained constant during storage followed by a drop at d 60 due to identical reasons to the previous cheeses.

8.3.5.3 Cheese Springiness

Figure 8.10 indicates the change in springiness in cheeses over 60 d of storage. Springiness was greatest in the EPS only cheeses. These were also the hardest cheeses with the lowest moisture and showed greatest cohesiveness. This parameter remained constant throughout the storage period. Microstructural examinations of this cheese showed a porous structure after 45 d of storage (Figure 8.2, B) which allowed this cheese variable to 'spring' back to its original length as it maintained its integrity and resisted deformation unlike other cheeses. FR1 and FR2 pre-acidified cheeses showed slightly less springiness. Furthermore,

they showed reduction in springiness with time which was significantly lower ($P < 0.05$) after 60 d and after 28 d of storage, respectively. These cheeses had a similar moisture content and the microstructure showed a lack of porosity as observed with the EPS control cheeses after 45 d of storage. FR1 pre-acidified cheeses showed the lowest springiness values and had a significant reduction in springiness at d 60 ($P < 0.05$). As with cohesiveness, the relationship between moisture and hardness and their effects on the protein microstructure existed for springiness and are responsible for the loss in the ability of the cheese to recover to its original state.

8.3.5.3 Cheese Chewiness

The changes in chewiness in cheeses over 60 d of storage are shown in Figure 8.11. As a secondary parameter, chewiness was calculated as hardness × cohesiveness × springiness. The hardness of cheeses is the dominant factor influencing the chewiness of cheeses and the trend in results is similar between the two variables. Chewiness was greatest in EPS cheeses ($P < 0.05$) and lower in FR1 based and FR2 pre-acidified cheese. FR1 pre-acidified cheeses showed least chewiness ($P < 0.05$). Chewiness significantly reduced with storage time ($P < 0.05$).

8.3.6 Cheese Stretch

Figure 8.12 shows the stretch performance of cheeses over 60 d of storage. Cheeses containing FR1 had a maximum stretch of 450 mm during the first 14 days of storage. At d 7, FR1 only cheeses, however, exhibited a thick and unappealing type of stretch before becoming smooth and fibrous at d 14 upon hydration of the protein matrix. The appearance of FR1 pre-acidified cheese showed exceptional stretch characteristics during this period of time. At 28 d, the stretch characteristics of FR1 pre-acidified cheeses began to deteriorate and stretched cheese appeared very thin and there was some loss in stretch distance. Cheeses made with FR1 alone

continued to show maximum stretch with a smooth appearance. At d 45, there was a slight reduction in the stretch distance recorded for both FR1 variables before degrading further to show a significant reduction in stretch performance after 60 d of storage. At these times, both types of cheeses became very thin and threadlike in appearance when stretched. The FR1 pre-acidified cheeses showed most deterioration. The loss in the ability to stretch with time can be attributed to the increase in protein breakdown and the effect on the cross-linking of the proteins associated with maintaining the structure making the cheese more viscous as was detected with TPA analysis.

EPS only cheeses showed the least amount of stretch over the first 14 d, however, the stretch distance gradually increased to d 28 before stabilising to show a notable amount of stretch to over 400 mm that was comparable to FR1 cheeses after a 60 d period. When stretched the appearance of the melted cheese strands were fibrous and exceptionally good after 28 d of maturation.

FR2 pre-acidified cheeses had poor stretch characteristics. Although a substantial stretch distance of FR2 cheeses was observed over the initial 14 d of storage, the stretch distance rapidly decreased to below 200 mm by d 28. After melting of cheeses to prepare for the stretch test, FR2 based cheeses were very porous in appearance as was observed when performing the melt test. The porous consistency of melted cheese may have physically disrupted the continuous flow of cheese when stretched resulting in a low stretch distance. Furthermore, excessive seepage of liquid was detected at the bottom of the beaker at d 7. The seepage associated with these cheeses suggests that FR2 had a lower water holding capacity to that of FR1. The microstructure of FR2 appeared to show a denser protein matrix possibly due to increased coalescence of casein strands (Figure 8.2, G) as compared to the protein matrix of other cheeses and was likely to contributed to syneresis.

8.3.7 Yield Point

The yield point of melted cheese is a functional characteristic and is a measure of the perception an individual would attain (*e.g.* while eating a pizza). On the contrary, the measurements of TPA of unmelted low-fat Mozzarella cheeses often depict the textural behaviour of cheeses.

As indicated by TPA analysis observations in reference to the loss of protein network, leading to the loss in integrity of unmelted cheese, a similar loss in bonding strength was shown to exist in melted cheese as measured by the yield point. The changes in the yield point of cheeses over 60 d of storage are illustrated in Figure 8.13. Melted cheeses showed a similar trend in their yield point resistance to force before reaching the irreversible point of deformation to that observed in the hardness measurements of unmelted cheeses. Results show that the initial deformation force had a greater resistance in the EPS cheeses that were consequently the hardest, followed by the FR1 and FR2 pre-acidified cheeses, respectively, and finally the FR1 pre-acidified cheeses showing the least resistance to force before deformation. A gradual reduction in force required before the yield point was reached was observed in all melted cheeses.

8.3.8 Pizza Bake

A pizza bake test was carried out to understand the browning behaviour, melt and flow of cheeses during pizza baking (Metzger *et al.*, 2000a; Rudan and Barbano, 1998a; Rudan and Barbano, 1998b). The concept of an oil barrier applied to shredded low fat Mozzarella before baking was introduced by Rudan and Barbano (1998a,b). It was found to reduce moisture loss from the shred surface and subsequent skin formation leading to improved melting and browning.

8.3.8.1 L,a,b values: Before Cooking

Table 8.2 shows the L-values (whiteness) of cheese before cooking, when warm and after cooling at 28 and 45 d of storage. Cheeses lacking an oil coating at d 28 had a higher L-value before cooking than the cheeses coated with oil with the exception of FR1 pre-acidified cheeses that showed no significant difference between the two treatments ($P > 0.05$), possibly due to the extremely high moisture content. The oil coating therefore reduced the whiteness of the cheese before cooking. At d 45, L-values became similar between the oil and no-oil treatments for all variables following the hydration of the protein matrix and effects of proteolysis allowing uniform light distribution as was the case with FR1 pre-acidified cheeses at d 28. EPS control cheeses, on the contrary, maintained greater L-values when untreated, presumably due to the low moisture content that had little effect on light scattering upon hydration of the protein matrix.

Table 8.3 shows the a-values (redness) of cheese before cooking, when warm and after cooling at 28 and 45 d of storage. The a-values were similar between cheese types and in the green section of the colour spectrum before baking.

Table 8.4 shows the b-values (yellowness) of cheese before cooking, when warm and after cooling at 28 and 45 d of storage. The b-values were generally similar between cheese types and between treatments ($P > 0.05$). Yellowness did not alter over time between d 28 and d 45 before cooking ($P > 0.05$).

8.3.8.2 L,a,b values: Warm

The baking behaviour of the four types of cheese is shown in Figure 8.14, A to D. The whiteness increased significantly ($P < 0.05$) immediately after cooking while pizzas were still warm (Table 8.2). EPS and FR1 only cheeses (Figure 8.14, A and B) appeared similar after baking showing a golden brown uniform bake with complete shred fusion when coated with oil, however, both cheeses lacked complete shred fusion and these were scorched individual shreds

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creating an unappealing appearance in the absence of an oil coating. FR1 pre-acidified cheeses (Figure 8.14, C) with the highest moisture content were very light showing little surface browning when coated with oil. Shred fusion and cheese melt were greatly improved without the use of a oil coating. Due to a higher moisture content retained in the hydrated protein matrix, FR1 cheeses, however, appeared lighter with less browning. This was confirmed as both FR1 cheeses exhibited the greatest L-values in general. Similar to before cooking, cheese whiteness (L-values) remained higher ($P < 0.05$) in the absence of an oil coating (Table 8.2), with the exception of FR2 pre-acidified cheeses. The FR2 cheeses (Figure 8.14, D) showed poor baking characteristics for both treatments at d 28 by displaying poor shred fusion and melt with a high degree of localised surface scorching of individual shreds of cheese even in the presence of an external oil coating. A high degree of surface scorching in these cheeses may be related to the β-lactoglobulin content of FR2 which may increase non-enzymatic Maillard browning by reacting with lactose and its constituents. Between d 28 and d 45 of maturation within a cheese type, the L-values of oil coated and non-coated cheeses remained unchanged with the exception of FR2 oil treated cheeses that showed a vast improvement when baked and had greater L-values. Ultimately, an external oil coating remained necessary to achieve an acceptable pizza bake appearance with complete shred fusion of low fat Mozzarella cheeses.

The a-values also increased after baking ($P < 0.05$) due to surface browning. FR2 based untreated cheeses showed the greatest a-values at d 28, confirming the scorching previously observed. EPS and FR1 untreated cheeses had lower a-values and FR1 pre-acidified cheeses had the lowest. At d 45, FR2 cheeses (no-oil) showed similar a-values to EPS and FR1 cheeses signifying the improvement in baking quality after maturation. FR2 pre-acidified cheeses had lower a-values. Oil treatment improved baking (lower a-values than untreated cheeses).

The increase in whiteness (L-values) observed when pizzas were baked was also evident with yellowness and b-values increased significantly (P < 0.05). Oil coated cheeses generally

had greater b-values than untreated cheeses and they did not alter between d 28 and d 45 of storage.

8.3.8.3 L,a,b values: Cool

L-values were lower after cooling of the baked pizzas, and oil coated cheeses showed significantly greater L-values at both time points to those lacking an oil coating. However, all cheeses became translucent and shiny. The oil coating influences the light scattering properties of the cheeses, contributes to the whiteness by providing the physical barrier to oxygen and prevent excessive moisture loss. EPS control cheeses lacking an oil coating had the lowest L-values after cooling and whiteness remained greatest in the FR1 pre-acidified cheeses.

Both a- and b-values remained higher when cooled to those observed before baking. The b-values did, however, reduce after cooling.

8.4. CONCLUSIONS

Moisture in low fat Mozzarella cheeses made with EPS producing starter cultures was increased with the use of FR1 and FR2 fat replacers leading to improved yield and textural characteristics. Pre-acidification of the cheese milk and use of FR1 further increased the moisture content and yield. Pre-acidified cheeses also had an increased level of proteolysis and subsequent hydration of the protein matrix improved their functional behaviour. The nature of the fat replacer, however, had the greatest influence on the microstructure of cheeses and the impact had on the textural and functional characteristics. FR1 containing cheeses showed better melt, stretch and pizza bake performance as compared to FR2 containing cheeses. By combining EPS cultures with the appropriate fat replacer and pre-acidification, it is possible to increase the yield of low fat Mozzarella cheeses, achieve textural and behavioural attributes

superior to those of untreated low fat Mozzarella cheeses, and reduce maturation time, thereby reducing storage periods.

Table 8.1. Average yield and composition of low fat Mozzarella cheeses (mean ± standard error).

Cheese	Yield (kg/100kg) (n = 3)	Moisture (%) (n = 9)	Fat (%) (n = 6)	[1]MNFS (%) (n = 6)	[2]FDM (%) (n = 6)	Protein (%) (n = 6)	[3]M:P
EPS[4]	7.71 ± 0.07[c]	52.84 ± 0.32[d]	6.5 ± 0.00[a]	56.51 ± 0.15[d]	13.78 ± 0.16[a]	34.03 ± 0.29[a]	1.55
EPS [5]FR1	8.72 ± 0.20[b]	56.21 ± 0.28[c]	6.0 ± 0.50[abc]	59.80 ± 0.33[c]	13.70 ± 0.35[a]	31.19 ± 0.36[b]	1.80
EPS FR1 [6]Pre-acid	9.44 ± 0.13[a]	60.25 ± 0.39[a]	5.7 ± 0.17[c]	63.89 ± 0.25[a]	14.33 ± 0.22[a]	28.59 ± 0.34[d]	2.11
EPS FR2 Pre-acid	9.11 ± 0.13[a]	57.89 ± 0.32[b]	6.0 ± 0.00[bc]	61.59 ± 0.16[b]	14.25 ± 0.15[a]	30.98 ± 0.24[bc]	1.87

[abc]One-way ANOVA of means in the same column with different superscript are significantly different ($P < 0.05$).

[1]MNFS = Moisture in non-fat substance.

[2]FDM = Fat in dry matter.

[3]M:P = Moisture to protein ratio based on final (average) moisture and protein contents.

[4]EPS = Capsular exopolysaccharides (EPS) produced by *S. thermophilus* 285.

[5]FR = Fat replacer.

[6]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

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Table 8.2. Mean Hunter L-values (n = 15 to 25 ± SE) for whiteness measurements of low fat Mozzarella cheeses for given variables, treatments and processing stages.

	¹EPS		EPS ²FR1		EPS FR1 ³Pre-acid		EPS FR2 Pre-acid	
	No Oil	Oil	No Oil	Oil	No Oil	Oil	No Oil	Oil
Before Cook$_{28}$[4]	64.48 ± 0.54^{cdefg,B}	60.88 ± 0.61^{h,DE}	66.78 ± 0.35^{a,B}	64.42 ± 0.33^{de,DE}	63.54 ± 0.73^{efg,CDE}	62.89 ± 0.58^{g,DE}	65.48 ± 0.28^{bcd,BCD}	63.10 ± 0.55^{fg,F}
Before Cook$_{45}$	62.50 ± 0.58^{bcd,C}	60.16 ± 0.16^{e,E}	62.88 ± 0.68^{abcd,CDE}	63.65 ± 0.38^{ab,E}	62.39 ± 0.37^{cd,DE}	62.35 ± 0.37^{d,E}	64.22 ± 0.24^{a,CD}	63.74 ± 0.42^{ab,EF}
Warm$_{28}$	71.60 ± 1.14^{cdef,A}	67.64 ± 0.89^{g,A}	73.32 ± 0.88^{abc,A}	70.42 ± 0.90^{def,A}	74.54 ± 0.44^{a,A}	71.87 ± 0.40^{bcde,A}	69.93 ± 1.08^{efg,A}	69.30 ± 0.81^{fg,BCD}
Warm$_{45}$	70.34 ± 0.84^{bcd,A}	68.74 ± 0.85^{d,A}	71.17 ± 1.00^{abcd,A}	69.33 ± 0.60^{cd,A}	72.28 ± 0.97^{ab,A}	72.43 ± 0.31^{a,A}	71.83 ± 1.12^{ab,A}	72.24 ± 0.53^{ab,A}
Cool$_{28}$	58.27 ± 0.72^{g,E}	62.15 ± 1.07^{ef,CDE}	61.27 ± 0.84^{f,E}	64.92 ± 1.03^{bcde,CDE}	64.85 ± 0.75^{cd,BC}	68.17 ± 0.52^{a,BC}	63.79 ± 0.94^{def,DE}	68.11 ± 0.75^{a,D}
Cool$_{45}$	58.46 ± 1.19^{g,DE}	65.14 ± 0.61^{c,B}	62.12 ± 0.63^{f,DE}	65.59 ± 0.76^{bc,BCD}	62.13 ± 0.59^{ef,E}	67.58 ± 0.46^{a,C}	62.41 ± 0.72^{def,E}	68.29 ± 0.79^{a,CD}

[abc]One-way ANOVA of means across a row and [ABC]one-way ANOVA of means down a column with different superscript are significantly different ($P < 0.05$).

¹EPS = Capsular exopolysaccharides (EPS) produced by *S. thermophilus* 285.

²FR = Fat replacer.

³Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

⁴$_{28,45}$Pizza bake performed after 28 d and 45 d of storage at 4°C.

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Table 8.3. Mean Hunter a-values (n = 15 to 25 ± SE) for redness measurements of low fat Mozzarella cheeses for given variables, treatments and processing stages.

	EPS[1]		EPS[2]FR1		EPS FR1[3]Pre-acid		EPS FR2 Pre-acid	
	No Oil	Oil	No Oil	Oil	No Oil	Oil	No Oil	Oil
Before Cook[4]$_{28}$	-1.62 ± 0.07[cd,C]	-1.23 ± 0.12[a,CD]	-1.66 ± 0.06[d,C]	-1.57 ± 0.07[bcd,D]	-1.23 ± 0.09[a,DE]	-1.44 ± 0.07[abc,DE]	-1.26 ± 0.06[a,C]	-1.34 ± 0.15[abcd,E]
Before Cook$_{45}$	-1.43 ± 0.08[bc,BC]	-1.23 ± 0.08[ab,D]	-1.19 ± 0.11[ab,B]	-1.49 ± 0.06[c,CD]	-1.30 ± 0.05[ab,E]	-1.37 ± 0.07[abc,CDE]	-1.19 ± 0.06[a,BC]	-1.34 ± 0.07[abc,DE]
Warm$_{28}$	1.68 ± 0.77[bcde,A]	2.38 ± 0.75[abcd,AB]	0.96 ± 0.55[cde,A]	0.46 ± 0.60[def,B]	-0.71 ± 0.20[f,BC]	0.03 ± 0.72[ef,ABCD]	2.70 ± 0.53[ab,A]	3.92 ± 0.76[a,A]
Warm$_{45}$	1.61 ± 0.60[a,A]	1.36 ± 0.81[a,AB]	1.13 ± 0.63[a,A]	1.44 ± 0.55[a,AB]	-0.80 ± 0.30[b,CDE]	-1.71 ± 0.12[c,E]	1.33 ± 0.70[a,A]	0.94 ± 0.53[a,C]
Cool$_{28}$	2.52 ± 0.41[a,A]	3.18 ± 77[a,A]	1.26 ± 0.44[bcd,A]	2.08 ± 0.82[abcd,AB]	0.60 ± 0.56[cd,A]	0.19 ± 0.41[d,A]	2.25 ± 0.62[ab,A]	3.03 ± 0.74[ab,AB]
Cool$_{45}$	1.68 ± 0.59[a,A]	0.98 ± 0.43[ab,B]	0.77 ± 0.45[ab,A]	2.25 ± 0.59[a,A]	0.03 ± 0.34[b,ABC]	-1.35 ± 0.20[c,BCDE]	1.50 ± 0.65[ab,A]	1.38 ± 0.76[ab,BC]

[abc]One-way ANOVA of means across a row with different superscript and [ABC]one-way ANOVA of means down a column with different superscript are significantly different ($P < 0.05$).
[1]EPS = Capsular exopolysaccharides (EPS) produced by *S. thermophilus* 285.
[2]FR = Fat replacer.
[3]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.
[4]$_{28,45}$Pizza bake performed after 28 d and 45 d of storage at 4°C.

Table 8.4. Mean Hunter b-values (n = 15 to 25 ± SE) for yellowness measurements of low fat Mozzarella cheeses for given variables, treatments and processing stages.

	EPS[1]		EPS[2] FR1		EPS FR1[3] Pre-acid		EPS FR2 Pre-acid	
	No Oil	Oil	No Oil	Oil	No Oil	Oil	No Oil	Oil
Before Cook[4]$_{28}$	$19.92 \pm 0.38^{cdef,E}$	$18.96 \pm 0.35^{fgh,E}$	$21.43 \pm 0.26^{a,D}$	$19.74 \pm 0.28^{def,DE}$	$19.05 \pm 0.32^{efgh,E}$	$18.40 \pm 0.23^{g,h,E}$	$20.23 \pm 0.30^{bcd,DE}$	$18.40 \pm 0.35^{gh,C}$
Before Cook$_{45}$	$20.19 \pm 0.32^{a,DE}$	$19.24 \pm 0.39^{a,DE}$	$19.92 \pm 0.25^{a,E}$	$19.30 \pm 0.23^{a,E}$	$19.85 \pm 0.25^{a,D,E}$	$19.78 \pm 0.25^{a,D,E}$	$19.78 \pm 0.25^{a,E}$	$19.82 \pm 0.21^{a,BC}$
Warm$_{28}$	$28.19 \pm 0.63^{defg,A}$	$32.35 \pm 1.07^{a,A}$	$27.42 \pm 0.57^{fg,A}$	$28.54 \pm 0.75^{cdefg,ABC}$	$25.95 \pm 0.39^{h,A}$	$26.48 \pm 0.97^{gh,A}$	$27.71 \pm 0.43^{efg,A}$	$28.60 \pm 0.58^{bcdefg,A}$
Warm$_{45}$	$28.43 \pm 0.38^{bcd,A}$	$30.45 \pm 0.82^{a,A}$	$27.54 \pm 0.42^{cd,A}$	$29.59 \pm 0.48^{a,A}$	$24.47 \pm 0.33^{e,f,B}$	$23.73 \pm 0.32^{f,C}$	$27.44 \pm 0.63^{d,A}$	$28.53 \pm 0.63^{abcd,A}$
Cool$_{28}$	$25.56 \pm 0.33^{def,BC}$	$27.47 \pm 0.42^{a,BC}$	$25.20 \pm 0.42^{ef,BC}$	$27.40 \pm 0.27^{a,C}$	$25.07 \pm 0.34^{f,A,B}$	$25.77 \pm 0.61^{c,d,e,f,A}$	$25.80 \pm 0.45^{bcdef,BC}$	$27.92 \pm 0.45^{a,A}$
Cool$_{45}$	$24.52 \pm 0.49^{de,C}$	$27.22 \pm 0.40^{a,C}$	$25.19 \pm 0.31^{cd,C}$	$27.47 \pm 0.21^{a,BC}$	$22.64 \pm 0.44^{f,C}$	$23.78 \pm 0.32^{e,B,C}$	$25.41 \pm 0.52^{bcd,C}$	$27.72 \pm 0.42^{a,A}$

[abc]One-way ANOVA of means across a row with different superscript and [ABC]one-way ANOVA of means down a column with different superscript are significantly different ($P < 0.05$).

[1]EPS = Capsular exopolysaccharides (EPS) produced by *S. thermophilus* 285.

[2]FR = Fat replacer.

[3]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

[4]$_{28,45}$Pizza bake performed after 28 d and 45 d of storage at 4°C.
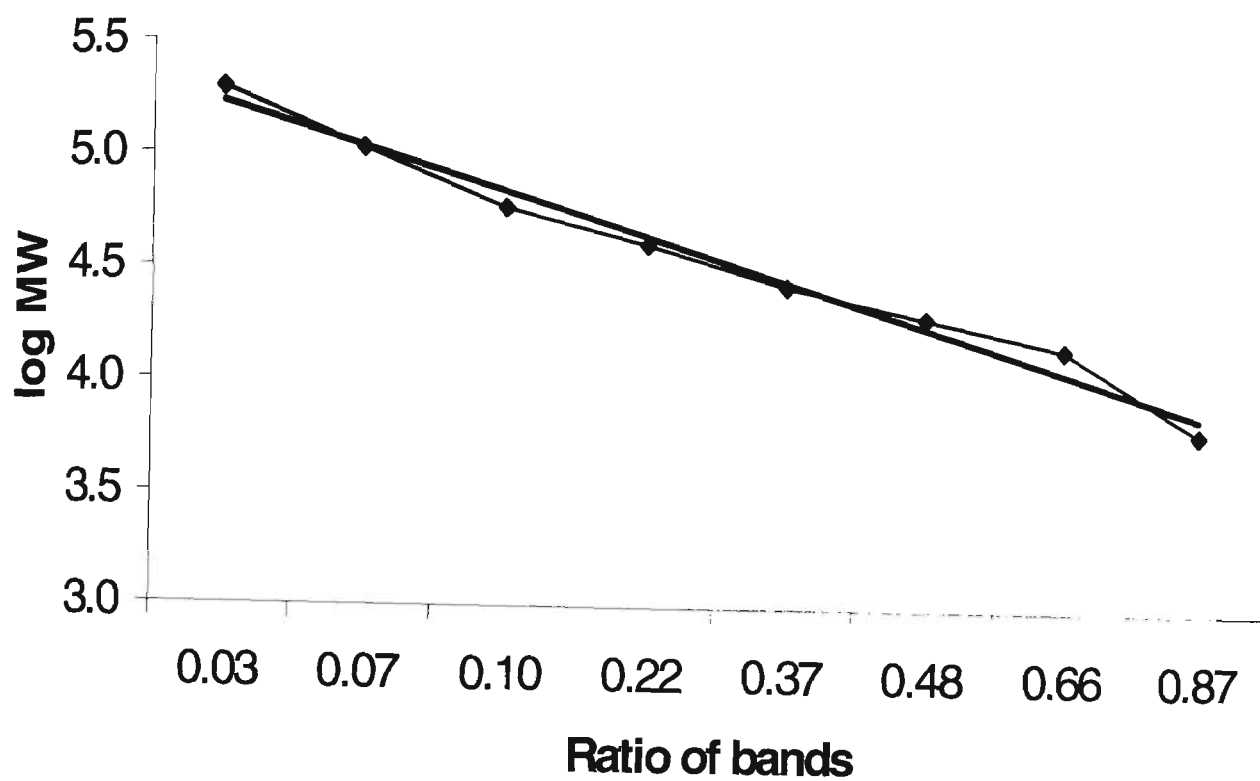
Figure 8.1. SDS-PAGE standard curve used to calculate the molecular weight of caseins in low fat Mozzarella cheeses.
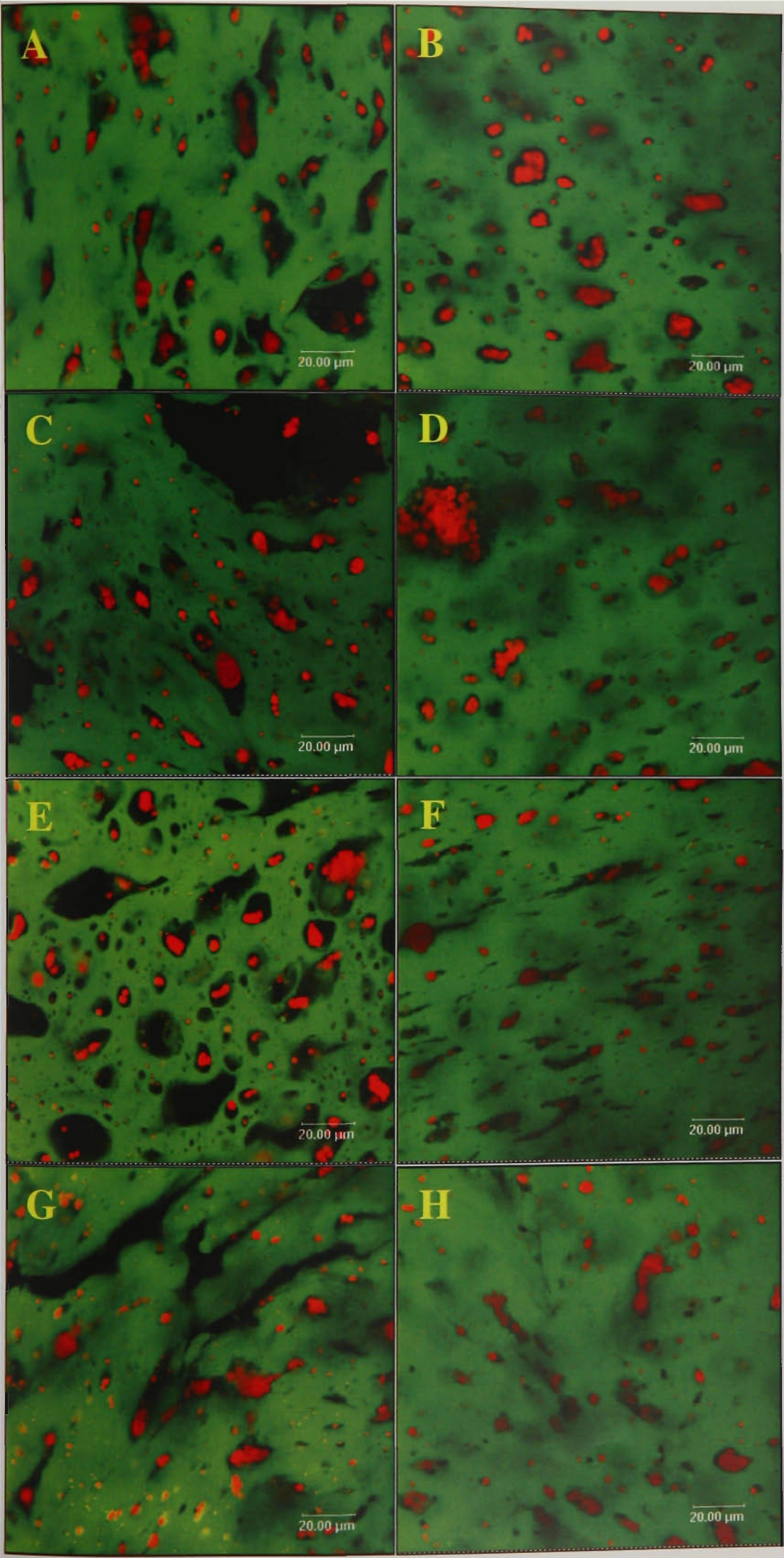
Figure 8.2. Distribution of fat in the protein matrix of low fat Mozzarella cheese at d 7 and d 45 respectively, made with capsular exopolysaccharide forming *S. thermophilus* 285 (EPS) (A and B), EPS and fat replacer 1 (FR1) (C and D), EPS and FR1 pre-acidified (E and F), and EPS and FR2 pre-acidified (G and H).
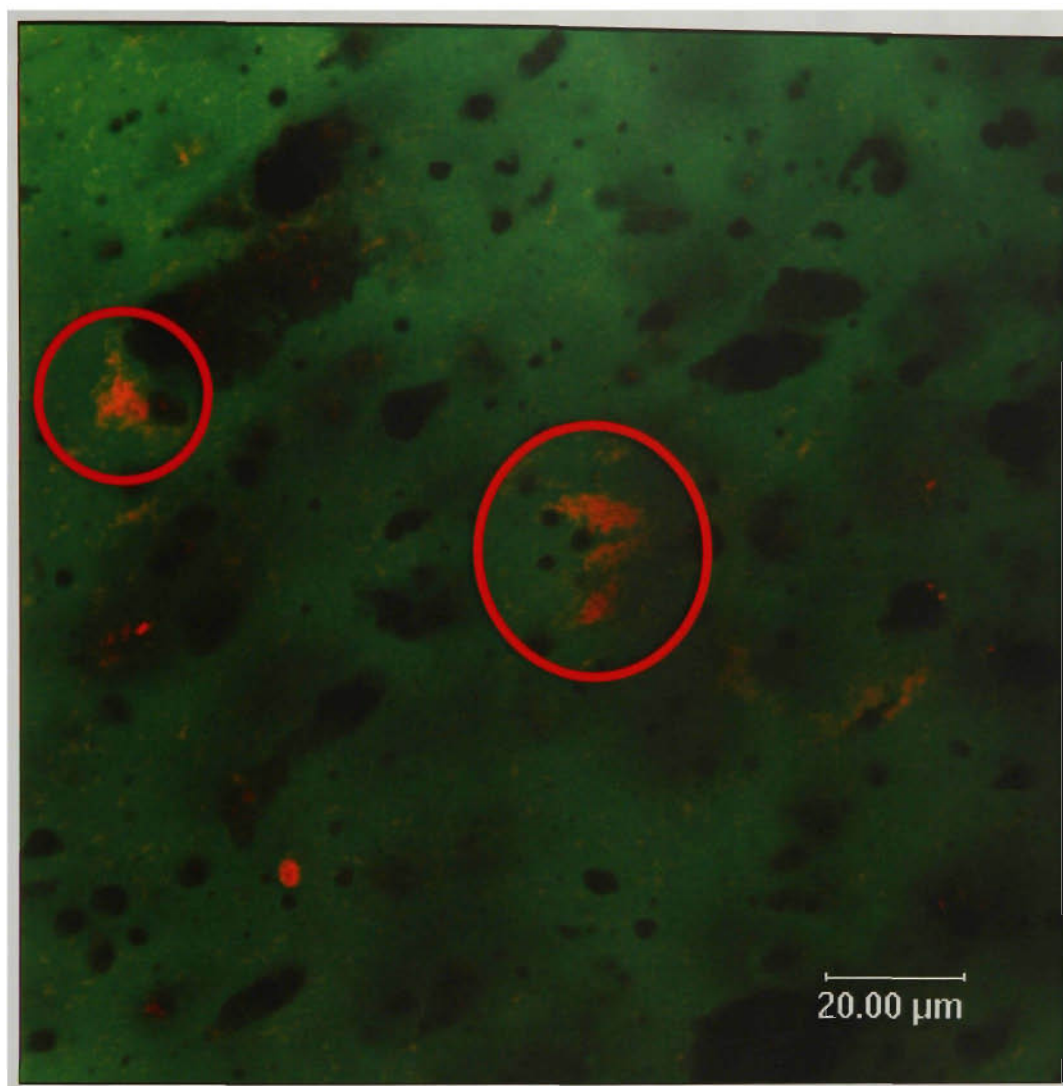
Figure 8.3. Distribution of microbial exopolysaccharides (EPS) in the protein matrix of low fat Mozzarella cheese. Dense EPS concentration is emphasised in circles.
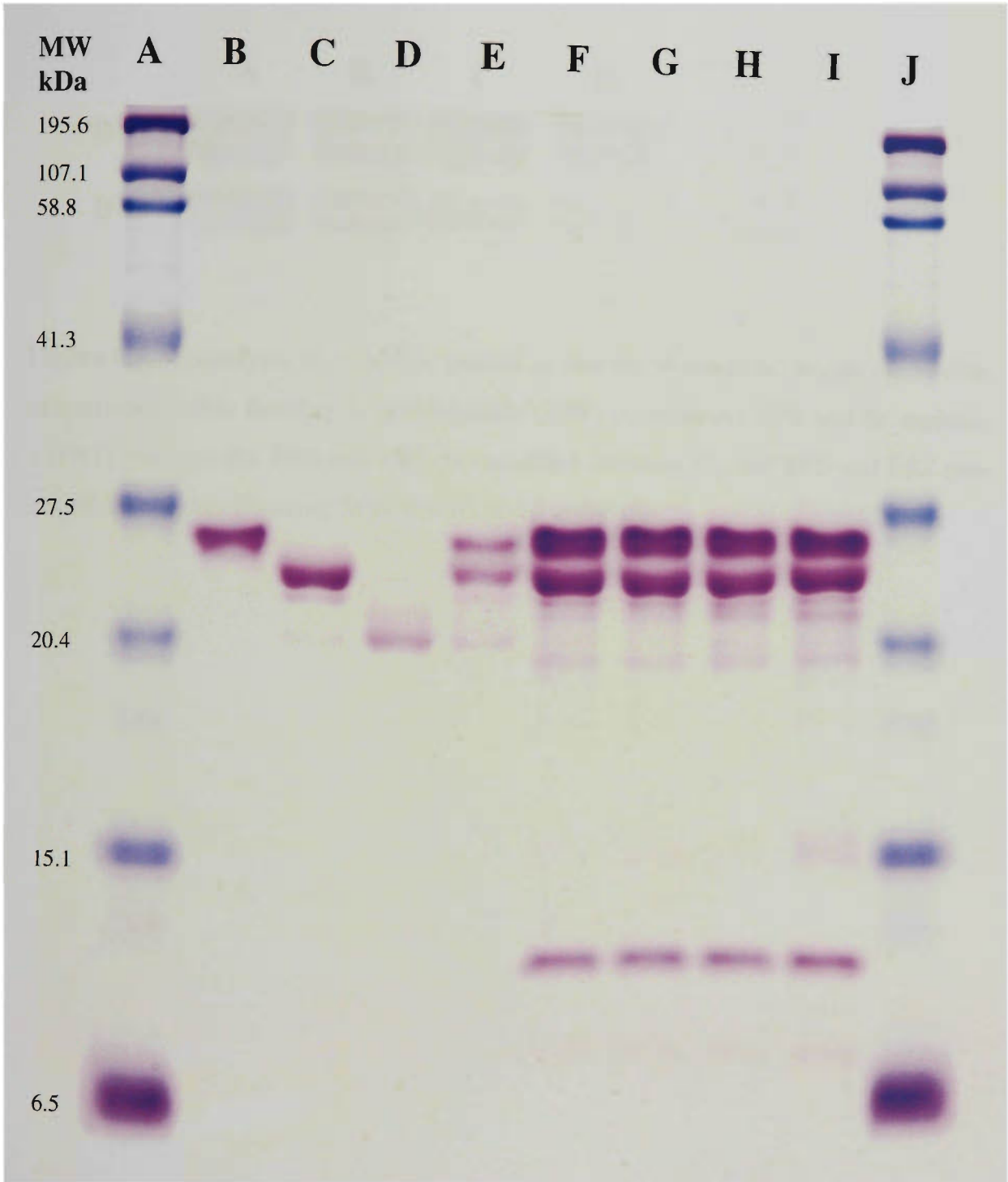
Figure 8.4. SDS-PAGE profile of caseins in low fat Mozzarella cheeses made with exopolysaccharide forming *S. thermophilus* 285 (EPS) (column F), EPS and fat replacer 1 (FR1) (column G), EPS and FR1 pre-acidified (column H), and EPS and FR2 pre-acidified (column I). Proteins verified using a broad range standard (columns A and J), α- (column B), β- (column C), κ- (column D) and whole casein (column E).
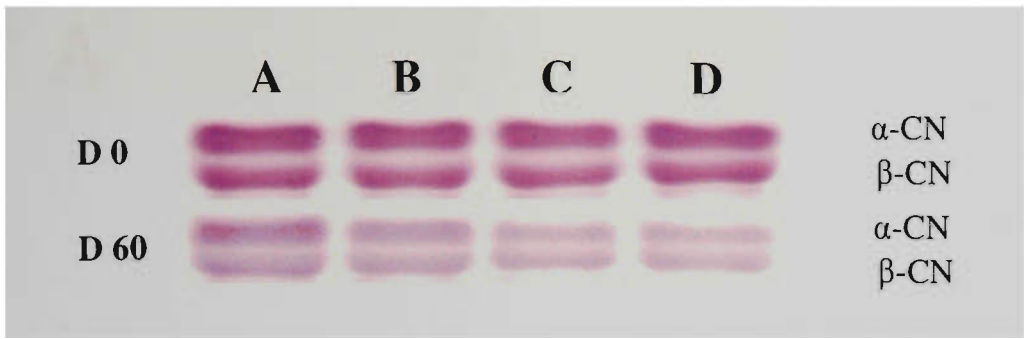
Figure 8.5. Proteolysis of α- and β- caseins in low fat Mozzarella cheeses made with exopolysaccharide forming *S. thermophilus* (EPS) (column A), EPS and fat replacer 1 (FR1) (column B), EPS and FR1 pre-acidified (column C), and EPS and FR2 pre-acidified (column D) using SDS-PAGE at d 0 and d 60.
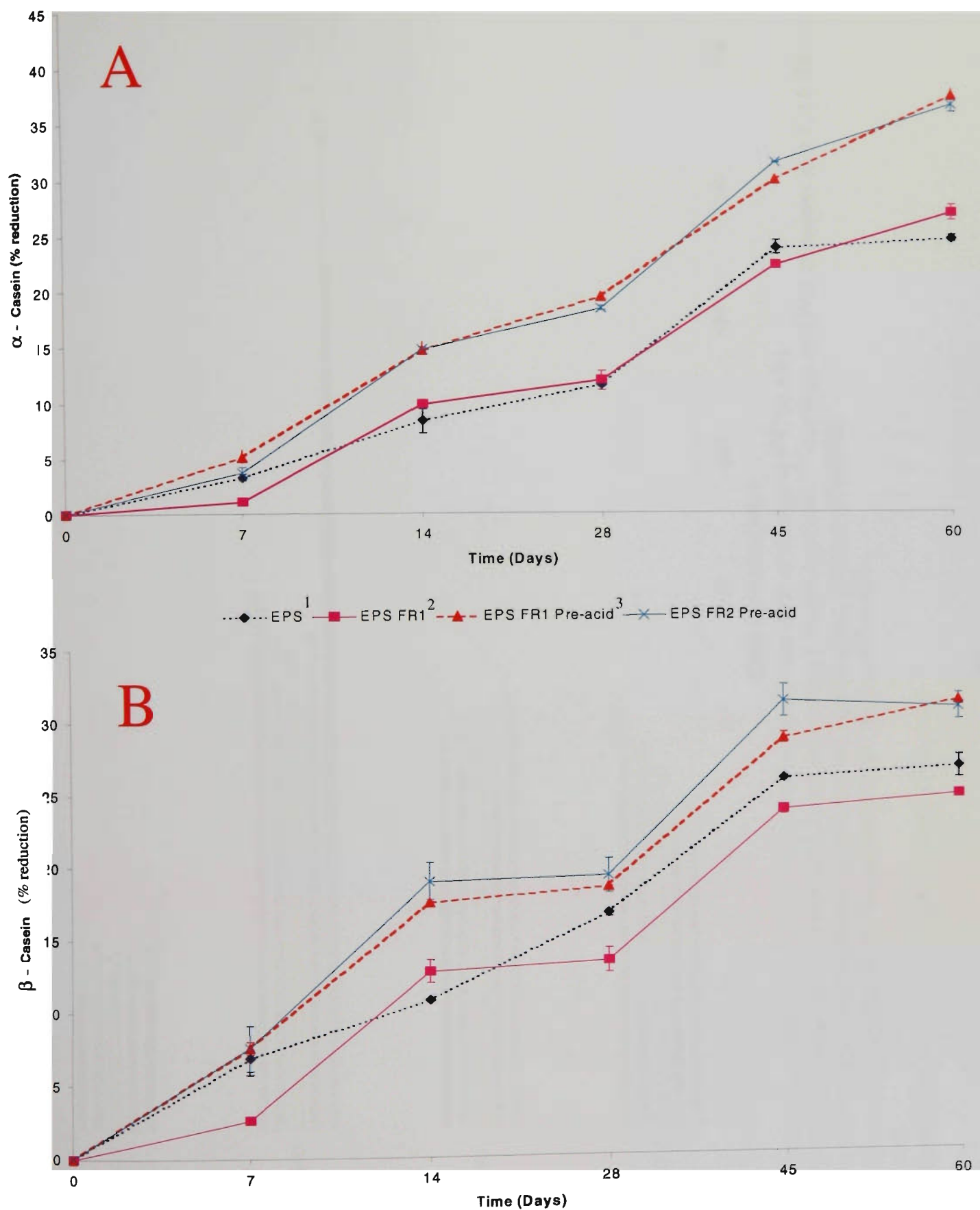
Figure 8.6. Reduction in α-casein (A) and β-casein (B) in low fat Mozzarella cheese as a percentage of d 0 over 60 d of storage at 4°C (n = 2).
[1]EPS = Capsular exopolysaccharides (EPS) produced by *S. thermophilus* 285.
[2]FR = Fat replacer.
[3]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.
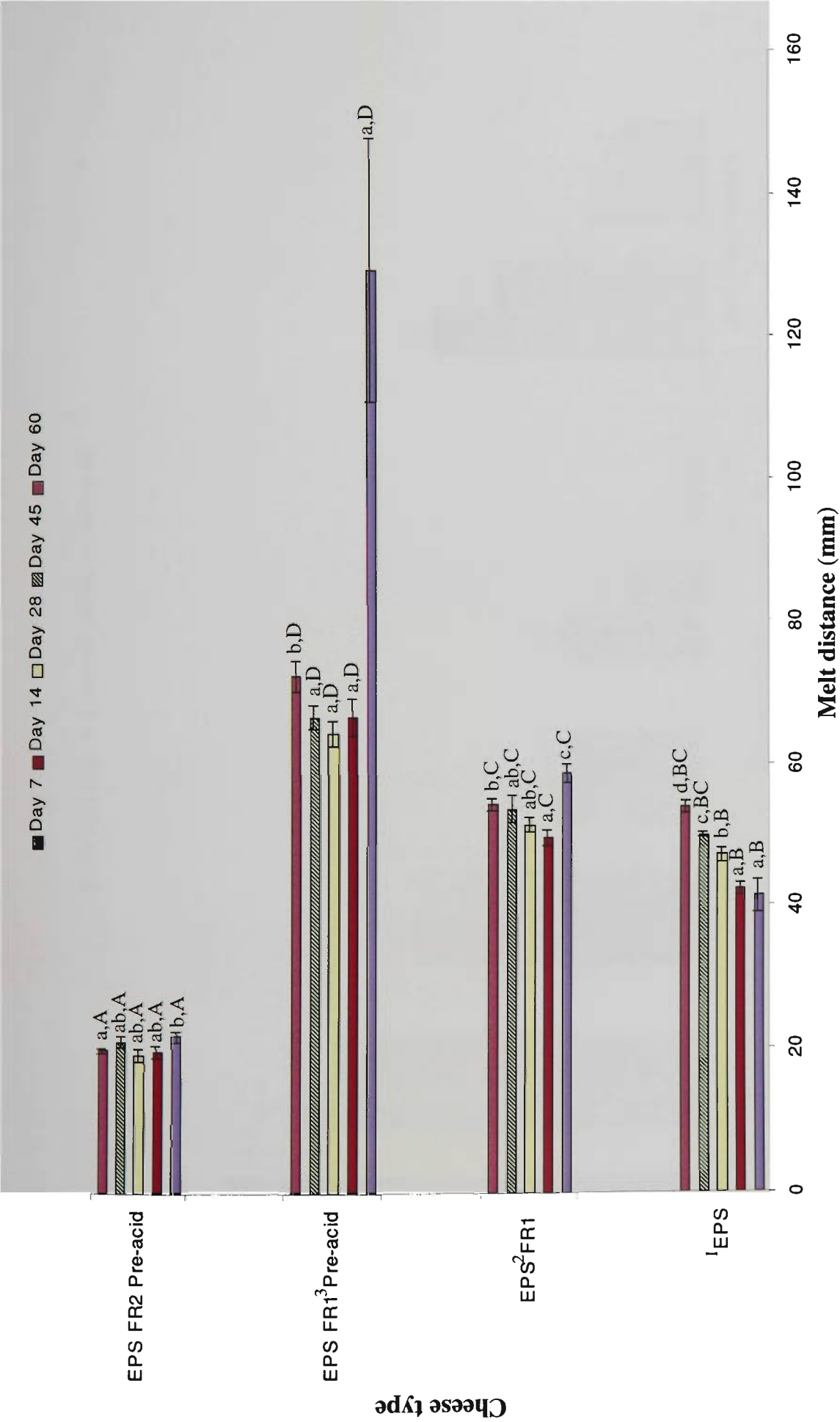
Figure 8.7. Changes in meltability of low fat Mozzarella cheeses over 60 d of storage at 4°C (n = 6). [a,b,c]One-way ANOVA of means (n = 6) between d 7 and d 60 within a cheese type and [A,B,C]one-way ANOVA of means at d 7, 14, 28, 45 and 60 between cheese types with different superscripts are significantly different ($P < 0.05$).

[1]EPS = Capsular exopolysaccharides (EPS) produced by *S. thermophilus* 285.

[2]FR = Fat replacer.

[3]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

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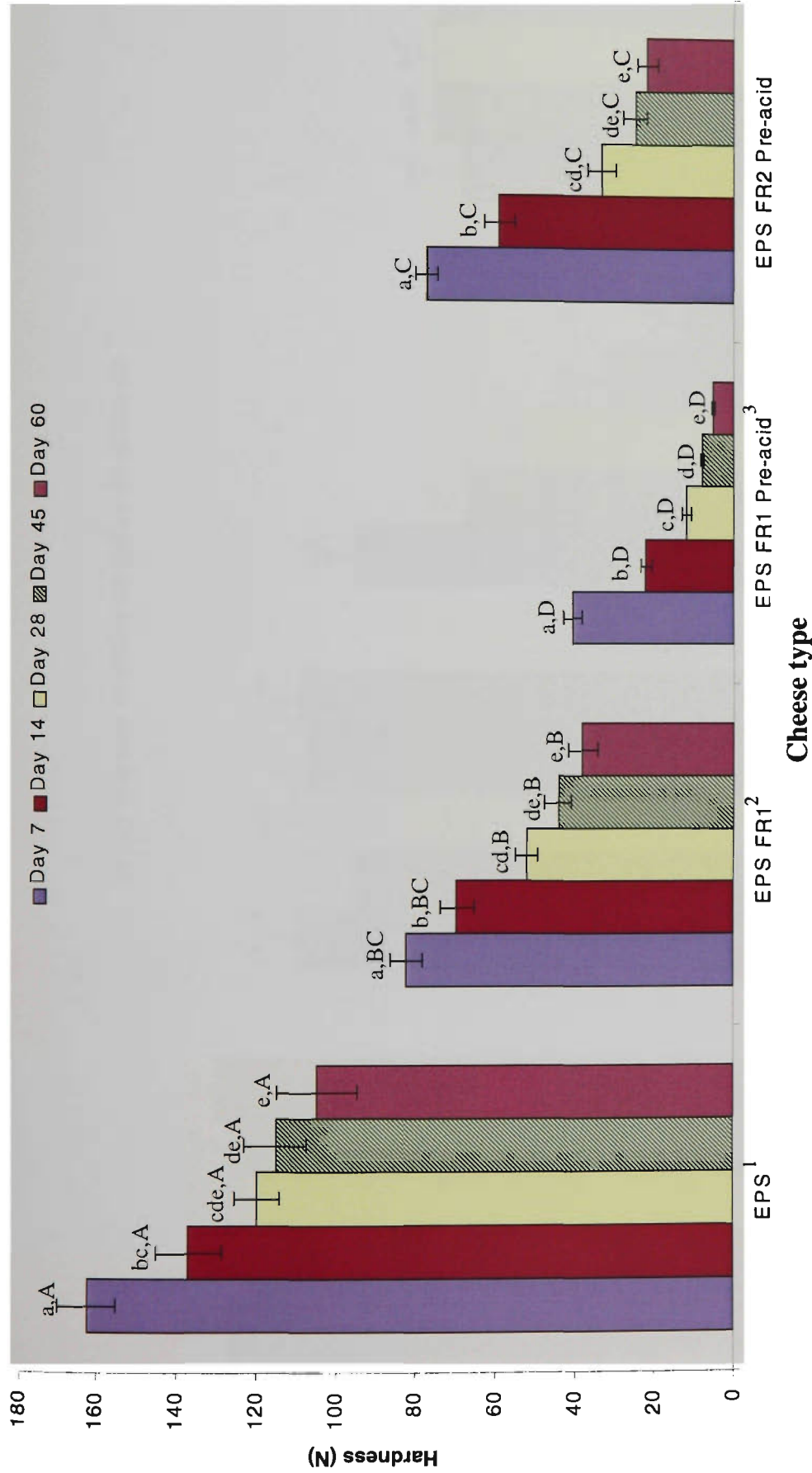
Figure 8.8. Changes in hardness of low fat Mozzarella cheeses over 60 d of storage at 4°C (n = 9). [abc]One-way ANOVA of means (n = 6) between d 7 and d 60 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28, 45 and 60 between cheese types with different superscripts are significantly different (*P* < 0.05).

[1]EPS = Capsular exopolysaccharides (EPS) produced by *S. thermophilus* 285.

[2]FR = Fat replacer.

[3]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

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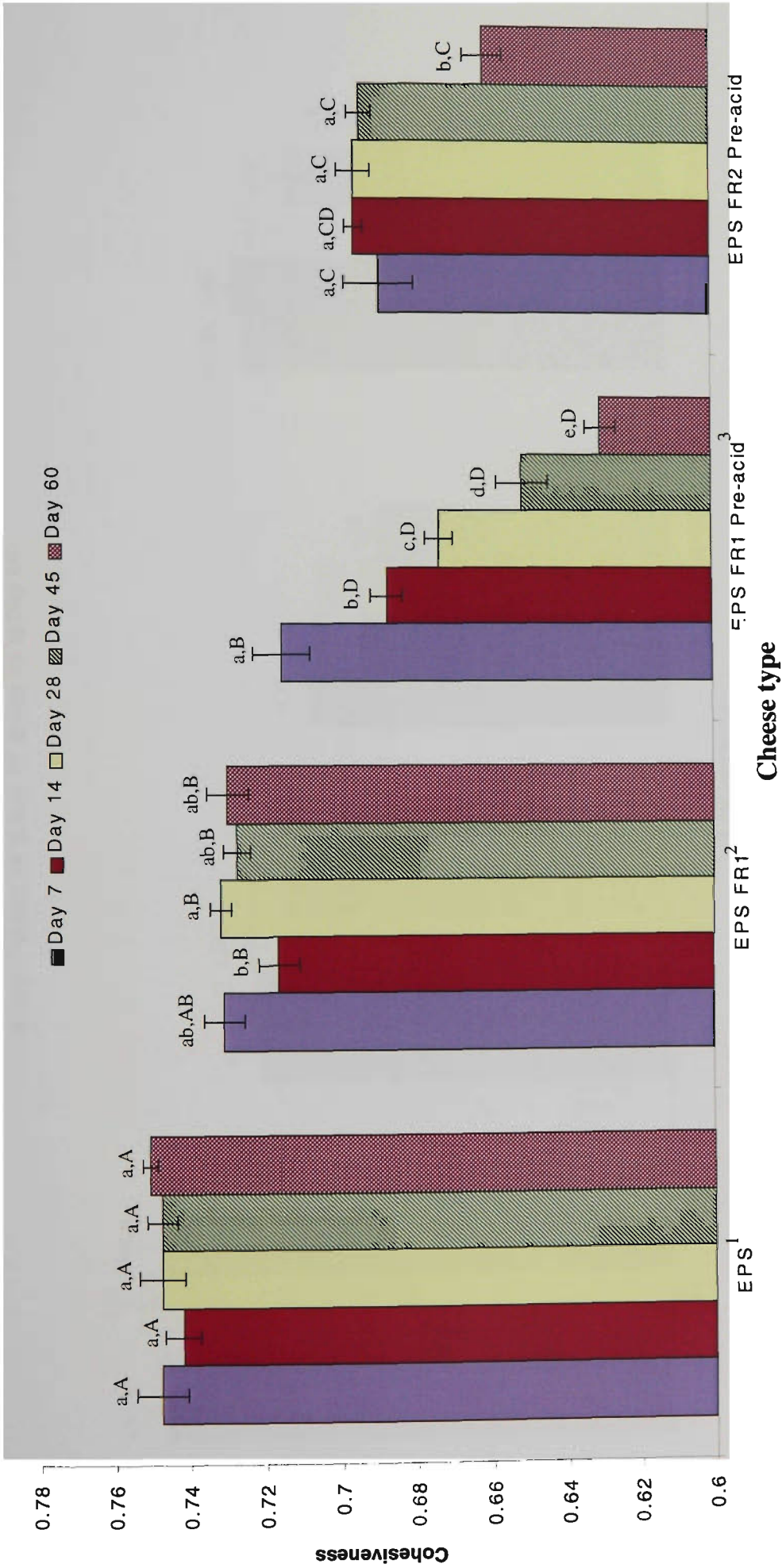
Figure 8.9. Changes in cohesiveness of low fat Mozzarella cheeses over 60 d of storage at 4°C (n = 9). [abc]One-way ANOVA of means (n = 6) between d 7 and d 60 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28, 45 and 60 between cheese types with different superscripts are significantly different ($P < 0.05$).

[1]EPS = Capsular exopolysaccharides (EPS) produced by *S. thermophilus* 285.

[2]FR = Fat replacer.

[3]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.
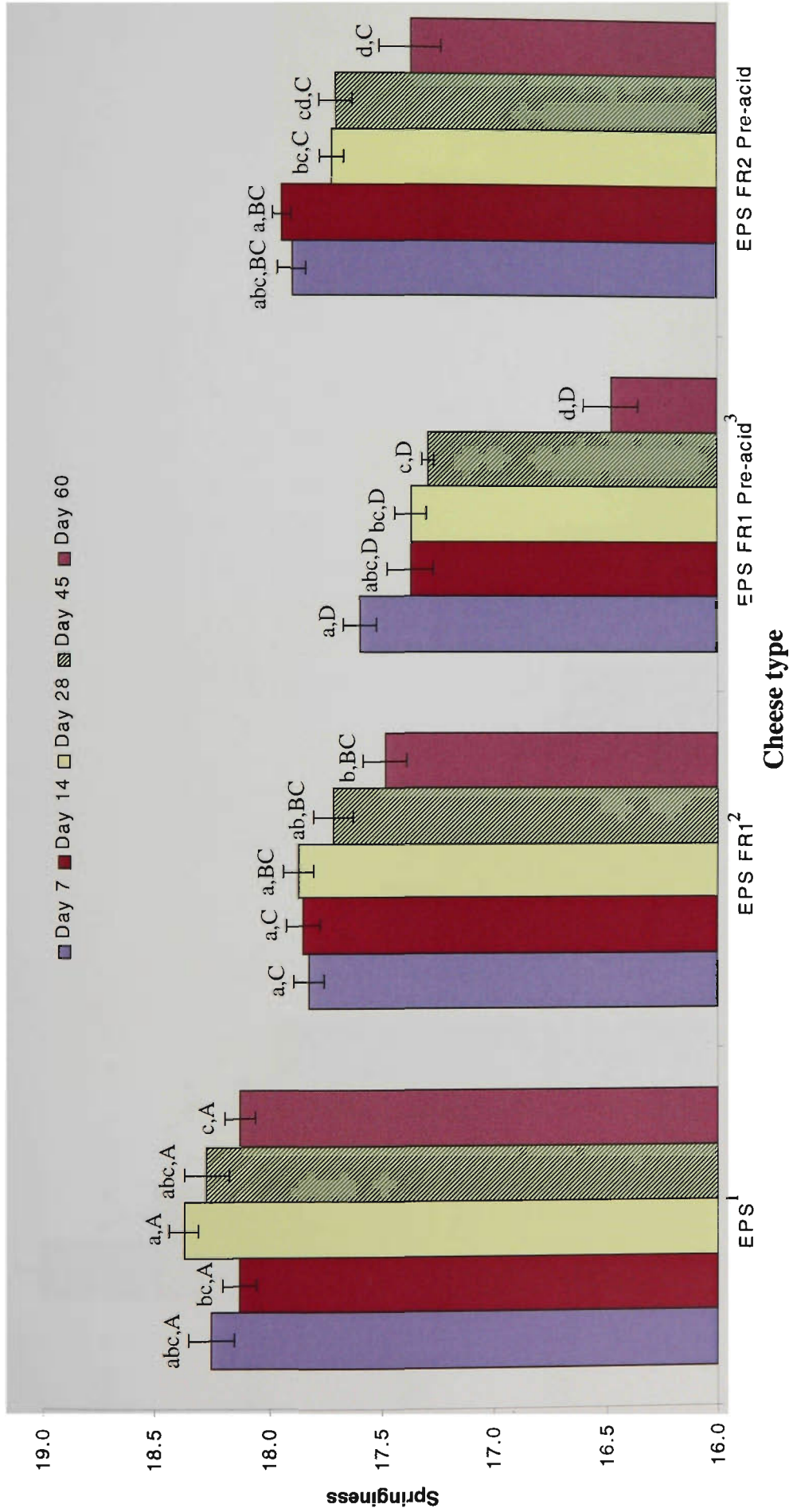
Figure 8.10. Changes in springiness of low fat Mozzarella cheeses over 60 d of storage at 4°C (n = 9). [abc]One-way ANOVA of means (n = 6) between d 7 and d 60 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28, 45 and 60 between cheese types with different superscripts are significantly different ($P < 0.05$).
[1]EPS = Capsular exopolysaccharides (EPS) produced by *S. thermophilus* 285.
[2]FR = Fat replacer.
[3]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

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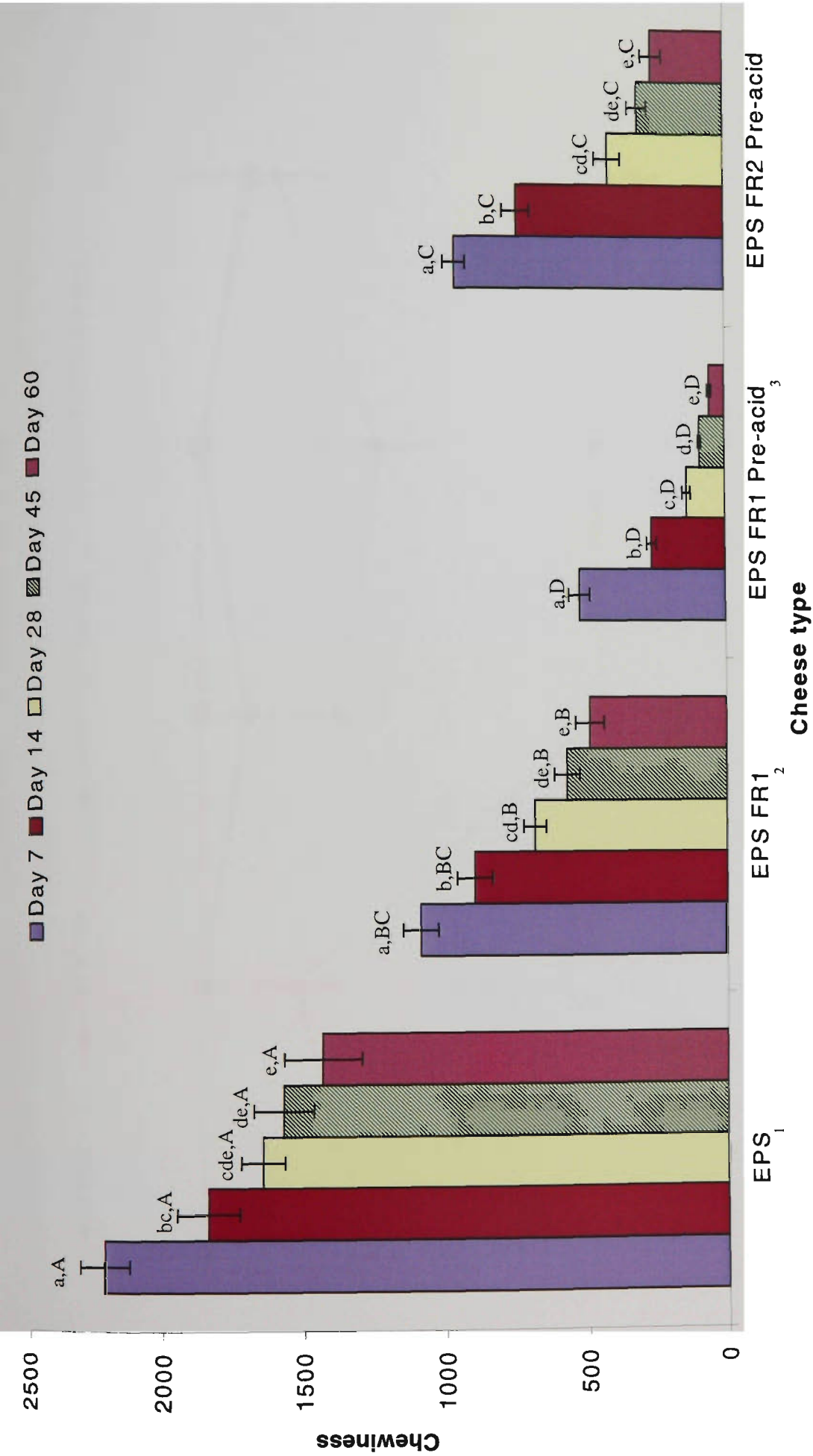
Figure 8.11. Changes in chewiness of low fat Mozzarella cheeses over 60 d of storage at 4°C (n = 9). [abc]One-way ANOVA of means (n = 6) between d 7 and d 60 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28, 45 and 60 between cheese types with different superscripts are significantly different (*P* <0.05).

[1]EPS = Capsular exopolysaccharides (EPS) produced by *S. thermophilus* 285.

[2]FR = Fat replacer.

[3]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

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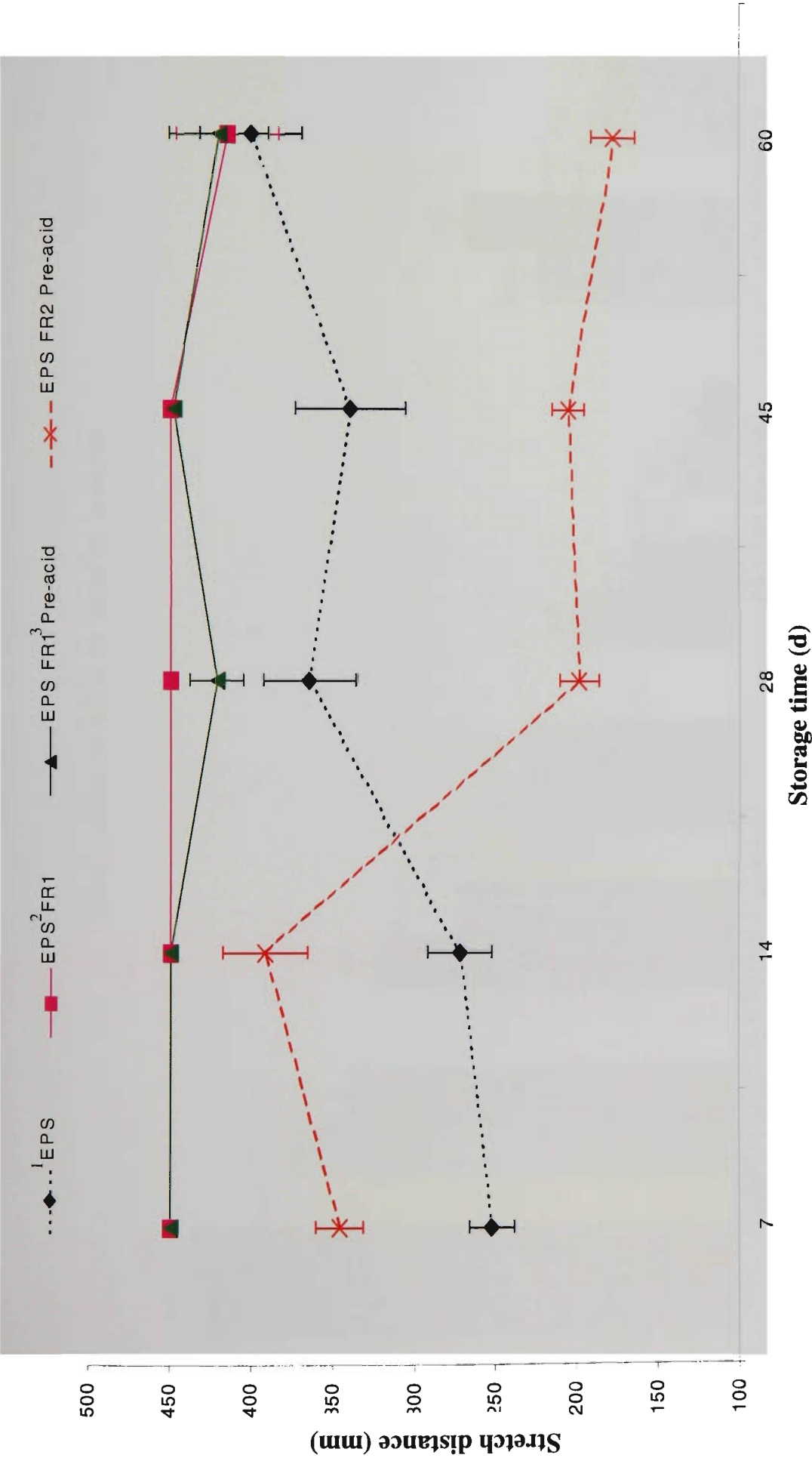
Figure 8.12. Changes in stretch distance of low fat Mozzarella cheeses over 60 d of storage at 4°C (n = 6).
[1]EPS = Capsular exopolysaccharides (EPS) produced by *S. thermophilus* 285.
[2]FR = Fat replacer.
[3]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.
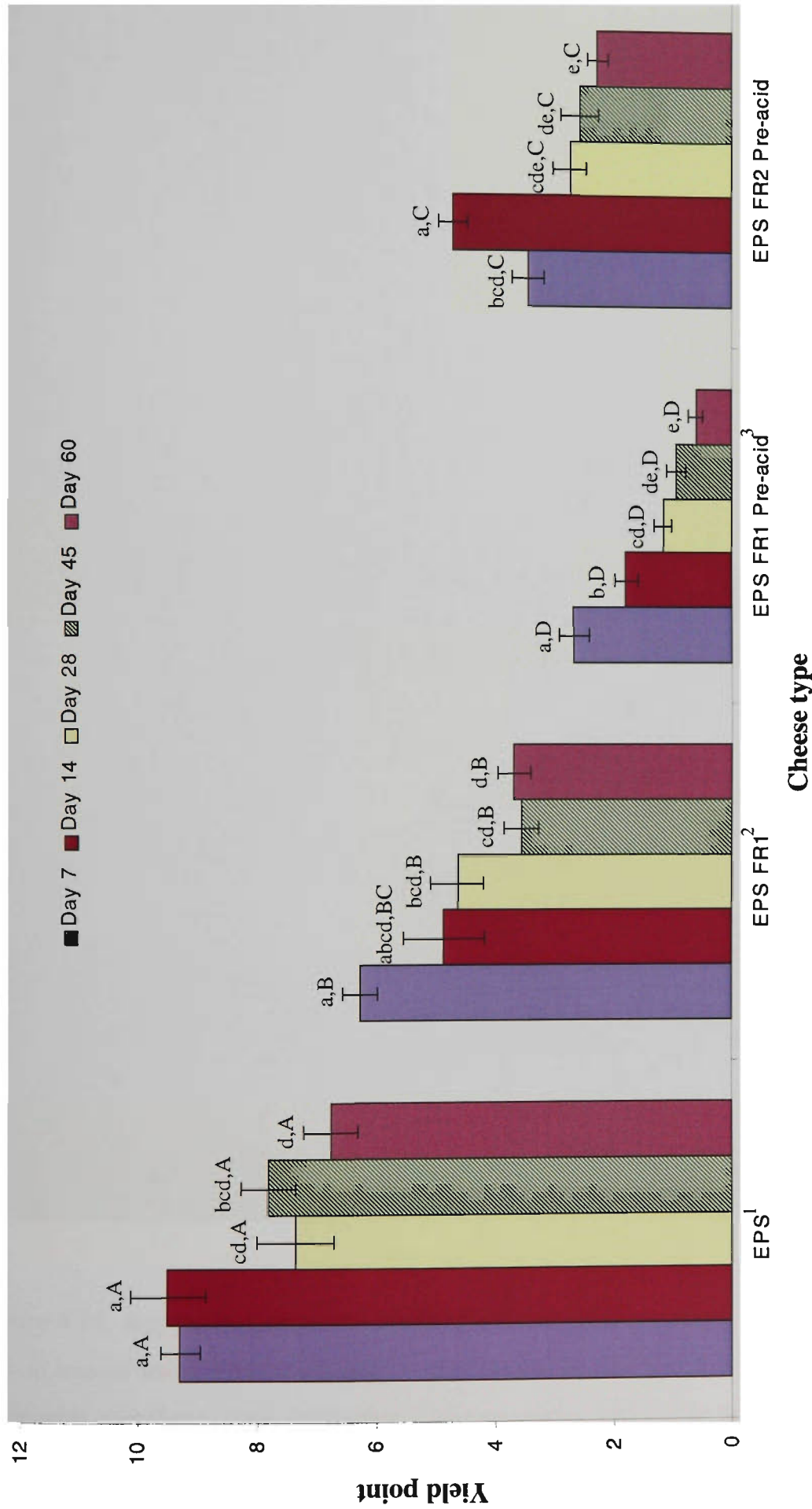
Figure 8.13. Changes in yield point of low fat Mozzarella cheeses over 60 d of storage at 4°C (n = 6). [a,b,c] One-way ANOVA of means (n = 6) between d 7 and d 60 within a cheese type and [A,B,C] one-way ANOVA of means at d 7, 14, 28, 45 and 60 between cheese types with different superscripts are significantly different ($P < 0.05$).

[1] EPS = Capsular exopolysaccharides (EPS) produced by *S. thermophilus* 285.

[2] FR = Fat replacer.

[3] Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

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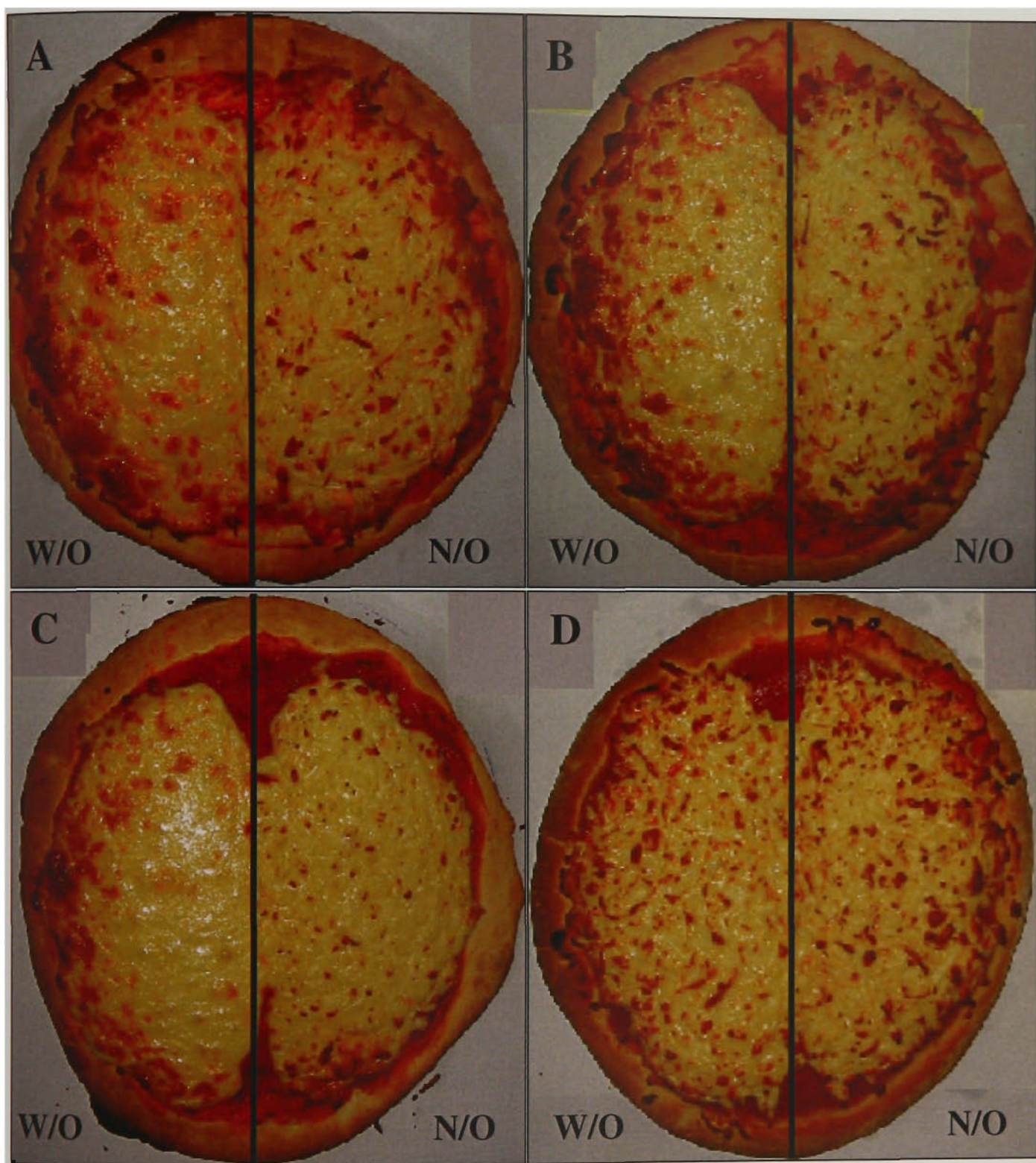
Figure 8.14. Appearance of pizzas topped with low fat Mozzarella cheese; untreated half (N/O) and oil treated half (W/O) immediately after baking at 262°C for 7 min. Cheese on pizzas were made with capsular exopolysaccharide *S. thermophilus* 285 (EPS) only (A), EPS and fat replacer 1 (FR1) (B), EPS and FR1 pre-acidified (C), EPS and FR2 pre-acidified (D).

CHAPTER 9.0

Effects of Pre-acidification and use of EPS Starter Culture on Moisture Retention, Texture and Functional Characteristics of Low Fat Mozzarella Cheeses: Scale-Up Study

9.1 INTRODUCTION

The stretching ability of Mozzarella cheeses when melted has made Mozzarella the choice ingredient for use on pizzas. The fat allows moisture retention in cheeses and provides a lubricating effect upon heating. When the fat content is reduced to make low fat Mozzarella cheeses, the texture and functionality are affected. This also reduces the amount of free oil released during heating and causes a reduction in the pizza baking performance. Fife _et al._ (1996) and Rudan and Barbano (1998a) have reported that low fat Mozzarella cheeses have inferior melting and browning characteristics as compared to the high fat counterpart. A hydrophobic barrier to coat the surface of cheese shreds has been used successfully to improve baking performance (Chapters 7.0 and 8.0; Rudan and Barbano, 1998b; Zisu and Shah, 2005).

Low fat Mozzarella cheeses have a low moisture to protein ratio. We have found that low fat Mozzarella cheeses containing 6% fat have reduced moisture retention and higher protein content (Chapters 5.0 to 8.0). Low fat cheeses were hard and showed greater chewiness and springiness and reduced cohesiveness and adhesiveness. Low fat cheeses were also associated with poor melting properties. These were also tough, more brittle and less pliable when stretched. Other researchers have reported similar results (Konstance and Holsinger, 1992; Masi and Addeo, 1986; McMahon _et al._, 1993; Merrill _et al._, 1994; Mistry and Anderson, 1993; Rudan _et al._, 1994; Tunick _et al._, 1991).

Fat and unbound water act as a lubricant and increase the ability of cheese particles to flow. This has prompted research into increasing the moisture content of low fat Mozzarella cheeses (Chapters 5.0 to 8.0; Low _et al._, 1998; McMahon _et al._, 1996; Perry _et al._, 1997; 1998; Petersen _et al._, 2000; Zisu and Shah, 2005). Research has shown that moisture content of Mozzarella cheeses affects functionality and increasing the moisture to protein ratio results in cheeses with greater melt. In our earlier study (Chapter 5.0; Zisu and Shah, 2002), we have used capsular exopolysaccharide (EPS) producing _Streptococcus thermophilus_ 285 and the mixed

capsular/ropy EPS producing _S. thermophilus_ 1275 to make low fat Mozzarella cheeses and improve moisture retention. These cheeses had higher moisture retention with enhanced yield and improved textural and functional characteristics. Encapsulated EPS synthesised by _S thermophilus_ 285 showed no adverse effects in cheeses whereas ropy EPS cheeses were coated in a layer of slime. EPS producing cultures have also been used in improving texture of fermented milks (Hassan _et al._, 1996b; Marshall and Rawson, 1999).

Calcium also affects texture and functionality of low fat Mozzarella cheeses (Chapter 6.0; Joshi _et al._, 2003; Metzger _et al._, 2000; Metzger _et al._, 2001). Removing some calcium from casein micelles by pre-acidification of milk prior to enzymic coagulation is believed to increase proteolysis and improve meltability (Chapter 8.0; Feeney _et al._, 2002; Joshi _et al._, 2003; Zisu and Shah, 2005).

Meltability of Mozzarella cheeses was shown to improve as a result of protein degradation (Chapter 8.0; Fife _et al._, 1996). We have found that co-culturing an EPS producing strain of _S. thermophilus_ with a non-EPS _S. thermophilus_ increased melt and softened low fat Mozzarella cheeses to a greater extent than using starter cultures individually (Chapter 7.0).

The objective of this study was to examine the effects of pre-acidification and use of EPS starter cultures singly or as a co-culture on moisture retention, texture and functionality of low fat Mozzarella cheeses in a semi-automated system at a semi-commercial quantity.

9.2 MATERIALS AND METHODS

9.2.1 Starter Cultures

Capsular EPS producing _S. thermophilus_ strain 285 (described in Chapter 3.0) and non-EPS 372 and _L. delbrueckii_ ssp. _bulgaricus_ strain 474 were used in this study. _S. thermophilus_ 372 and _L. delbrueckii_ ssp. _bulgaricus_ 474 were used as bulk starter (Dairy Farmers,

Toowoomba, QLD, Australia). Working culture of _S. thermophilus_ 285 was prepared as described in Chapter 3.0, section 3.2.1.

9.2.2 Cheese Making

Each batch of cheese was made from a total of 200 L of full cream, raw milk obtained from Dairy Farmers (Toowoomba, QLD, Australia). Cheeses were made using the research and development cheese making facilities of Dairy Farmers (Toowoomba, QLD, Australia). Full cream milk was separated and the resulting skim milk (~0.01 to 0.05% fat) and cream (~37 to 45% fat) were used to standardise the cheese milk to 0.5% fat. The fat contents of cream, and full cream-, skim- and cheese- milk were analysed using a MilkoScan S50 (Foss Pacific Pty. Ltd., Chatswood, NSW, Australia). Cheese milk was pasteurised at 72°C for 15 sec and used to make cheeses with a final fat content of 6% as indicated below:

Batch 1: _S. thermophilus_ 372 (non-EPS) + _L. delbrueckii_ ssp. _bulgaricus_ 474 (non-EPS) (Control),

Batch 2: _S. thermophilus_ 285 (EPS) + _L. delbrueckii_ ssp. _bulgaricus_ 474,

Batch 3: Pre-acidification + _S. thermophilus_ 285 + _L. delbrueckii_ ssp. _bulgaricus_ 474,

Batch 4: Pre-acidification + _S. thermophilus_ 285 (75%) + _S. thermophilus_ 372 (25%) + _L. delbrueckii_ ssp. _bulgaricus_ 474.

The starter bacteria were inoculated at the rate of 2.4% consisting of 1.6% of _S. thermophilus_ and 0.8% of _L. delbrueckii_ ssp. _bulgaricus_. The cheese milk for batch 4 was inoculated with 1.6% of _S. thermophilus_ at a ratio of 75:25 (0.75% of EPS _S. thermophilus_ 285 and 0.25% of non-EPS _S. thermophilus_ 372). The pasteurised cheese milk was tempered to 35°C for half an hour. The cheese milk for batches 3 and 4 was pre-acidified to pH 6.1 using 50% (wt/vol) citric acid. A 100 mL aliquot of milk was stored at –20°C for later analysis. Chymosin (Chymax 570; Chr. Hansen, Pty. Ltd., Bayswater, VIC, Australia) was added at the

rate of 8 mL per 200 L of milk. A setting time of 25 min was used for batches 1 and 2 while a setting time of 15 min was applied for pre-acidified milks (batches 3 and 4). The curd was cut using a cheese knife (1 cm^2 wire grid) based on setting time and subjective assessment of the coagulum with a knife. The curd was cooked for 20 min, gradually increasing the temperature to 40°C. The whey was drained at pH 6.2 (pH 6.1 for pre-acidified cheeses; batches 3 and 4) and an aliquot (500 mL) was stored at –20°C for later analysis. The curd was cheddered into slabs before milling at pH 5.2. Salt was added to the curd at the rate of 1.5% (wt/wt) and the salted curd held for 30 min. An aliquot of curd (40 g) was collected and stored at –20°C for later analysis. The remaining curd was stretched for 7 min in hot water maintained at 75°C containing 3% salt (wt/wt). The volume of stretching water was 2.5 times the weight of the curd. An aliquot (500 mL) of stretching water was frozen at –20°C for later analysis.

Cheese blocks were immediately placed into barrier bags and vacuum sealed using a Multivac A300/16 machine (Multivac Sepp Haggenmuller KG, Wolfertschwenden, Germany) before storing at 4°C in a fan forced cold room. An appropriate aliquot from each block cheese was removed on the following day and frozen at –20°C for quantification of EPS and calcium determination.

9.2.3 Cheese Analysis

An appropriate sample size was removed from cheese blocks on the day following production and each cheese was analysed for fat and moisture contents using an InfraLab analyser (NDC Infrared Engineering, Maldon, Essex, UK). The protein content was determined using the Kjeldahl method as per AOAC methods (AOAC, 1999). The salt content was determined by mixing 1 g of sample with 50 mL of water and titrated with 1N silver nitrate solution using a Mettler Toledo DL50 apparatus (Mettler-Toledo International Inc., Greifensee, Switzerland). The pH was measured by homogenising 10 g of cheese sample with 10 mL of

water using a pH meter (Hanna Instruments Pty. Ltd., Tullamarine, VIC, Australia). Textural characteristics, stretch and meltability of each cheese were performed at d 7, 14, 28, 45, 60 and 90. A pizza bake test was carried out a d 28, 45, 60 and 90.

9.2.4 Isolation and Quantification of Exopolysaccharides

Each of the milk, whey, stretch water, cheese and curd samples were prepared according to the procedure detailed in Chapter 6.0, section 6.2.4. EPS was extracted and quantified in each sample according to the procedure described in Chapter 3.0, section 3.2.5.

9.2.5 Determination of Calcium

An atomic absorption spectrophotometer (Spectra AA400, Varian Australia Pty. Ltd., Mulgrave, VIC, Australia) was used to determine the calcium content in cheese, curd, milk, whey and stretch water as described in Chapter 6.0, section 6.2.5. A standard curve for the determination of calcium is shown in Figure 9.1.

9.2.6 Cheese Melt

The meltability of Mozzarella cheeses was determined in 250 mm long glass tubes with a diameter of 24 mm and a thickness of 3 mm (R.B. Instruments, Mt. Eliza, VIC, Australia) using 10 g of finely grated cheese and heating to 110°C for 100 min as described in Chapter 5.0, section 5.2.5.

9.2.7 Texture Profile Analysis

The textural characteristics of cheeses including hardness, cohesiveness, springiness and chewiness were analysed with an Instron Universal Testing Machine (UTM) (5564, Instron Ltd., London, England) at room temperature (~22°C) as described in Chapter 5.0, section 5.2.7. The details of the texture parameters have been described in detail by Pons and Fiszman (1996).

9.2.8 Cheese Stretch

Cheese stretch was performed using the UTM as described in Chapter 5.0, section 5.2.6. The stretching distance of the melted cheese was increased to 450 mm and the stretching speed was increased to 450 mm/min.

9.2.9 Pizza Bake

Shredded Mozzarella cheese (300 g) was baked on a pizza base at 262°C for 7 min according to the procedure described in Chapter 7.0, section 7.2.9 at 28, 45, 60 and 90 d of storage.

2.2.10 Statistical Analysis

Each batch of cheese was made in triplicate and results are presented as a mean ± standard error of replicates. Hardness, cohesiveness, springiness and chewiness results are an average of 12 analyses, while the results of moisture and fat contents are an average of nine readings. Protein, melt, stretch and EPS quantification are representative of six analyses. The salt content and pH of each batch were measured three times. Calcium content of each batch

was analysed six to eighteen times. The pizza bake of each batch was replicated three times, and the Hunter L,a,b values obtained from the pizza bake are representative of 15 to 25 readings. To find significant differences between analyses, the means were analyzed using one-way analysis of variance (ANOVA) with a 95% confidence interval using Microsoft® Excel StatPro™ (Palaside Corporation, Newfield, NY). The ANOVA data with a $P < 0.05$ was classified as statistically significant.

9.3 RESULTS AND DISCUSSION

9.3.1 Composition

Table 9.1 shows the average composition of various batches of low fat Mozzarella cheeses. The moisture contents of control cheeses and those made with EPS and pre-acidified milk were lowest at 50.07% (53.55% as MNFS) and 50.08% (53.64% as MNFS), respectively. The combination of co-culturing at the ratio of 75% of EPS producing _S. thermophilus_ 285 and 25% of non-EPS _S. thermophilus_ 372 and pre-acidification significantly increased the moisture retention over control cheeses by 0.71% to 50.78% (54.39% as MNFS) $(P < 0.05)$. The moisture content of co-cultured cheeses was, however, similar to that of cheeses made with EPS and pre-acidified milk $(P > 0.05)$. The moisture content of cheeses made with EPS cultures and without acid treatment increased by 1.3% to 51.37% (55.05% as MNFS). In general, these results correspond with those in cheeses made at pilot scale (Chapters 5.0 to 8.0). However, all cheeses made with EPS had significantly greater moisture contents. Furthermore, EPS in cheeses made at a pilot scale in general increased the moisture retention by a greater amount in comparison to the moisture contents in cheeses made at semi-commercial scale. Higher moisture expulsion occurred in curds made at scale-up in comparison to pilot trials possibly due to a greater weight

of curds. The extra weight of the curd in cheese vats caused more compression when piled during cheddering resulting in greater syneresis.

The fat and salt contents as well as the pH among cheeses were similar ($P > 0.05$). Hence, the fat, salt and pH were not expected to influence the texture and functionality of low fat Mozzarella cheeses between variables. The protein content of EPS cheeses was lower than other cheese types ($P < 0.05$) as a consequence of higher moisture retention.

Table 9.2 displays the amount of EPS extracted from cheese, curd, whey, stretch water and milk. Control cheeses and the curd, whey, stretch water and milk collected from cheese making did not contain EPS. Cheeses made entirely with EPS producing *S. thermophilus* 285 had significantly higher concentrations of EPS at 79.29 mg/g of cheese ($P < 0.05$) and this was reflected in the moisture content. EPS cheeses made by pre-acidification of milk resulted in less EPS production at 28.69 mg/g of cheese. EPS concentrations were found to diminish in a similar manner in cheeses made with pre-acidification at pilot trials (Chapter 6.0, section 6.3.1). Due to EPS synthesis being growth associated and the capsular form of EPS remaining attached to the cell surface, the reduction in EPS was believed to occur due to pre-acidification as a result of reduced fermentation time. Cell numbers of the EPS producing bacteria were unlikely to reach the levels attained in the pure EPS cheeses. Cheeses made by pre-acidification and co-culturing *S. thermophilus* 285 (75%) with a non-EPS producer (25%), showed a lower amount of EPS at 16.19 mg/g of cheese. During cheese manufacture at a pilot scale (Chapter 7.0), this was attributed to using 25% less of the capsular EPS producing strain and pre-acidification.

EPS concentrations in curd made with EPS cultures only were significantly greater than those in cheeses ($P < 0.05$). Although, the curds of the batches made with EPS pre-acidified and co-cultured milks had more EPS than cheeses, this was not significant ($P > 0.05$). Higher EPS concentrations appeared to occur in the curd due to higher moisture contents.

The amount of EPS lost in the whey was a measure of EPS expelled during draining. Higher amount of EPS was quantified in the whey in pre-acidified batches. Although more EPS

was produced in cheeses and curds of untreated EPS cheeses, a similar concentration of EPS was isolated from the whey in all variables ($P > 0.05$). The whey of cheeses made with EPS producing starter culture contained 23.73 mg/mL, 26.67 mg/mL and 23.82 mg/mL of EPS for EPS, EPS pre-acidified and pre-acidified cheeses made with 75% *S. thermophilus* 285 and 25% *S. thermophilus* 372, respectively.

No EPS was detected in samples of stretch water and milk. Because EPS was absent in milk, its production by *S. thermophilus* 285 occurred during cheese manufacture. The observations of EPS production correlated with those at pilot trials (Chapters 6.0 and 7.0).

Table 9.3 gives the calcium contents in cheese, curd, whey, stretch water and milk. The control (at 959.07 mg/100 g; 13.96 mg/g of protein) and EPS cheeses (at 931.84 mg/100 g; 13.40 mg/g of protein) showed a greater calcium content than those made with EPS and pre-acidification (at 864.78 mg/100 g; 12.41 mg/g of protein) and by co-culturing and pre-acidification (at 782.03 mg/100 g; 11.20 mg/g of protein). The calcium contents in the curd of corresponding variables showed a similar pattern. The calcium contents in the curd were slightly lower than those in cheeses due to a greater moisture content. Correspondingly, the calcium content of whey was lower in the control (at 23.80 mg/100 g) and EPS cheeses (at 20.44 mg/100 g) and higher in those made with EPS and pre-acidification (at 27.71 mg/100 g) and by co-culturing and pre-acidification (at 26.24 mg/100 g). Calcium concentrations in stretch water remained unaffected by pre-acidification. To eliminate variations in the initial calcium content in the milk, sampling was performed prior to pre-acidification and the results showed similar calcium contents between variables ($P > 0.05$). Pre-acidification reduced calcium levels in cheeses; this was expected to influence the texture and functionality of cheeses.

9.3.2 Cheese Melt

Figure 9.2 shows the meltability of cheeses over 90 d of storage at 4°C. In general, the melt distance increased over the storage period for all cheese types ($P < 0.05$). Control cheeses

increased melt distance by 8.4 mm to reach 64.1 mm at d 90. A significant improvement in melt distance occurred between d 14 and d 45 only ($P < 0.05$). EPS only cheeses did not result in better melt than the control cheeses ($P > 0.05$), and the latter showed a greater melt distance at d 14 and 45 ($P < 0.05$). EPS cheeses improved melt between d 14, 28 and 90 ($P < 0.05$), by 10.6 mm to reach a maximum of 63.1 mm. Pre-acidified cheeses in general exhibited better meltability than the non-treated variables. The EPS pre-acidified cheeses had better melt than the controls at d 28 and 45 and showed greater melt than EPS cheeses at d 45 and 90 ($P < 0.05$). The EPS cheeses made from pre-acidified milk had a significant improvement in melt between d 14, 28, 45 and 90 ($P < 0.05$). They had a peak flow distance of 65.6 mm, representing an increase by 12.2 mm. The combination of pre-acidification and co-culturing showed the greatest melt. This variable had significantly greater melt throughout the storage period ($P < 0.05$). Meltability improved between d 14 and 90 ($P < 0.05$) to reach 69.1 mm, representing an increased melt distance of 9.7 mm. The co-culturing combination of 75% EPS and 25% non-EPS producing strains of *S. thermophilus* appeared to be complementary. The symbiotic relationship of *S. thermophilus* 285 and *S. thermophilus* 372 displayed favourable effects towards the maturation and meltability of low fat Mozzarella cheeses. Secondary proteolysis induced by the starter proteases and peptidases may have been responsible. Similar results were observed in cheeses made on a smaller scale during pilot trials (Chapter 7.0). Pre-acidification was shown to improve meltability and co-culturing with a non-EPS producing strain of *S. thermophilus* resulted in a superior melt distance.

9.3.3 Texture Profile Analysis

9.3.3.1 Cheese hardness

The hardness values of cheeses stored at 4°C over 90 d are shown in Figure 9.3. Hardness was greatest at d 7 and 14 for all cheese types ($P < 0.05$). Cheeses showed reduced

hardness during storage, and were softest at d 90. The first significant reduction in the hardness occurred at d 28 ($P < 0.05$). The softening at d 28 was subtle in control cheeses but occurred abruptly in cheeses containing EPS. The reduction in hardness between d 14 and 28 correlated with the increase in melt distance in EPS containing cheeses (Figure 9.2), but not in control batches.

Control cheeses were hardest overall and the hardness values were significantly greater at all time points ($P < 0.05$). The maximum resistance force occurring in control cheeses remained at over 100 N after 90 d. EPS in cheeses created softer cheeses and as a result lowered the maximum force occurring after compression. At d 7 and 14, all cheeses containing EPS showed similar hardness ($P > 0.05$), except EPS pre-treated cheeses, which were harder than co-cultured cheeses at d 14 ($P < 0.05$). After 28 d of storage, the EPS pre-acidified cheeses appeared harder than EPS- and co-cultured-cheeses in general. Cheeses made with pre-acidified milk containing EPS were significantly harder than EPS only cheeses at d 28 and 60 and co-cultured cheeses at d 28, 60 and 90 ($P < 0.05$). The co-cultured cheeses were the softest at d 60 and 90 ($P < 0.05$). EPS only cheeses contained the greatest amount of EPS and had the highest moisture retention, which contributed to the softness. EPS pre-acidified cheeses were harder due to less EPS production and lower moisture content. Co-cultured pre-acidified cheeses made with the starter culture ratio of 75% EPS _S. thermophilus_ and 25% non-EPS _S. thermophilus_ showed accelerated maturation at d 60 and 90 of storage resulting in substantial softening. This was possibly due to greater secondary proteolysis. A similar trend was also observed in cheeses made with EPS producing starter cultures on a smaller scale at pilot trials (Chapters 5.0 to 7.0).

9.3.3.2 Cheese springiness

The springiness of cheeses is shown in Figure 9.4. A compression of 50% was used and due to the resilient nature of Mozzarella cheeses, samples did not show fracture allowing the accurate determination of springiness. The control and EPS pre-acidified cheeses showed

resistance to deformation over 90 d of storage at 4°C. Unlike hardness, the reduced moisture content of these cheeses had an influence on springiness and there was no reduction in this parameter over time ($P > 0.05$). The EPS and co-cultured pre-treated cheeses, having higher moisture contents, showed a decrease in springiness. The loss in the ability to maintain structural integrity after compression became significant at d 90 of storage and springiness was significantly reduced ($P < 0.05$).

Control cheeses generally showed higher springiness than those made with EPS. Springiness was significantly lower in EPS cheeses at d 14, 45 and 90 ($P < 0.05$). This was evident at d 45 in EPS pre-acidified cheeses and d 45 and 90 in co-cultured cheeses.

9.3.3.3 Cheese cohesiveness

Figure 9.5 shows cohesiveness of cheeses over 90 d of storage at 4°C. The cohesiveness of cheeses in general appeared low in the control batch, however, this parameter was higher in co-cultured pre-acidified cheeses. Co-cultured cheeses exhibited significantly more cohesiveness at d 28, 60 and 90 ($P < 0.05$). EPS and EPS pre-treated cheeses had slightly greater cohesiveness than the control but this was only significant at d 45 ($P < 0.05$). The cohesiveness of these cheeses was lower than those made by co-culturing and pre-treatment. EPS only cheeses had lower cohesiveness than the co-cultured variety at d 7, 28 and 90 ($P < 0.05$), while EPS pre-treated cheeses showed lower cohesiveness at d 28 and 45 ($P < 0.05$).

There was no reduction in the cohesiveness of cheeses over time within each variable, however, this parameter was higher in cheeses at d 7.

9.3.3.4 Cheese chewiness

The change in chewiness of cheeses stored at 4°C is shown in Figure 9.6. Because the compression forces applied by the UTM were not destructive allowing samples to remain intact, cohesiveness was calculated by the primary textural characteristics of hardness × cohesiveness ×

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springiness. Similarly to hardness of cheeses, a reduction in chewiness occurred over time ($P <$ 0.05) and was the lowest at 90 d. Control cheeses exhibited the highest chewiness ($P < 0.05$). The chewiness between EPS cheeses was similar. EPS cheeses made from pre-acidified milk, however, showed more chewiness over EPS only cheeses at d 28 and 60 and co-cultured cheeses (also pre-acidified) at d 7, 60 and 90 ($P < 0.05$). The chewiness of co-cultured cheeses was lower than those made from EPS only starter cultures at d 60 and 90 ($P < 0.05$).

The TPA of low fat Mozzarella cheeses containing 6% fat made using a semi automated setup was in general similar to cheeses made manually at a pilot scale (Chapters 5.0 to 7.0).

9.3.4 Cheese Stretch

The stretch performance recorded over 90 d of storage at 4°C is given in Figure 9.7. At d 14, the control cheeses showed greater stretch than EPS pre-acidified and co-cultured cheeses ($P < 0.05$). Cheeses made entirely from EPS *S. thermophilus* culture showed similar stretch to other variables ($P > 0.05$). At d 28, EPS pre-acidified cheeses showed less stretch than the co-cultured cheeses ($P < 0.05$). The cheese stretch distance was similar for all variables beyond d 28 ($P > 0.05$). At d 90, the stretch performance was reduced from the greatest stretch distance achieved by each variable (either d 14 or d 28) ($P < 0.05$).

At a pilot scale (Chapter 5.0), the control cheeses had a lower stretch distance than EPS cheeses at d 28, and although this was apparent in the batches made at scale-up (Figure 9.7), the stretch distance between the cheeses was not significant ($P > 0.05$). In general, all cheese variables made at a pilot scale showed a similar stretch distance after 28 d of storage. There was no significant improvement after 28 d of storage. The appearance of the stretched cheese showed some deterioration with prolonged storage, however, this did not translate to a reduction in stretch distance.

9.3.5 Pizza Bake

The pizza bake performance of low fat Mozzarella cheeses at d 28 and d 90 is shown in Figure 9.8, A to H. Each pizza base was divided into halves and topped with untreated cheese shreds on one half and with oil coated cheese shreds on the remaining half. Oil was used to coat cheese shreds to artificially introduce fat at the surface of the shreds. Because low fat cheeses have insufficient fat on the surface, the fat does not provide a barrier to surface moisture dehydration and there is scorching and incomplete shred fusion. By applying canola oil to the surface of cheese shreds, the problem of surface moisture evaporation is overcome. At d 28 (Figure 9.8, A to D) and in the absence of an oil coating, all cheeses showed poor baking characteristics. The cheese shreds exhibited excessive scorching and incomplete fusion. This was particularly evident near the surface and around the margins of the pizzas where the moisture was first evaporated causing the formation of a crust and leading to subsequent burning. The control cheeses (Figure 9.8, A) showed most scorching, followed by the cheeses containing EPS made from pre-acidified milk (Figure 9.8, C), and co-cultured and pre-acidified cheeses (Figure 9.8, D). Pizzas baked with cheeses made with EPS only cultures (Figure 9.8, B) exhibited the most complete shred fusion and had the least scorching. Coating cheese shreds with 1% canola oil showed a vast improvement in baking performance and each cheese type showed complete shred fusion and uniform browning. Control cheeses and those made with EPS showed similar characteristics (Figure 9.8, A and B, respectively). Cheeses made with pre-acidified milk (Figure 9.8, C and D) showed less browning and those made by co-culturing an EPS strain of _S. thermophilus_ with a non-EPS strain (Figure 9.8, D) had the least browning. This is possibly due to stimulation effects of co-culturing leading to better utilisation of lactose. Lactose was not measured in this study.

After 90 d of storage (Figure 9.8, E to H), the baking performance observed for all types of cheeses was vastly improved with less scorching and an uniform golden bake and greater

shred fusion. Without coating the cheese shreds with oil, evidence of incomplete shred fusion remained, although shred fusion was improved from initial observations at d 28. The cheeses made with EPS only culture exhibited the best pizza bake performance (Figure 9.8, F). In the presence of an oil coating, the control and EPS pre-acidified cheeses (Figure 9.8, E and G, respectively) demonstrated similar baking attributes with a uniform golden bake. EPS only cheeses showed the least amount of browning and were light in appearance (Figure 9.8, F). Figure 9.8 H displays the baking characteristics of the co-cultured EPS and non-EPS strains of _S. thermophilus_. These cheeses showed unique bake after 90 d of storage. A high level of golden browning occurred at this time point, yet scorching remained absent. Cheese shreds showed greater melt and flow towards the outer edges of the pizza bases, exhibiting a degree of puffiness when hot.

Pizza bake analysis was also conducted at d 45 and d 60 of storage, however, images from these tests are not provided. Observations at theses time points showed an improvement in baking characteristics between d 28 and d 45 and again between d 45 and d 60. EPS only cheeses were most appealing, presumably due to the higher moisture content. It was necessary to coat cheese shreds in oil in all batches to achieve complete shred fusion and reduce scorching.

9.3.5.1 Hunter L,a,b values

Tables 9.4, 9.5 and 9.6 show the Hunter L-values (representing whiteness), a-values (representing redness) and b-values (representing yellowness) of cheeses, respectively before cooking, after baking when warm and upon cooling.

Cheeses not coated in oil, in general, had greater L-values than the oil coated cheeses before baking, however, this was not significant ($P > 0.05$). Similarly, a- and b-values were alike between oil coated and non-coated cheeses prior to baking ($P > 0.05$). L- and a-values reduced between d 28 and d 90 in all cheese types and with both treatments ($P < 0.05$). The reduction in L-values was not significant in pre-acidified cheeses when coated with oil ($P >$

0.05). In general, there was no difference in L- and b-values between the cheese types before cooking ($P > 0.05$). Control cheeses had the lowest a-values in both treatments ($P < 0.05$) and the remaining variables were similar.

When cheeses were warm immediately after baking, L- and b-values increased ($P < 0.05$). With an increase in browning, a-values also increased ($P < 0.05$). At d 28, the oil coated cheeses had greater L-values than non-coated cheeses in the control and EPS cheeses ($P < 0.05$). The control untreated cheeses had the lowest L-values and greatest a-values at this time point. Pre-acidified cheeses showed greater L-values with oil treated cheeses up to d 45 ($P < 0.05$). As the baking performance improved with time and scorching was reduced in the untreated cheeses, L-values became similar between the treatments. Pre-acidified cheeses showed lower L-values in oil coated cheeses over the untreated cheeses at d 90 ($P < 0.05$) as browning became uniform across the face of the pizzas topped with the oil coated cheese shreds. Correspondingly, a-values in control and EPS cheeses were greater in untreated cheeses at d 28 ($P < 0.05$) before becoming similar at d 45 and beyond ($P > 0.05$). Pre-acidified cheeses continued to show greater a-values when untreated up to d 45, became similar at d 60, and reduced at d 90. Cheeses treated with oil had greater b-values than non-coated cheeses, particularly after longer storage. Untreated cheeses had similar b-values between d 28 and d 90 ($P > 0.05$). Cheeses coated with oil showed an increase in b-values with time but the increase was significant in pre-acidified cheeses only ($P < 0.05$).

After cooling for half-an-hour, L-values reduced in both treatments. The oil coated cheeses in general maintained greater L-values and were similar over time. Due to scorching of untreated cheese shreds and browning of oil coated cheeses, a-values remained similar to those recorded when warm. The b-values of cheeses made without pre-acidification reduced when cooled as was observed with the L-values, particularly after extensive storage. Cheeses made with pre-acidified milk showed no changes in b-values after cooling. Oil coated cheeses had greater b-values than non-coated cheeses in general.

9.4 CONCLUSIONS

The moisture content of the control cheeses made with non-EPS producing starter cultures and without pre-acidification was lowest. EPS in cheeses increased the moisture content, although the moisture content in cheeses made with EPS and pre-acidification was similar to the control batches. The moisture retention in Mozzarella cheeses containing EPS was lower than that observed in the corresponding cheeses made during pilot trials. This was attributed to the slight modifications in the manufacturing procedure, particularly during the cheddaring stage. Low fat Mozzarella cheeses made at scale-up in a semi-automated setup exhibited similar textural and functional characteristics to those made manually on a smaller scale at a pilot scale setup. EPS reduced the hardness and chewiness of cheeses and pre-acidification resulted in improved meltability. Stretchability was not influenced by the manufacturing procedure. The pizza bake characteristics were best in cheeses made with EPS only cultures. Although pizza baking was substantially improved during the storage period, a surface coating of oil was necessary.

Table 9.1. Average composition of low fat Mozzarella cheeses (mean ± standard error).

Cheese	Moisture (%) (n = 9)	Fat (%) (n = 6)	MNFS[1] (%) (n = 6)	FDM[2] (%) (n = 6)	Protein (%) (n = 6)	Salt (%) (n = 3)	S/M[3] (%) (n = 3)	pH (n = 3)
Control	50.07 ± 0.11^c	6.50 ± 0.09^a	53.55 ± 0.10^c	13.02 ± 0.10^a	34.31 ± 0.43^{ab}	1.04 ± 0.00^a	2.08 ± 0.05^a	5.37 ± 0.02^a
[4]EPS	51.37 ± 0.15^a	6.68 ± 0.14^a	55.05 ± 0.15^a	13.74 ± 0.14^a	33.82 ± 0.20^b	1.03 ± 0.03^a	2.01 ± 0.07^a	5.33 ± 0.02^a
EPS[5] pre-acid	50.08 ± 0.25^{bc}	6.63 ± 0.41^a	53.64 ± 0.32^{bc}	13.28 ± 0.34^a	34.79 ± 0.26^a	1.01 ± 0.06^a	2.02 ± 0.15^a	5.34 ± 0.01^a
[6]75:25 pre-acid	50.78 ± 0.26^{ab}	6.63 ± 0.33^a	54.39 ± 0.29^{ab}	13.47 ± 0.31^a	34.37 ± 0.24^{ab}	1.04 ± 0.02^a	2.05 ± 0.13^a	5.33 ± 0.01^a

[abc]One-way ANOVA of means in a column with different superscript are significantly different ($P < 0.05$).

[1]MNFS = Moisture in non-fat substance.

[2]FDM = Fat in dry matter.

[3]S/M = Salt in moisture.

[4]EPS = Capsular exopolysaccharides (EPS) produced by *S. thermophilus* 285.

[5]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

[6]75:25 = Mixture of EPS producing *S. thermophilus* 285 and 25% non-EPS *S. thermophilus* 372.

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Table 9.2. EPS extracted from cheese, curd, whey, stretch water and milk (n = 6 ± standard error).

	Control	[1]EPS	EPS[2] pre-acid	[3]75:25 pre-acid
Cheese (mg/g)	[4]ND^{d,A}	79.29 ± 2.46^{a,B}	28.69 ± 2.00^{b,A}	16.19 ± 0.51^{c,B}
Curd (mg/g)	ND^{d,A}	88.10 ± 1.66^{a,A}	34.53 ± 2.78^{b,A}	19.64 ± 1.50^{c,AB}
Whey (mg/ml)	ND^{b,A}	23.73 ± 2.00^{a,C}	26.67 ± 2.24^{a,A}	23.82 ± 2.48^{a,A}
Stretch water (mg/ml)	ND^{a,A}	ND^{a,D}	ND^{a,B}	ND^{a,C}
Milk (mg/ml)	ND^{a,A}	ND^{a,D}	ND^{a,B}	ND^{a,C}

abcOne-way ANOVA of means in a column with different superscript are significantly different ($P < 0.05$).

[1]EPS = Capsular exopolysaccharides (EPS) produced by S. thermophilus 285.

[2]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

[3]75:25 = Mixture of EPS producing S. thermophilus 285 and 25% non-EPS S. thermophilus 372.

[4]ND = Not detected.

Table 9.3. Calcium content in cheese, curd, whey, stretch water and milk (n = 6 to 18 ± standard error).

Sample (mg/100g)	Control	[1]EPS	EPS [2]pre-acid	[3]75:25 pre-acid
Cheese	959.07 ± 6.10[a]	931.84 ± 6.22[b]	864.78 ± 10.64[c]	782.03 ± 3.31[d]
Cheese (mg/g protein)	13.96 ± 0.51[a]	13.40 ± 0.58[a]	12.41 ± 0.61[bc]	11.20 ± 0.31[c]
Curd	899.74 ± 37.81[a]	898.64 ± 19.29[a]	786.84 ± 12.43[b]	733.51 ± 7.49[c]
Whey	23.80 ± 0.34[b]	20.44 ± 0.33[c]	27.71 ± 0.86[a]	26.24 ± 0.71[a]
Stretch water	13.36 ± 0.49[a]	11.39 ± 0.37[a]	12.44 ± 0.35[a]	11.14 ± 0.92[a]
Milk	76.24 ± 2.20[a]	68.77 ± 1.55[a]	76.61 ± 3.71[a]	71.59 ± 0.75[a]

[abc]One-way ANOVA of means in a column with different superscript are significantly different ($P < 0.05$).

[1]EPS = Capsular exopolysaccharides (EPS) produced by S. thermophilus 285.

[2]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

[3]75:25 = Mixture of EPS producing S. thermophilus 285 and 25% non-EPS S. thermophilus 372.

Table 9.4. Mean Hunter L-values (n = 15 to 25) for whiteness measurements of low fat Mozzarella cheeses for given variables, treatments and processing stages.

	Control		EPS[1]		EPS[2] pre-acid		75:25[3] pre-acid	
	No Oil	Oil	No Oil	Oil	No Oil	Oil	No Oil	Oil
B.C.-28[4]	65.09 ± 0.72[abc,BCDE]	64.02 ± 0.76[abc,FGH]	66.08 ± 0.99[a,BCDEF]	65.84 ± 0.61[a,GHI]	64.18 ± 0.72[abc,EFGHI]	62.85 ± 0.94[c,IJKL]	65.00 ± 0.78[abc,FGH]	63.44 ± 0.50[bc,HIJ]
B.C.45	62.92 ± 0.89[abc,DEFGH]	60.79 ± 0.97[c,JK]	64.46 ± 1.06[ab,CDEFGH]	62.66 ± 0.81[abc,JK]	64.77 ± 0.80[a,DEFGH]	62.64 ± 0.66[abc,JKL]	64.35 ± 1.16[ab,HI]	62.47 ± 0.73[bc,IJ]
B.C.60	62.38 ± 1.14[abcde,EFGH]	62.10 ± 0.64[bcde,HIJK]	64.30 ± 0.78[a,DEFGH]	63.53 ± 0.42[ab,HIJK]	63.22 ± 0.54[abcd,FGHI]	61.55 ± 0.60[cde,L]	61.11 ± 0.94[de,J]	60.96 ± 0.63[e,K]
B.C.90	58.71 ± 0.95[b,J]	61.61 ± 0.83[a,IJK]	62.17 ± 0.89[a,H]	61.94 ± 1.03[a,K]	62.08 ± 0.89[a,I]	62.61 ± 0.55[a,KL]	62.11 ± 0.85[ab,IJ]	61.17 ± 0.81[ab,JK]
Warm28	63.94 ± 1.97[f,CDEFGH]	71.99 ± 0.80[abcde,A]	69.33 ± 1.02[de,A]	71.94 ± 0.71[abc,AB]	66.28 ± 1.52[ef,BCDEFG]	70.06 ± 0.97[bcd,CD]	69.97 ± 1.51[cde,ABCD]	72.65 ± 0.64[a,AB]
Warm45	70.41 ± 0.98[de,A]	68.64 ± 0.60[e,C]	71.93 ± 1.67[abcde,A]	73.54 ± 0.76[a,A]	70.50 ± 1.03[cde,A]	73.66 ± 0.25[a,A]	71.21 ± 0.70[bcd,A]	73.93 ± 0.49[a,A]
Warm60	69.57 ± 1.10[a,A]	69.47 ± 0.84[a,BC]	70.48 ± 0.90[a,A]	69.29 ± 1.02[a,DEF]	68.51 ± 0.99[a,ABC]	68.00 ± 0.91[a,DE]	68.86 ± 1.11[a,ABCDE]	69.55 ± 0.95[a,CD]
Warm90	68.99 ± 0.99[ab,A]	69.95 ± 0.90[a,ABC]	69.94 ± 0.81[a,A]	70.36 ± 0.64[a,BCD]	70.81 ± 1.11[a,A]	67.08 ± 0.95[bc,EFG]	68.21 ± 0.95[abc,BCDE]	65.80 ± 1.18[c,FGH]
Cool28	60.38 ± 1.44[f,HIJ]	64.01 ± 0.92[bcde,GHI]	63.11 ± 1.53[ef,FG]	68.12 ± 0.77[a,EF]	63.16 ± 1.50[def,GHI]	63.91 ± 1.06[cdef,HIJKL]	67.69 ± 1.51[c,DEFGH]	69.46 ± 0.57[a,D]
Cool45	61.83 ± 1.20[d,FGHIJ]	64.86 ± 1.20[cd,DEFG]	69.14 ± 1.09[ab,AB]	69.84 ± 0.62[a,CDE]	70.47 ± 1.18[a,AB]	71.08 ± 0.46[a,BC]	67.50 ± 0.75[bc,DEFG]	71.19 ± 0.62[a,BCD]
Cool60	60.92 ± 1.11[de,GHIJ]	60.66 ± 1.07[e,K]	63.41 ± 0.85[bcde,EFGH]	62.78 ± 1.71[cde,IJ]	66.02 ± 0.73[abc,CDEFG]	66.07 ± 0.97[abc,FG]	65.68 ± 1.15[abc,EFGH]	66.56 ± 0.94[a,EFG]
Cool90	59.20 ± 0.89[f,IJ]	64.24 ± 1.02[bcde,EFGHI]	62.58 ± 0.78[de,GH]	67.05 ± 0.66[a,FG]	62.20 ± 1.26[ef,HI]	65.76 ± 1.06[abc,GHI]	64.50 ± 1.19[abcde,GHI]	63.89 ± 1.13[cde,GHIJ]

[abc] One-way ANOVA of means across a row and [ABC] one-way ANOVA of means down a column with different superscript are significantly different ($P < 0.05$).

[1] EPS = Capsular exopolysaccharides (EPS) produced by S. thermophilus 285.

[2] Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

[3] 75:25 = Mixture of EPS producing S. thermophilus 285 and 25% non-EPS S. thermophilus 372.

[4] B.C. = Before cook.

[28,45,60,90] Pizza Bake performed at x d of storage at 4°C.

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Table 9.5. Mean Hunter a-values (n = 15 to 25) for redness measurements of low fat Mozzarella cheeses for given variables, treatments and processing stages.

	Control		[1]EPS		EPS[2] pre-acid		[3]75:25 pre-acid	
	No Oil	Oil	No Oil	Oil	No Oil	Oil	No Oil	Oil
[4]B.C.[-28]	$-3.48 \pm 0.17^{de,G}$	$-3.68 \pm 0.05^{e,E}$	$-2.50 \pm 0.11^{ab,G}$	$-2.43 \pm 0.09^{a,EF}$	$-2.93 \pm 0.10^{a,HI}$	$-2.79 \pm 0.12^{bc,GH}$	$-2.44 \pm 0.16^{ab,C}$	$-2.63 \pm 0.08^{ab,E}$
B.C.[-45]	$-4.95 \pm 0.12^{c,J}$	$-4.47 \pm 0.14^{bc,GH}$	$-3.61 \pm 0.21^{a,J}$	$-3.74 \pm 0.14^{a,I}$	$-3.83 \pm 0.16^{a,K}$	$-3.65 \pm 0.39^{ab,IJK}$	$-3.88 \pm 0.19^{a,F}$	$-3.81 \pm 0.16^{a,I}$
B.C.[-60]	$-4.51 \pm 0.17^{e,HIJ}$	$-4.34 \pm 0.12^{de,FGH}$	$-3.23 \pm 0.12^{a,HIJ}$	$-3.47 \pm 0.12^{abc,HI}$	$-3.65 \pm 0.14^{bc,IJK}$	$-3.68 \pm 0.09^{c,JK}$	$-3.16 \pm 0.18^{a,DE}$	$-3.30 \pm 0.18^{ab,FGH}$
B.C.[-90]	$-4.52 \pm 0.18^{d,IJ}$	$-4.47 \pm 0.15^{cd,H}$	$-3.45 \pm 0.13^{a,IJ}$	$-3.43 \pm 0.13^{a,GHI}$	$-3.79 \pm 0.13^{ab,JK}$	$-3.93 \pm 0.10^{b,K}$	$-3.41 \pm 0.16^{a,EF}$	$-3.32 \pm 0.29^{a,GHI}$
Warm[28]	$7.55 \pm 1.61^{a,A}$	$1.82 \pm 0.94^{de,ABCD}$	$4.75 \pm 1.01^{ab,A}$	$1.89 \pm 0.61^{cde,ABC}$	$7.33 \pm 1.33^{a,A}$	$3.38 \pm 0.94^{bcde,BCDE}$	$3.86 \pm 1.32^{abcde,A}$	$0.75 \pm 0.73^{e,C}$
Warm[45]	$-0.47 \pm 0.78^{a,F}$	$1.28 \pm 0.81^{a,CD}$	$-0.70 \pm 1.67^{ab,FG}$	$-2.78 \pm 0.35^{bcd,FGH}$	$0.85 \pm 0.97^{a,EF}$	$-3.28 \pm 0.24^{cd,HIJ}$	$-0.32 \pm 0.50^{a,B}$	$-3.58 \pm 0.27^{d,HI}$
Warm[60]	$3.34 \pm 1.50^{a,ABCDEF}$	$3.01 \pm 1.64^{a,ABCD}$	$2.40 \pm 1.11^{a,ABCDEF}$	$2.67 \pm 1.41^{a,ABC}$	$2.55 \pm 1.06^{a,DEF}$	$1.74 \pm 0.91^{a,EF}$	$2.19 \pm 1.02^{a,AB}$	$2.42 \pm 1.03^{a,ABC}$
Warm[90]	$1.23 \pm 1.09^{abc,DEF}$	$1.13 \pm 1.01^{abc,D}$	$1.66 \pm 1.34^{abc,CDEF}$	$0.32 \pm 0.85^{c,BCD}$	$0.57 \pm 1.05^{c,BCD}$	$3.75 \pm 1.10^{a,CDE}$	$2.62 \pm 0.84^{abc,A}$	$3.90 \pm 0.88^{a,AB}$
Cool[28]	$4.50 \pm 1.07^{abcd,ABCD}$	$4.26 \pm 0.87^{bcd,A}$	$5.46 \pm 1.24^{abcd,A}$	$3.60 \pm 0.82^{cde,A}$	$5.05 \pm 1.21^{abcd,ABCD}$	$6.94 \pm 0.86^{a,A}$	$2.29 \pm 1.09^{de,AB}$	$1.91 \pm 0.62^{e,BC}$
Cool[45]	$2.17 \pm 1.08^{a,CDEF}$	$1.94 \pm 1.15^{a,ABCD}$	$-0.26 \pm 0.96^{abcd,EF}$	$-1.81 \pm 0.35^{cd,DEF}$	$-2.62 \pm 0.72^{d,GHI}$	$-0.22 \pm 1.20^{abcd,F}$	$1.45 \pm 0.90^{a,AB}$	$-1.41 \pm 0.61^{bcd,D}$
Cool[60]	$2.72 \pm 1.60^{a,BCDEF}$	$4.83 \pm 1.23^{a,A}$	$2.20 \pm 0.68^{a,BCD}$	$4.19 \pm 1.23^{a,A}$	$2.64 \pm 0.76^{a,CDEF}$	$4.77 \pm 1.09^{a,ABCD}$	$3.93 \pm 0.97^{a,A}$	$3.61 \pm 0.82^{a,AB}$
Cool[90]	$1.03 \pm 0.69^{de,EF}$	$1.35 \pm 1.04^{bcde,BCD}$	$1.04 \pm 1.00^{cde,DEF}$	$0.28 \pm 0.85^{e,CD}$	$3.92 \pm 0.57^{ab,BCD}$	$3.16 \pm 0.98^{abcd,DE}$	$3.08 \pm 0.73^{abcd,A}$	$4.72 \pm 0.87^{a,A}$

[abc]One-way ANOVA of means across a row and [ABC]one-way ANOVA of means down a column with different superscript are significantly different ($P < 0.05$).

[1]EPS = Capsular exopolysaccharides (EPS) produced by S. thermophilus 285.

[2]Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

[3]75:25 = Mixture of EPS producing S. thermophilus 285 and 25% non-EPS S. thermophilus 372.

[4]B.C. = Before cook.

[28, 45, 60, 90]Pizza Bake performed at x d of storage at 4°C.

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Table 9.6. Mean Hunter b-values (n = 15 to 25) for yellowness measurements of low fat Mozzarella cheeses for given variables, treatments and processing stages.

	Control		EPS[1]		EPS pre-acid[2]		75:25[3] pre-acid	
	No Oil	Oil	No Oil	Oil	No Oil	Oil	No Oil	Oil
$B.C._{28}$[4]	13.68 ± 0.54^{def,H}	13.78 ± 0.22^{cde,I}	15.31 ± 0.27^{a,G}	14.70 ± 0.39^{abcd,K}	12.62 ± 0.26^{fg,G}	11.96 ± 0.25^{g,J}	13.45 ± 0.44^{ef,H}	14.17 ± 0.27^{bcde,HI}
$B.C._{45}$	15.67 ± 0.23^{ab,EFG}	14.26 ± 0.36^{de,HI}	16.20 ± 0.29^{a,DEF}	15.60 ± 0.39^{ab,IJK}	13.52 ± 0.50^{ef,EFG}	12.97 ± 0.29^{f,HI}	14.84 ± 0.435^{bcde,FG}	14.48 ± 0.35^{cde,GHI}
$B.C._{60}$	15.46 ± 0.27^{a,FG}	14.37 ± 0.29^{bcd,GHI}	16.10 ± 0.27^{a,EFG}	15.90 ± 0.27^{a,HIJ}	13.39 ± 0.26^{ef,FG}	12.60 ± 0.26^{f,IJ}	14.19 ± 0.35^{cde,GH}	14.15 ± 0.23^{d,I}
$B.C._{90}$	14.99 ± 0.34^{abcd,G}	14.61 ± 0.24^{de,FGH}	15.67 ± 0.25^{a,FG}	15.30 ± 0.19^{abc,JK}	13.93 ± 0.32^{ef,DEF}	13.36 ± 0.18^{f,GH}	14.85 ± 0.24^{bcd,EFG}	14.80 ± 0.23^{cd,FGHI}
$Warm_{28}$	26.20 ± 1.35^{ab,ABC}	29.72 ± 1.36^{a,ABCDE}	28.25 ± 1.03^{ab,ABC}	29.32 ± 0.84^{ab,CDE}	27.66 ± 0.97^{ab,A}	28.90 ± 1.04^{ab,D}	26.63 ± 1.08^{ab,BCD}	25.76 ± 1.37^{b,DE}
$Warm_{45}$	27.11 ± 1.58^{ab,AB}	29.41 ± 1.68^{a,ABCDE}	25.98 ± 1.42^{abc,ABC}	23.77 ± 1.17^{bcd,G}	26.25 ± 1.57^{abc,ABC}	21.67 ± 0.59^{d,F}	25.49 ± 0.78^{ab,CD}	22.90 ± 0.58^{cd,E}
$Warm_{60}$	27.47 ± 1.01^{cde,A}	32.35 ± 1.47^{ab,A}	29.31 ± 0.97^{cde,A}	32.51 ± 0.91^{a,A}	27.05 ± 1.52^{c,ABC}	31.18 ± 1.13^{ab,ABC}	27.46 ± 1.45^{c,ABCD}	31.84 ± 0.63^{a,A}
$Warm_{90}$	26.65 ± 0.82^{ef,AB}	32.78 ± 1.14^{ab,A}	28.85 ± 0.81^{cdef,A}	30.96 ± 0.90^{bcd,ABCD}	26.43 ± 1.14^{f,ABC}	33.8 ± 01.09^{ab,A}	28.66 ± 1.09^{def,ABC}	34.05 ± 1.00^{a,A}
$Cool_{28}$	23.89 ± 1.07^{f,BCD}	27.47 ± 0.73^{abc,CDE}	26.59 ± 0.70^{bcd,ABC}	28.63 ± 0.76^{bcd,DE}	24.77 ± 0.81^{def,BC}	28.72 ± 0.39^{a,D}	24.50 ± 0.47^{ef,D}	26.56 ± 0.83^{cdef,BCD}
$Cool_{45}$	26.18 ± 0.81^{ab,ABCD}	27.73 ± 1.10^{a,BCDE}	26.12 ± 1.01^{abc,BC}	24.89 ± 0.86^{bc,FG}	23.42 ± 0.76^{c,C}	25.72 ± 1.29^{abc,E}	27.64 ± 0.68^{a,ABC}	26.15 ± 1.07^{abc,CD}
$Cool_{60}$	22.68 ± 0.74^{c,D}	27.25 ± 0.97^{d,DE}	27.29 ± 0.67^{cd,ABC}	29.75 ± 0.57^{cd,BCD}	27.77 ± 0.76^{bcd,A}	31.00 ± 0.69^{a,ABC}	30.08 ± 1.20^{abcd,A}	31.35 ± 0.59^{a,A}
$Cool_{90}$	23.52 ± 0.46^{c,CD}	27.00 ± 0.72^{bcd,E}	25.43 ± 0.97^{d,C}	27.43 ± 0.74^{abcd,E}	26.39 ± 0.83^{cd,ABC}	29.90 ± 0.95^{a,BCD}	27.44 ± 1.01^{a,ABC}	30.80 ± 1.46^{a,A}

^{abc}One-way ANOVA of means across a row and ^{ABC}one-way ANOVA of means down a column with different superscript are significantly different ($P < 0.05$).

[1] EPS = Capsular exopolysaccharides (EPS) produced by S. thermophilus 285.

[2] Pre-acid = Cheese milk pre-acidified to pH 6.1 with citric acid.

[3] 75:25 = Mixture of EPS producing S. thermophilus 285 and 25% non-EPS S. thermophilus 372.

[4] B.C. = Before cook.

$_{28, 45, 60, 90}$Pizza Bake performed at x d of storage at 4°C.

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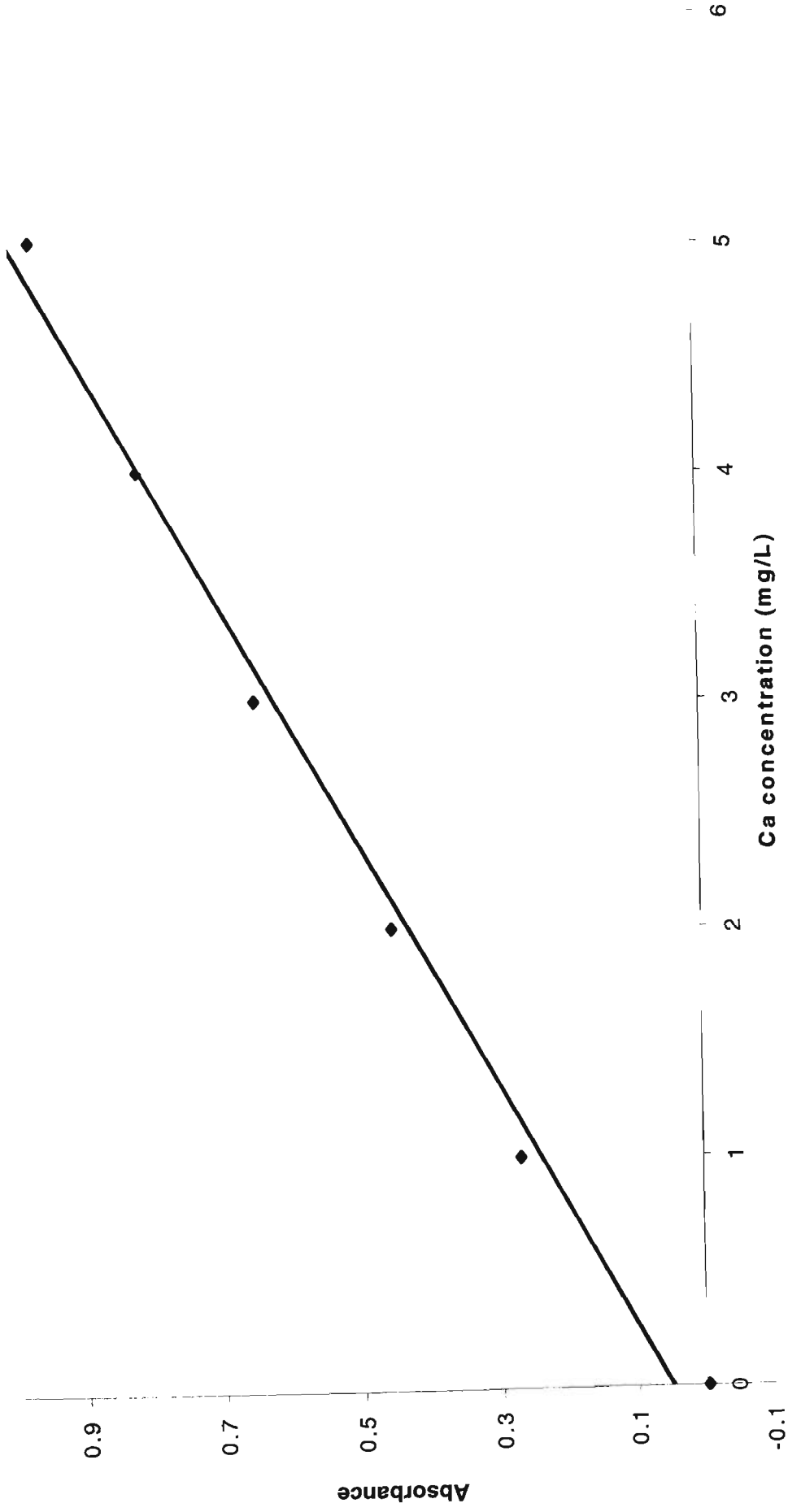
Figure 9.1. Standard curve for the determination of calcium in cheese, curd, milk, whey and stretching water using atomic absorption spectroscopy.

Figure 9.2. Cheese melt of control, [1]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285), EPS [2]pre-acid (cheese milk pre-acidified to pH 6.1), and [3]75:25 (mixture of 75% EPS producing *S. thermophilus* 285 and 25% non-EPS *S. thermophilus* 372) pre-acid cheeses over 90 d of storage at 4°C.

[abc]One-way ANOVA of means (n = 6) between d 7 and d 90 within a cheese type and [ABC]one-way ANOVA of means at d 14, 28, 45, 60 and 90 between cheese types with different superscript are significantly different ($P < 0.05$).

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Figure 9.3. Hardness of control, [1]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285), EPS [2]pre-acid (cheese milk pre-acidified to pH 6.1) and [3]75:25 (mixture of 75% EPS producing *S. thermophilus* 285 and 25% non-EPS *S. thermophilus* 372) cheeses over 90 d of storage at 4°C.

[abc]One-way ANOVA of means (n = 12) between d 7 and d 90 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28, 45, 60 and 90 between cheese types with different superscript are significantly different (*P* <0.05).

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Figure 9.4. Springiness of control, [1]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285), EPS [2]pre-acid (cheese milk pre-acidified to pH 6.1) and [3]75:25 (mixture of 75% EPS producing *S. thermophilus* 285 and 25% non-EPS *S. thermophilus* 372) cheeses over 90 d of storage at 4°C.
[abc]One-way ANOVA of means (n = 12) between d 7 and d 90 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28 and 45 between cheese types with different superscript are significantly different (P < 0.05).
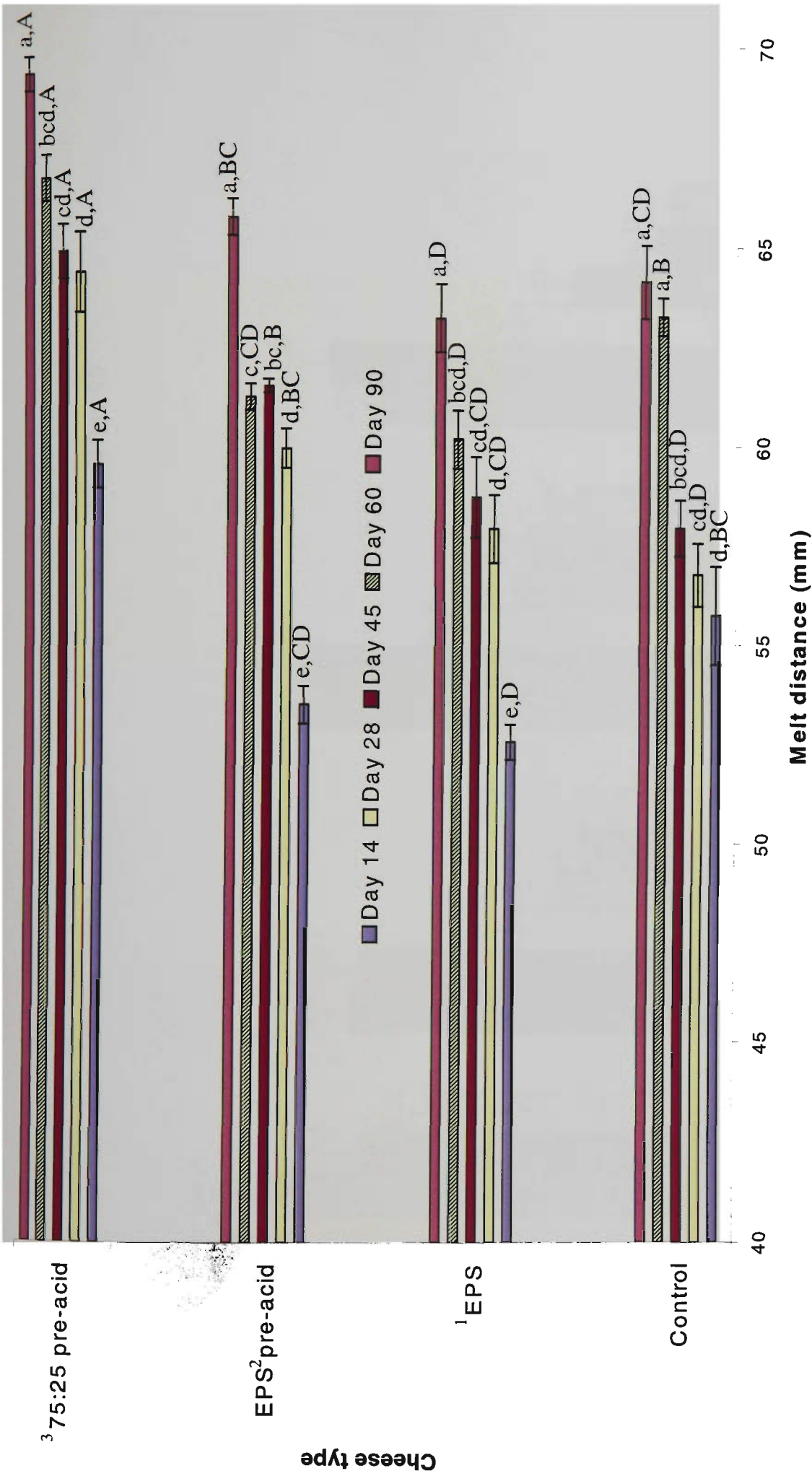
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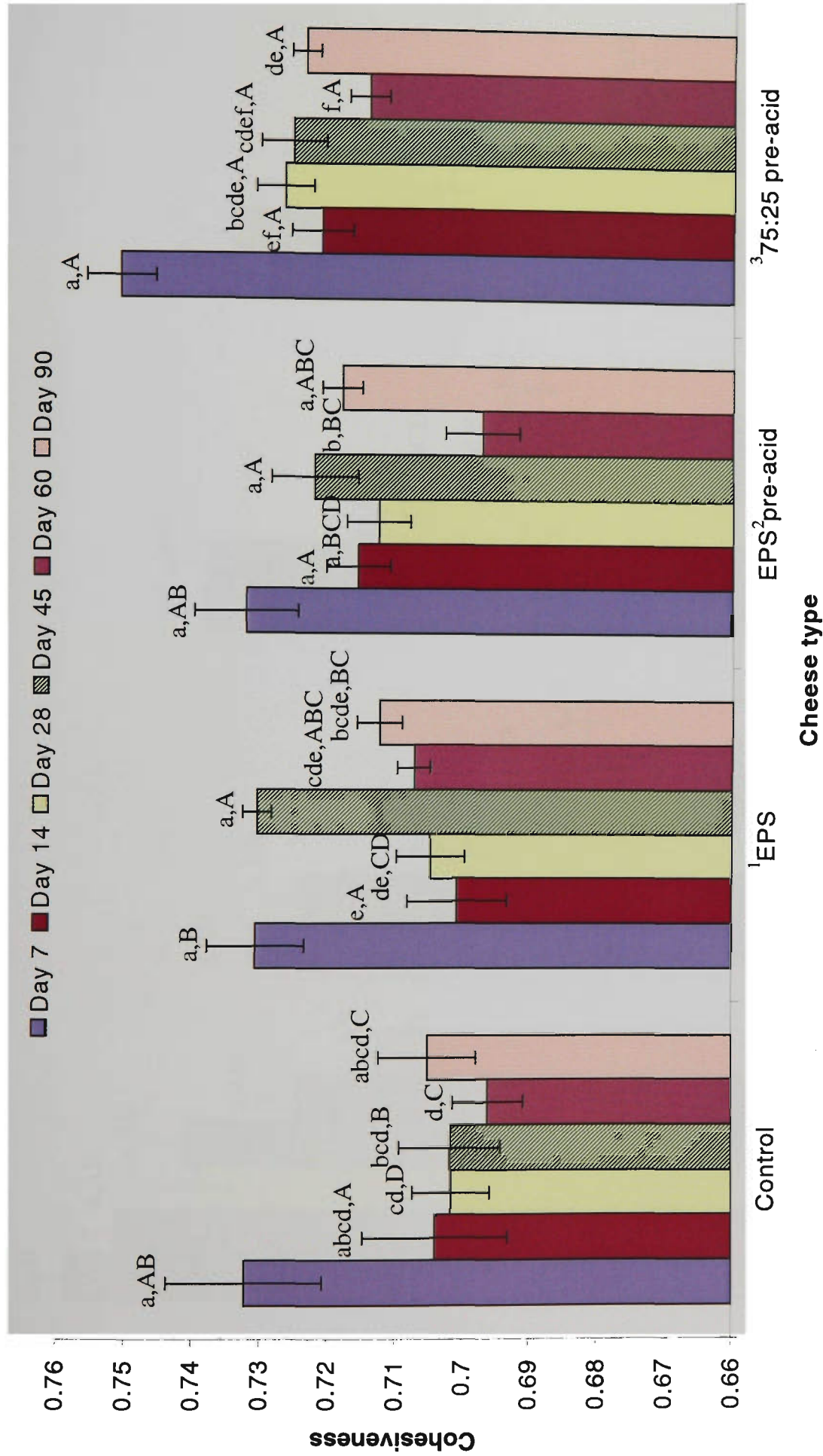
Figure 9.5. Cohesiveness of control, [1]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285), EPS [2]pre-acid (cheese milk pre-acidified to pH 6.1) and [3]75:25 (mixture of 75% EPS producing *S. thermophilus* 285 and 25% non-EPS *S. thermophilus* 372) cheeses over 90 d of storage at 4°C.
[abc]One-way ANOVA of means (n = 12) between d 7 and d 90 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28, 45, 60 and 90 between cheese types with different superscript are significantly different (*P* < 0.05).

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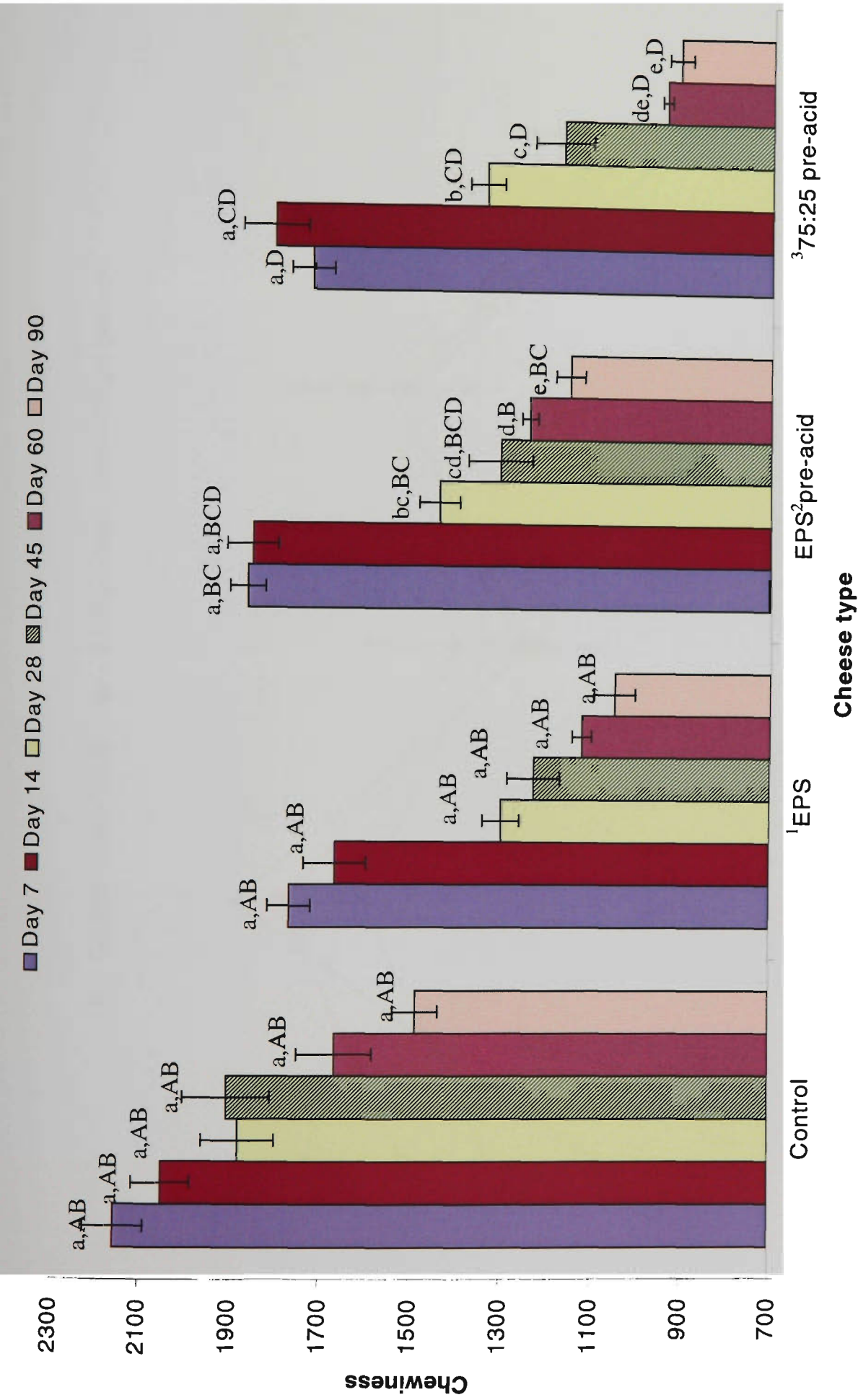
Figure 9.6. Chewiness of control, [1]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285), EPS [2]pre-acid (cheese milk pre-acidified to pH 6.1) and [3]75:25 (mixture of 75% EPS producing *S. thermophilus* 285 and 25% non-EPS *S. thermophilus* 372) cheeses over 90 d of storage at 4°C.

[abc]One-way ANOVA of means (n = 12) between d 7 and d 90 within a cheese type and [ABC]one-way ANOVA of means at d 7, 14, 28, 45, 60 and 90 between cheese types with different superscript are significantly different (*P* < 0.05).
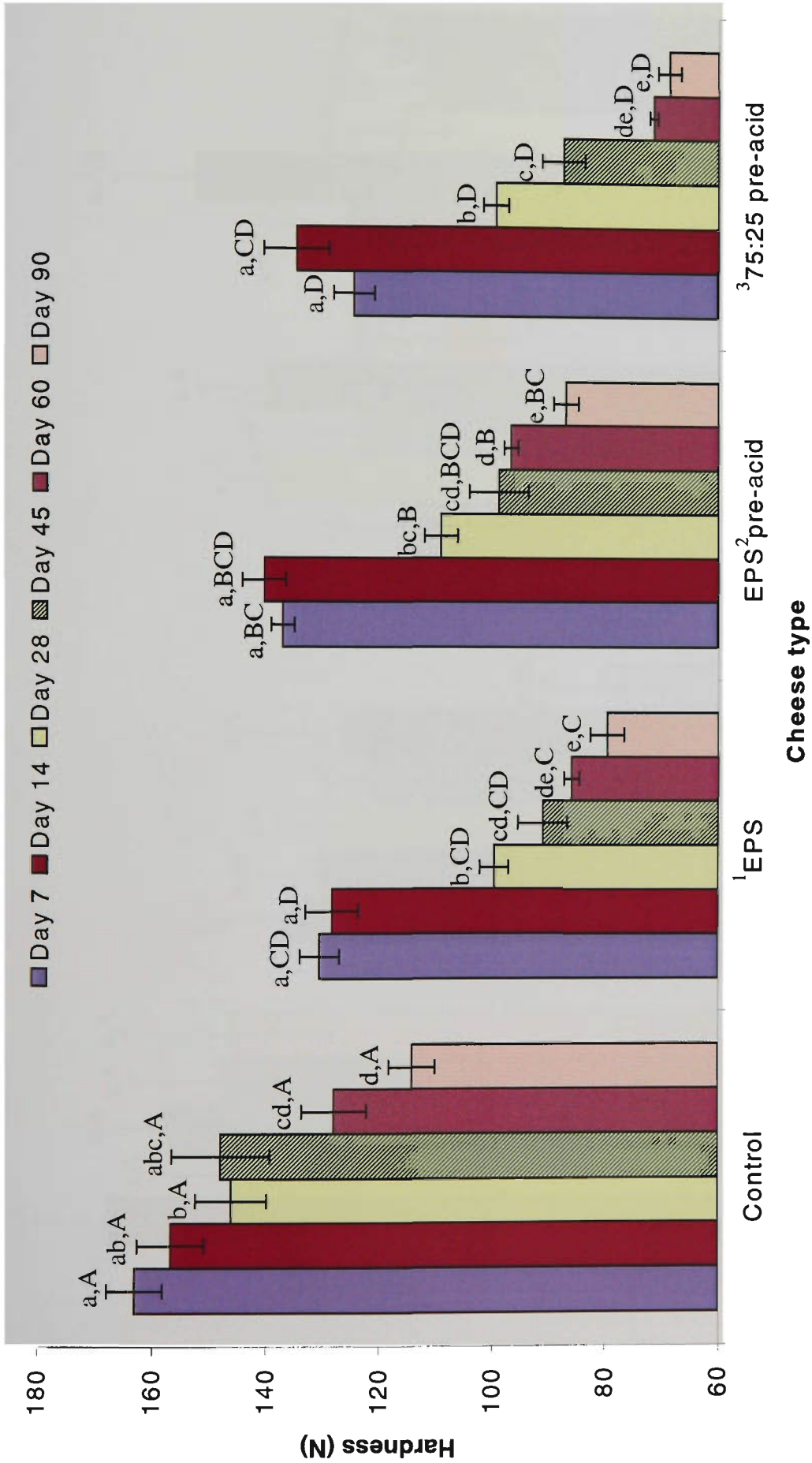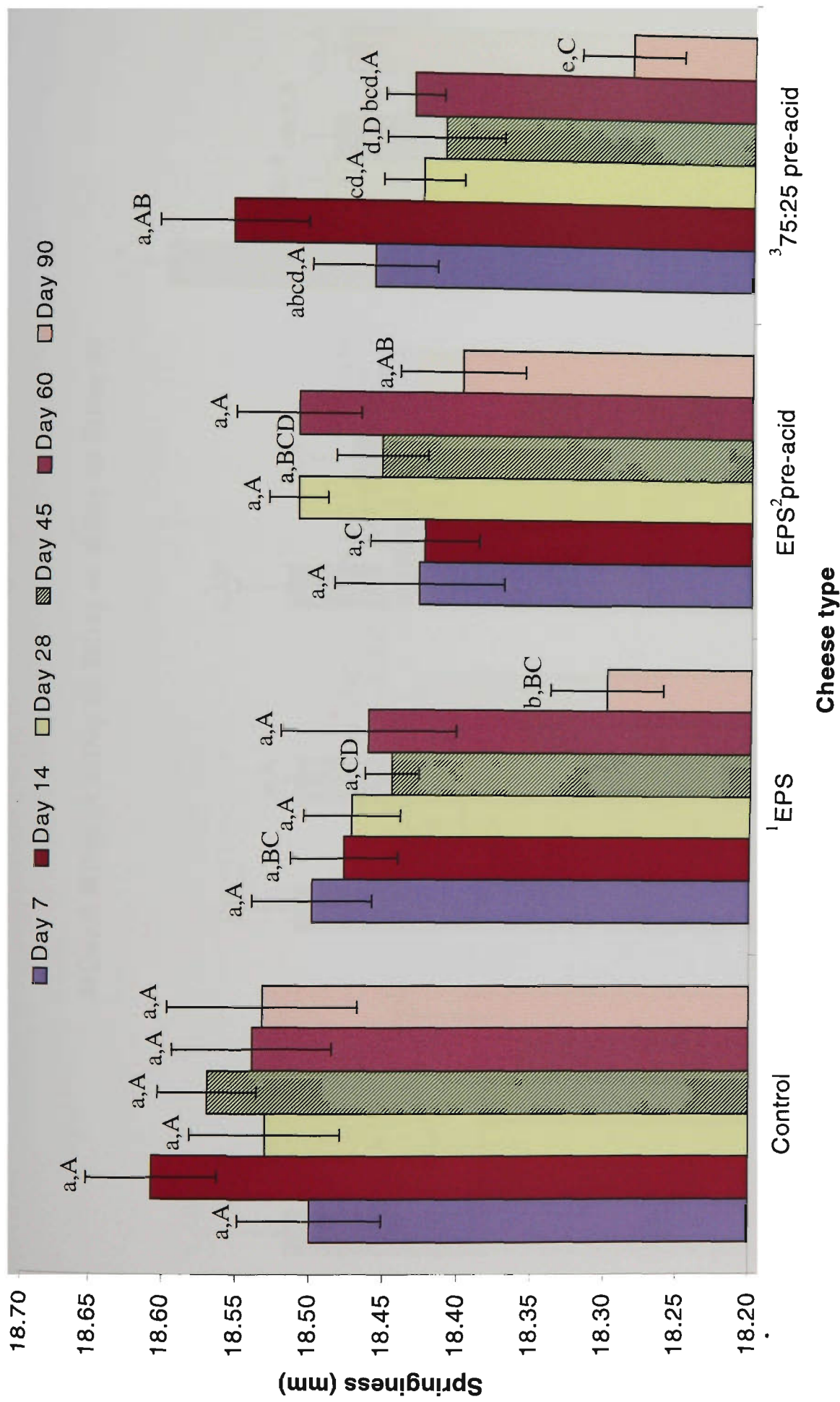
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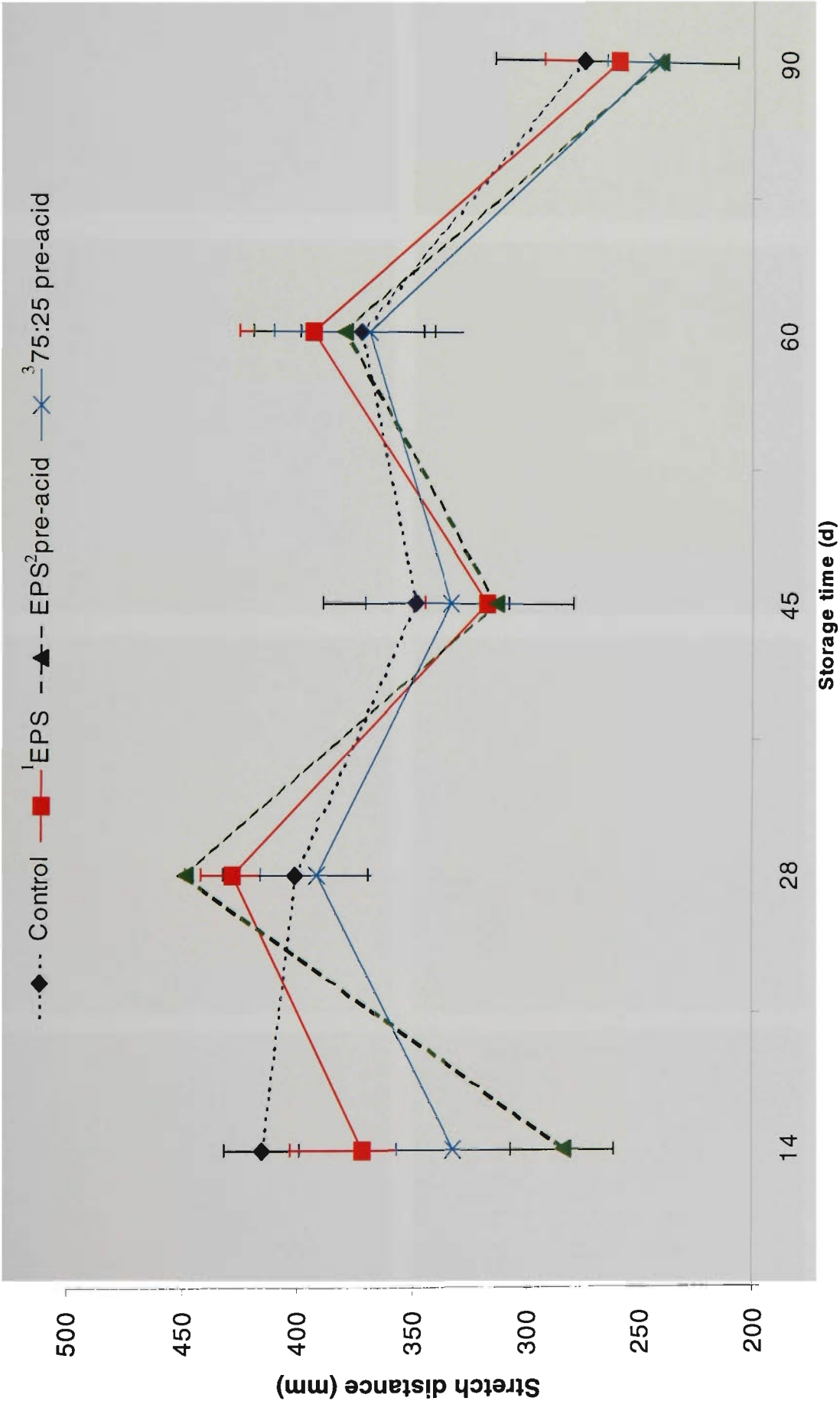
Figure 9.7. Stretch characteristics of control, [1]EPS (capsular exopolysaccharides produced by *S. thermophilus* 285), EPS [2]pre-acid (cheese milk pre-acidified to pH 6.1), and [3]75:25 (mixture of 75% EPS producing *S. thermophilus* 285 and 25% non-EPS *S. thermophilus* 372) pre-acid cheeses over 90 d of storage at 4°C (n = 6).

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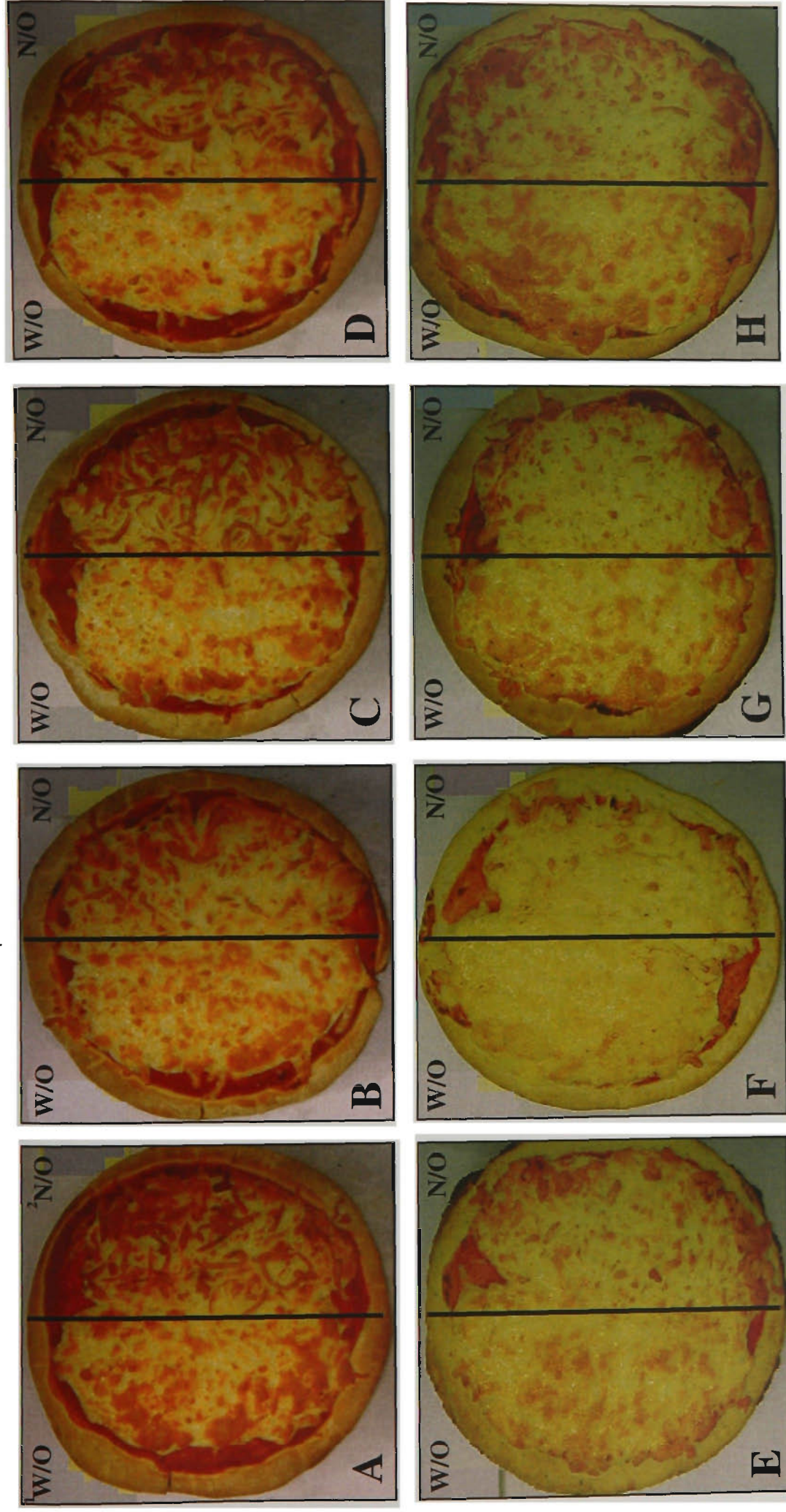
Figure 9.8. Pizza bake characteristics for control cheeses baked at 262°C / 7 min at d 28 and d 45 of storage, respectively (A and E), EPS (capsular EPS produced by *S. thermophilus* 285) cheeses (B and F), EPS pre-acidified (cheese milk pre-acidified to pH 6.1) cheeses (C and G) and 75 % (EPS *S. thermophilus*) : 25 % (non-EPS *S. thermophilus*) co-cultured and pre-acidified cheeses (D and H).

[1]W/O = With oil.
[2]N/O = No oil.

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CHAPTER 10.0

Summary of Results

Eighty strains of lactic acid cocci and twenty strains of lactobacilli were evaluated for their ability to produce microbial exopolysaccharides. Eleven strains were identified as *S. thermophilus* (801, 285, 371, 760, 1275, 751, 753, 1439, 287, 288, 486) and produced capsular EPS. *S. thermophilus* 1275 was a mixed capsular/ropy EPS producer. Results indicate that a significant number of EPS producing LAB exist within a genus, many of which are genetically diverse. Two strains were identified as *L. delbrueckii* ssp. *bulgaricus* (756, 840) and showed the ability to synthesise capsular EPS. Strain 820 was a capsular EPS yielding *Lactococcus lactis* ssp. *lactis*. Two genetically similar groups of bacteria were identified within the *S. thermophilus* genus by PFGE. The first of these groups consisted of strains 287, 288, 371 and 801 and the second group consisted of strains 751 and 753. The remaining strains of EPS producing *S. thermophilus* had individual restriction patterns and were genetically different from each other and from those identified in the two groups.

EPS production was greatest in milk medium by *S. thermophilus* 1275 at 360 mg/L after 24 h of fermentation. Capsular EPS producing *S. thermophilus* strains 753, 1439, 285 produced 90, 99 and 84 mg/L of EPS, respectively, and *S. thermophilus* 287, 288 and 486 synthesised a more modest 11, 18 and 19 mg/L of EPS after 24 h. When ropy *S. thermophilus* 1275 was grown in milk, the viscosity was greater than that of milk in which capsular LAB were cultured. The viscosity of milk in which capsular and non-EPS *S. thermophilus* strains were cultured was generally similar. The capsular EPS producing strain *S. thermophilus* 285 and the ropy/capsular producing *S. thermophilus* 1275 were selected for the manufacture of low fat Mozzarella cheese making trials.

Because of the high amount of EPS synthesised by *S. thermophilus* 1275, the factors influencing production of the polysaccharide were studied in this strain. The temperature of incubation, pH of the medium, supplementation with WPC and co-culturing with non-EPS adjunct *S. thermophilus* were investigated in milk fermentation studies.

EPS production, cell growth, metabolism of lactose and lactic acid production by *S. thermophilus* 1275 were influenced by the factors mentioned earlier. EPS production by *S. thermophilus* 1275 was shown to be growth associated at an optimum temperature of 40°C and pH 5.5, yielding 458 mg/L of EPS. The production of EPS was reduced at a temperature and pH above and below the optimum. At pH 4.5, no EPS synthesis took place. Addition of WPC and adjunct starter cultures increased production of EPS. Supplementation with WPC at the optimum temperature and pH yielded 1029 mg/L of EPS. However, when the pH was uncontrolled, the milk containing WPC fermented with this strain resulted in 491 mg/L of EPS, nevertheless, this was greater than milk without fortification. Co-culturing with non-EPS *S. thermophilus* at the ratio of 75:25 and 50:50, EPS to non-EPS culture at optimum temperature and pH yielded 832 mg/L and 676 mg/L of EPS, respectively.

Low fat Mozzarella cheeses containing 6% fat were made with EPS producing *S. thermophilus* 1275 and 285. The production time of cheeses was reduced when made with EPS producing *S. thermophilus* strains. Control cheeses made with non-EPS producing *S. thermophilus* 1303 had low moisture retention and poor yield. The moisture retention and yield of cheeses were significantly improved with capsular EPS producing *S. thermophilus* 285 and ropy EPS producing *S. thermophilus* 1275. Ropy EPS was isolated from curds at higher concentration and exhibited slightly greater moisture retention in cheeses than the capsular type. This did not significantly increase the yield. Capsular and ropy EPS reduced the hardness, springiness and chewiness of low fat Mozzarella cheeses and improved the melt and stretch characteristics. The adhesiveness and cohesiveness were greater in EPS cheeses. Because of the reduced fat content, there was a lack of free oil release during pizza baking, and a high degree of scorching of cheese shreds occurred at the surface.

Ropy EPS in cheeses did, however, show poor water holding capacity resulting in the seepage of moisture when cheeses were heated. Furthermore, ropy EPS resulted in a sticky layer of slime coating cheeses but dissipated with storage time.

To further improve the quality of low fat Mozzarella cheeses made with EPS cultures containing 6% fat, pre-acidification with citric acid to pH 6.1 was implemented into the cheese making procedure. The control cheeses made with non-EPS cultures and without pre-acidification showed lowest moisture content. Pre-acidification of the cheese milk did not increase moisture retention in cheeses unless made with EPS producing cultures, however, it did improve texture and functionality during the early stages of storage. The stretch distance of melted cheeses, although similar to that of the control cheeses, improved overall when milk was acid treated. The EPS concentrations in cheeses made with pre-acidified milk and capsular EPS producing _S. thermophilus_ 285 were similar to those in cheeses made with pre-acidified milk and ropy EPS producing _S. thermophilus_ 1275. Consequently, the moisture retention was similar between the two batches of cheeses. When baked on a pizza base the appearance of EPS cheeses remained poor after pre-acidification and 28 d of storage. EPS pre-acidified cheeses, however, exhibited good characteristics in general after 45 d of storage as a result of extensive proteolysis.

As was previously observed in cheeses made without pre-acidification, ropy EPS showed poor water holding capacity and excessive moisture seepage occurred when cheeses were heated during the first 28 d of storage. Similarly, the cheese surface was coated with a sticky layer of slime. Given that capsular and ropy EPS have similar beneficial effects on low fat Mozzarella cheeses when milk is pre-acidified, the selective use of the capsular EPS producing strains over the ropy type is rational when considering the implications associated with slime formation and utilisation of whey.

The next step in the development of a low fat Mozzarella cheese of acceptable quality containing 6% fat was to attempt to increase capsular EPS production of _S. thermophilus_ 285 during the manufacture of pre-acidified cheeses to increasing moisture retention. Based on the early fermentation results, supplementation with WPC into milk and co-culturing with 75% EPS _S. thermophilus_ 285 were carried out.

The moisture content of control (pre-acidified and non-EPS) cheeses was lowest. Moisture retention in pre-acidified cheeses increased with the use of capsular EPS producing *S. thermophilus* 285. This occurred when used as a starter culture singly, as a co-culture with a strain of non-EPS *S. thermophilus* or in the presence of WPC. Microbial EPS also improved the textural and functional characteristics of cheeses. Pizza bake characteristics were poor in all cheeses as has been observed to this point unless cheese shreds were coated with oil. The cheese shreds coated with canola oil did not dehydrate and all cheese types exhibited complete shred fusion, melt and flow after baking.

Co-culturing an EPS producing *S. thermophilus* with a non-EPS producing strain and the use of WPC did not increase EPS synthesis during cheese manufacture. A significant amount of EPS was lost through the whey. *S. thermophilus* 285 used as a co-culture limited the amount of EPS produced in cheese due to low initial EPS starter concentration, however, co-culturing led to improved melt and texture. On the contrary, the use of the WPC in cheeses interfered with the melt, texture and pizza bake performance. The aim to increase EPS synthesis in cheeses was not achieved, but the quality of cheeses in general was enhanced regardless.

In the final trials to develop a low fat Mozzarella cheese containing 6% fat at pilot scale, the carbohydrate based fat replacers OptaMax® (FR1) and Versagel® (FR2) were used together with capsular *S. thermophilus* 285 and pre-acidification. Moisture retention increased in EPS cheeses made with FR1 and FR2 fat replacers leading to improved yield and textural characteristics. FR1 based cheeses exhibited good stretch and melt properties, however, FR2 based cheeses did not show any improvement. Pre-acidification of the cheese milk and use of FR1 further increased the moisture content and yield. FR1 cheeses made with acid treated milk were the softest and had the highest stretch distance. Pre-acidified cheeses also had an increased level of α- and β-casein proteolysis and improved the cheeses functional and textural behaviour, particularly the melt distance.

The nature of the fat replacer had the greatest influence on the microstructure of cheeses and there was an impact on the textural and functional characteristics. FR1 based cheeses had a more open casein matrix (most porous when pre-acidified) and showed better melt and stretch as compared to FR2 containing cheeses. FR2 cheeses had the densest protein network that almost lacked porosity. The subsequent hydration of the protein matrix observed at a microstructural level improved the cheeses functional behaviour. Unlike fat, which was found in the serum channels of the casein matrix as expected, EPS existed within the matrix.

The pizza bake performance of all cheese types was poor initially unless cheese shreds were coated with oil. The characteristics of baked cheeses improved with storage time, however, FR1 based cheeses were superior to those that were FR2 based. By combining EPS cultures with the appropriate fat replacer (FR1) and pre-acidification of the cheese milk, it is possible to significantly increase the yield of low fat Mozzarella cheeses, achieve textural and behavioural attributes superior to those of untreated low fat Mozzarella cheeses, and reduce maturation time.

Based on the results obtained from cheeses made at pilot scale, the most successful low fat Mozzarella cheese variables containing EPS and 6% fat were made at semi-commercial quantities in a semi-automated system. The moisture content of the control cheeses made with non-EPS producing starter cultures and without pre-acidification was the lowest. Capsular EPS of *S. thermophilus* 285 in cheeses increased the moisture content, although the moisture content in cheeses made with EPS and pre-acidification was not significantly different to the control batches.

The moisture retention in Mozzarella cheeses containing EPS was lower than that observed in the corresponding cheeses made during pilot trials. This was attributed to slight modifications in the manufacturing procedure, particularly during the cheddaring stage. Pre-acid of the cheese milk also resulted in less EPS than that isolated from cheeses made without an acid treatment due to insufficient time provided for the starter culture population to develop.

Low fat Mozzarella cheeses made in a semi-automated setup exhibited similar textural and functional characteristics to those made manually on a smaller scale at a pilot scale setup. Capsular EPS reduced the hardness and chewiness of cheeses and pre-acidification improved the cheese melt distance. Stretchability was not influenced by the manufacturing procedure and was in general similar between cheese types. As has been evident through the experiments, the pizza bake performance was poor in the absence of an oil coating to prevent the evaporation of surface moisture, but improved with storage time. The pizza bake characteristics were, however, best in cheeses made with EPS only cultures.

Overall, a low fat Mozzarella cheese containing 6% fat of acceptable quality in terms of texture and functionality was developed. Combining capsular microbial exopolysaccharides produced by *S. thermophilus* 285 together with the carbohydrate based fat replacer, OptaMax® and by pre-acidification the most successful cheeses were made. The yield was significantly increased and the hardness of cheeses was reduced substantially. Cheeses exhibited a high melt distance and had a superior pizza bake performance showing complete shred fusion in the absence of surface scorching when coated in canola oil.

CHAPTER 11.0

Future Research Directions

We found that a substantial number of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* strains produced EPS. A concern raised by industry and the reluctance to use such strains of bacteria as starter cultures is the unreliable nature of EPS production. Although we have examined EPS production under various conditions to some degree, there is a need for further research on genetic mutations that may cause the loss of EPS production. Co-culturing the EPS producing *S. thermophilus* 1275 with a strain of non-EPS producing *S. thermophilus* increased EPS synthesis (Chapter 4.0). It was speculated that the non-EPS co-culture had a symbiotic relationship with the EPS producer and there was an increased availability to nutrients. Further research must be conducted to understand this relationship.

The popularity of low fat dairy products has increased in recent years due to consumer awareness of reducing the total dietary fat intake. Simultaneously, the demand for Mozzarella cheese as a functional ingredient to be used on pizzas is increasing due to the popularity of fast and convenient food. In response to these trends, traditional Mozzarella cheese manufacturers have shown interest in developing a low fat product of acceptable quality. Presently, reduced fat Mozzarella cheeses available in supermarkets still contain high levels of fat. Low fat Mozzarella cheeses are bound to capture a large share of the current cheese market if we can overcome the problems associated with reducing fat content and if these low fat cheeses can meet the characteristics of a full fat Mozzarella cheese. Our study, as well as previous work conducted by others has demonstrated that there are possibilities to develop a reduced fat product. As a consequence of the complexity of the Mozzarella cheese system, there is still a scope of work to standardise methods of manufacture encompassing techniques on hand.

The work carried out as part of this project showed encouraging results, primarily for increasing the moisture content in cheeses by using polysaccharides of microbial and non-microbial origins and secondly by reducing the calcium content through pre-acidification. However, there is a need to expand upon this study in order to develop a deeper understanding of

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the mechanisms involved. Cheeses made with ropy EPS cultures (Chapters 5.0 and 6.0) were exceptional. Such cheeses had an unappealing coating of slime on the surface, however, it dissipated after a short storage period. It is not known how this occurs, and more importantly, the problems associated with processing of viscous whey containing EPS were not addressed. The viscosity of whey must be reduced to allow adequate ultrafiltration or other processes.

Pre-acidification of cheese milk did not allow sufficient time for starter cultures to produce ample amount of EPS, and the manufacturing procedure, specifically the time allowed for starters to grow should be addressed in the future.

The future research directions should also consider the potential to optimise the hydration of WPC and fat replacers into milk. In this study (Chapter 8.0), we used EPS and fat replacers to make low fat Mozzarella cheese, and by pre-acidification of the cheese milk containing OptaMax®, the moisture content increased significantly. Our speculation was based on increased gelation of starch as affected by the acid treatment, however, this is not completely understood. Furthermore, work is needed to understand the interactions between microbial exopolysaccharides, fat replacers and pre-acidification on the casein network. In the same experiment, we identified that individual and combined component had unique effect on the porosity of the casein network and its ability to retain moisture as well as on functionality of cheeses.

CHAPTER 12.0

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