Quantifying Calcium Losses During Manufacture of Low-Moisture Part-Skim Mozzarella and their Impact on Cheese Functionality

by

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A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy
(Food Science)

at the
University of Wisconsin-Madison
2023

Date of oral examination: 12/18/2023

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Dedicated to my parents

என் பெற்றோருக்கு அர்ப்பணம்

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ABSTRACT

The insoluble calcium content of low-moisture part-skim (LMPS) Mozzarella largely determines its functional properties, such as, melting and stretching. The amount of insoluble calcium in the cheese is dependent on various manufacturing factors and the amount of acid developed during the cheese manufacturing process. Although a few studies have been done in the past to understand the effects of certain factors on the final total calcium in cheese, none have measured the calcium losses (in real-time) during cheese manufacture. Measuring the state of calcium (amount or ratio of soluble to insoluble calcium) directly during cheese manufacturing can help us elucidate the importance of critical process steps and thus, better predict the functional properties of cheese. The main goals of our research were to study various critical manufacturing factors and their effects on calcium losses during cheese make and consequentially the impact on the rheological and functional properties of cheese, and (2) analyze the range of insoluble calcium values obtained in the final cheese to help predict the functionality of LMPS Mozzarella.

SUMMARY

Low-moisture part-skim (LMPS) Mozzarella consumption has been steadily increasing, especially since the 1970s due to the growing pizza market. It is critical to figure out more economical, easier, and faster ways of making this cheese with improved and consistent functionalities to meet the complex demands of the pizza market. The textural and functional performance of LMPS Mozzarella cheese is influenced by casein interactions (between and within casein molecules) and the amount of insoluble calcium associated with the micelles (Lucey et al., 2003). If the insoluble calcium content in the cheese is either too high or too low, the functional performance of cheese (e.g., on pizza) will be affected. Therefore, the insoluble calcium content in the cheese is a more important indicator of cheese functionality than the total calcium content (Lucey and Fox, 1993) but the insoluble calcium is not measured by manufacturers.

During cheesemaking, due to acid production and the decrease in pH, the colloidal (insoluble) calcium phosphate (CCP) partly solubilizes from casein. This soluble calcium, now in the aqueous phase of milk/curd, can be lost into the whey during cheese manufacture, thereby influencing the total (final) and insoluble calcium content in the cheese. The amount of soluble calcium generated from insoluble calcium solubilization in the milk (and curds) is dependent on how the cheese is manufactured. If there is not enough acid development in the curd during cheese manufacturing, much of the CCP remains intact (associated with the caseins). Various manufacturing factors affect the final (at 1 d) amount of insoluble calcium in cheese, with the most important parameters being the rate and the extent of acidification, the pH at whey drainage, the pre-acidification pH values, i.e., the pH at renneting, and the final pH value, i.e., at milling (Lucey and Fox, 1993; Lucey et al., 2003). The types of acid used during cheese make can also cause a

difference in the solubilization of CCP (Keller et al., 1973). Past research done in this area (Yun et al., 1993b; Yun et al., 1995; Metzger et al., 2001a and b; Mizuno et al., 2009; Sheehan and Guinee, 2004) has one or more limitations: (1) only total calcium, but not the insoluble calcium content, was measured after manufacture, (2) the amount of CCP dissolved and the amount of total calcium lost to whey due to individual manufacturing factors were unknown, (3) experiments to study the effects of one or more manufacturing factors were sometimes indiscernible (confounded with other factors), and (4) cheese makes from higher casein milk (≥ 4%) have not yet been explored. Therefore, tracking and quantifying calcium losses during cheese manufacture could lead to a better understanding of the importance that each of these factors plays in influencing the total (final) and insoluble calcium content in the cheeses and thereby determining its functional performance on pizzas.

Chapter 2 dealt with developing and validating a rapid water-soluble calcium (WSC) method to measure the insoluble calcium content in cheese (for use during cheese making). The parameters for the WSC method, such as, water temperature, homogenization time, and centrifugation speed involved during the sample preparation were optimized to reduce shifts in calcium equilibrium. When the WSC method was compared with other existing methods, it was found that although the WSC method had slightly different values compared to the cheese juice method (traditional method), it had similar and consistent trends amongst different cheese samples measured.

In Chapter 3, the influence of rates of pre-acidification (bench-scale work), pH at whey drainage, and the pH values for pre-acidification of cheese milk, on the calcium losses during cheese manufacture and their influence on rheological properties (pilot-scale experiments) were studied. The pH at whey drainage had a significant effect on the final insoluble calcium content in

the cheese, with a lower pH value resulting in more calcium losses during cheese manufacture. The differences in insoluble calcium content between cheese samples also affected their rheological properties. Varying the rate of pre-acidification (20 vs 60 min) slightly but significantly affected the insoluble calcium content in the milk dispersion. The pH at rennet addition seemed to slightly, but not significantly, impact the final insoluble calcium content in the cheese; as there was a lot of variability between trials, the differences in rheological properties of cheese were not significant between cheese samples.

In Chapter 4, the impact of the extent of pre-acidification (control no pre-acidification, pH 6.40, and pH 6.00) and types of acids (acetic, citric, and carbonic) on the calcium losses during the manufacture of LMPS Mozzarella (made from 4% casein milk), along with the changes in textural and functional properties of cheeses during refrigerated storage were studied. All the cheeses manufactured had similar compositions with similar moisture content. Pre-acidifying the cheese milk to pH 6.40 only slightly but not significantly impacted the final total and insoluble calcium content in the cheese; but when the cheese milk was pre-acidified to pH 6.00 the final total and insoluble calcium content in the cheese was significantly lower compared to control cheese, dramatically lower in the cheeses that were pre-acidified with citric acid due to its calcium chelating ability. Owing to these differences in calcium content between the samples, the functionality of the cheese was also affected where the cheese pre-acidified to pH 6.00 with citric acid had lower first chew hardness, lower chewiness, and lower strand thickness values compared to other cheeses when measured by sensory panelists as melted cheese on pizza.

Chapter 5 dealt with the impact of varying the casein content in the cheese milks (2.5, 4.0, and 5.5% CN) and milling (final) pH values (pH 5.40 and 5.10). The composition of the cheese was significantly different between cheeses with the cheeses made from milk with 5.5% casein

having significantly lower moisture (although it met the requirements for LMPS Mozzarella) and higher protein and fat contents as compared to cheeses made from milk with 2.5% casein. We found that the cheese made from milk with higher casein content dissolved less insoluble calcium during the cheese-making process, as a result of higher serum calcium and lower moisture content in the milk and curd samples compared to cheeses made from milk with 2.5% casein. Lowering the milling pH values only slightly reduced the insoluble calcium content in the final cheese but not the total calcium. As a result of differences in moisture and insoluble calcium content between the cheeses, the rheological, textural, and functional properties of the cheeses were also different. The cheese made from milk with 5.5% casein shredded well even at 3 mo of storage as a result of lower moisture and higher insoluble calcium content, compared to cheeses made from milk with 2.5% casein. These cheeses made from 5.5% casein milk also had smaller blisters on pizza, lower cohesiveness, and higher chewiness values as a result of higher insoluble calcium content after 1 and 3 mo of storage. The cheeses made from milk with the same casein content, but different milling pH values had slight differences in strand thickness values, with the cheeses exhibiting a thinner strand with lower insoluble calcium content in the final cheese.

In Chapter 6, data gathered from our research as influenced by critical manufacturing factors, in addition to the variability observed between our trials and cheese composition was used to identify the range of insoluble calcium obtained in the final cheese and thereby its functionality. Simple scatter plots and biplots were used to show correlations between compositional or manufacturing parameters against insoluble or soluble calcium content in the final cheese sample. The impact on the sensory parameters of cheese, measured as melted cheese on pizza, affected by the change in insoluble calcium content was identified and described for the individual parameter measured.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor Dr. John Lucey for giving me the greatest opportunity of my life. This opportunity paved the way for me to discover my passion for dairy science. His intellectual thinking, and intriguing questions, in addition to the guidance, support, and encouragement that I have received from him over the years have made me the researcher that I am today, and I am eternally grateful for that. I am thankful for all the advice, feedback, and different perspectives that I have received from my committee members- Dr. Rani Govindasamy-Lucey, Dr. Sundaram Gunasekaran, and Dr. Bradley Bolling. Many thanks to Søren Lillevang and Arla Foods for giving me the opportunity to work on a very interesting project with industrial significance.

I am grateful to John Jaeggi, Mike Molitor, and Mark Johnson for educating me on different areas of dairy products and technology and also for their insightful conversations. Special thanks to Kevin Sweeney from Saputo for sharing his valuable piece of knowledge from an industrial standpoint. I would like to thank our lab manager Yanjie Lu for training me on various analytical techniques and for helping me with anything and everything.

I consider myself lucky to have worked with many CDR staff and cheese makers- Gary Grossen, Joey Jaeggi, Mellisa Houfe, David Niemiec, Ben Mathers, Gina Mode, Jim Cropp, Jeremy Johnson, Luis Jimenez-Maroto, Juan Romero, Brad Harkins, and Derek Morrick. There is not one dull moment with these folks; they are warm, friendly, and helpful and they have all made CDR such a fun place to work in. I am very thankful to Rebecca Kimball-Kilgore, Aravind Chandrasekar, Jim Grisamore, Kristen Houck, Amy Bostley, and Nick Terebayza for all their help with analytical and micro testing. I am also thankful to Brandon Prochaska, our sensory

coordinator, for organizing sensory panels week after week to analyze the innumerable cheese samples with the right attitude. Many thanks to Rodrigo Ibanez for helping me with the capillary electrophoresis run. Special thanks to Srirupa for lending a helping hand with analytical testing when I needed it the most.

I would like to thank my wonderful lab mates - Daniel, Maggie, Jason, Ashlyn, Ben, Philip, Anastasia, Kelden, John, Grace, and Danielle for creating such a lively environment to work in. I am going to miss all the fun lab outings and birthday celebrations. I would like to thank all my friends from Madison – Suchita, Anjali, Shuvam, Ashwin, Aishwarya, Harshit, Guru, Venkat, Archana and Vigneshwar - they've all made my grad school journey much more enjoyable.

Lastly, I would like to thank my family - my dad for being a role model as a passionate scientist, my mom for her unconditional love, my brother for encouraging me to pursue higher education, my in-laws for their prayers and kind words of encouragement, and my husband, Dinesh, for being a pillar of support, walking with me side-by-side through ups and downs of life.

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ABBREVIATIONS

ANOVA	Analysis of Variance
C/F	Casein to Fat
Ca	Calcium
CCP	Colloidal calcium phosphate
CF	Correction Factor
CFR	Code of Federal Regulations
CLSM	Confocal laser scanning microscopy
CN	Casein
DA	Direct acid
dB/dpH	Buffering index
DF	Diafiltration
EDTA	Ethylene diamine tetra acetic acid
FDM	Fat-in-dry matter
G′	Storage modulus
G″	Loss modulus
GDL	Glucono-delta-lactone
HC	High concentration
HCl	Hydrochloric acid
HTST	High temperature short time
ICP-OES	Inductively coupled plasma- optical emission spectroscopy

LC	Low concentration
LMPS	Low-moisture part-skim
LT	Loss tangent
LT_{max}	Maximum loss tangent
MC	Medium concentration
MFGM	Milk fat globule membrane
NaOH	Sodium hydroxide
NFDM	Non-fat dry-milk
NPN	Non-protein nitrogen
P/F	Protein to Fat
PA	Pre-acidification
QDA	Quantitative Descriptive Analysis
RCBD	Randomized Complete Block Design
SC	Starter culture
UF	Ultrafiltration
WD	Whey drainage
WSC	Water soluble calcium
γ	Strain
δ	Phase angle
δ_{max}	Maximum phase angle
τ	Stress
F	Force

Chapter 1. Literature Review

1.1 Milk

Milk is defined as the secretion of the mammary glands of mammals, its primary biological function being nutrition of the young. Milk is a very variable biological fluid and is affected by genetic factors, including breed and individuality of the animal, stage of lactation, illness of the cow including mastitis and other diseases, feed, age, interval between milking, etc. (Walstra et al., 2006; Fox and McSweeney, 1998). Seasonality also affects the total solids in milk and has significant effects on individual milk components (Li et al., 2019). The approximate composition of bovine milk is shown in Table 1.1.

1.2 Milk Components

1.2.1 Milk Lipids

Milk is an oil-in-water emulsion where the fat in milk exists as globules dispersed in the aqueous phase of milk. These globules range in size from 2 to 6 μm and are surrounded by a surface layer called the milk fat globule membrane (MFGM) that is made up of polar lipids called phospholipids, proteins, and enzymes, allowing them to remain suspended in milk. The core of the fat globules mainly consists of triacylglycerol with minor amounts of diacylglycerols, monoacylglycerols, free fatty acids, polar lipids and sterols, and trace amounts of fat-soluble vitamins and β-carotene (Lucey et al., 2017). Milk fat is important in cheese making because it has a direct influence on the eating qualities of the cheese, it is directly related to the yield of cheese, and the characteristic flavor of cheese is strongly determined by the fatty acids released by the

hydrolytic action of lipase enzyme on milk fat. Other than flavor, milk fat also imparts mellowness and smoothness to the body of the cheese (Van Slyke and Price, 1949).

1.2.2 Milk Proteins

Milk proteins can be fractionated into two well defined groups: (1) the proteins called casein that precipitate at pH 4.6, its isoelectric pH; (2) the proteins called serum proteins or whey proteins that remain soluble at pH 4.6. Caseins constitute about 80% and the whey proteins constitute the remaining 20% of the total proteins in bovine milk. During cheese manufacture, the caseins undergo a slight alteration in their structure due to the specific action of rennet in the presence of calcium ions. This causes the caseins to coagulate and form a gel. Whey proteins do not undergo any significant alteration during normal cheese making conditions and are mostly lost in the whey stream during cheese manufacture (Fox and McSweeney, 1998). In addition to the caseins and whey proteins, milk contains two other proteinaceous materials: proteose peptone and non-protein nitrogen (NPN) (Fox and McSweeney, 2003).

1.2.1.1 Casein

Casein is classically defined as the protein precipitating from milk near pH 4.6, the isoelectric pH. Casein is not a globular protein, but a mixture of several components present as large aggregates called casein micelles.

Types of casein and primary structures

Caseins can be distinguished into α_{S1}- , α_{S2}-, β-, and κ-casein, but each of these occurs as a number of variants, most notable ones are the varying levels of phosphorylation of α_{S2}-casein, glycosylation of κ-casein and the proteolytic action of plasmin on β-casein, yielding γ-casein and

proteose-peptone. An experiment by Waugh and von Hippel (1956) to fractionate individual caseins from whole casein proved meaningful when they were able to classify caseins into calcium-sensitive and calcium-insensitive fractions. It was found that the calcium-insensitive fraction forms protein-calcium complexes with the calcium sensitive fraction (i.e., form a micelle) at Ca^{2+} concentrations comparable with those in milk. The calcium sensitive fraction is composed of α_{S1}-, α_{S2}-, and β-casein whereas the calcium insensitive fraction contains mostly κ-casein, which is known to be the protective colloid to stabilize the casein micelle (Fox and McSweeney, 2003).

Casein was initially considered to be a homogenous protein until heterogeneity was first demonstrated by Liderstrom-Lang and co-workers in 1920s. Caseins were originally defined as phosphoproteins. With the resolution of the primary structure, it became possible to classify them according to their chemical structure rather than on the basis of an operational definition or solubility (Whitney, 1988). The primary structure of all the caseins is not uniformly distributed but occurs in clusters, giving hydrophobic and hydrophilic regions as shown in Figure 1.1. The organic phosphates, which are attached to serine, occurs in clusters due to the mechanism by which phosphorylation occurs and the phosphate clusters bind calcium strongly. α_{S1}- Casein has a high net negative charge and a high phosphate content. α_{S2}-Casein is the most hydrophilic of the caseins and it has two cysteine residues forming disulfide bridges. β-Casein is the most hydrophobic of the caseins and it contains a large number of proline residues. β-Casein is somewhat like a soap molecule with a polar head and long chain apolar tail. In milk, some β-casein goes into solution at low temperature, thereby increasing the viscosity of the milk. κ-Casein has a C-terminal region that is strongly hydrophilic due to a high content of sugars, a few apolar residues and no aromatic residues, while the N-terminus is strongly hydrophobic. This detergent-like structure is probably important in micelle stabilization. κ-Casein has two cysteine residues that form intermolecular

disulfide bonds. The hydrophilic segment of κ-casein, i.e., the peptide bond between residues 105 and 106 is rapidly hydrolyzed and cleaved off by rennet during cheesemaking, rendering the residual caseins coagulable by Ca^{2+} and forming a gel (Fox and McSweeney, 1998; Walstra et al., 2006).

Structure of casein micelle

In milk, caseins exists as large colloidal particles known as micelles. On a dry matter basis, casein micelles contain about 94% protein and 6% low molecular weight species referred to as colloidal calcium phosphate (CCP), consisting of calcium, magnesium, phosphate, and citrate. The micelles are highly hydrated, binding > 2.0 g water/g protein. The micelles are generally spherical in shape with diameters ranging from 50 to 500 nm. Since the micelles are colloidal in nature, they are capable of scattering light, and the white color of the milk is largely due to light scattering by the micelles (Fox and McSweeney, 1998). For many years, various models for casein micelle structure have been proposed and refined, the most notable ones being sub-micellar, nanocluster, and dual binding models.

Sub-micellar model suggests that the casein micelles of bovine milk are composed of many variable sized cores (units) of insoluble salts of α_{S-} or β- caseins covered by a coat of κ-casein (Lucey et al., 2017). One view is that κ-casein deficient submicelles are located in the interior of the micelles with the κ-casein-rich submicelles concentrated at the surface, giving the micelle surface a κ-casein-rich layer but with some α_{S1-} , α_{S2-} and β-caseins also present at the surface. It was proposed that the hydrophilic C-terminal region of κ-casein protrudes from the surface forming a layer 5-10 nm thick and giving the micelles a hairy appearance as shown in the Figure 1.2a. This hairy layer is responsible for micelle stability through steric stabilization. If this

hairy layer is removed by specific hydrolysis, the colloidal stability of the micelles is destroyed and they coagulate or precipitate (Fox and McSweeney, 1998). Although this model explains principal features of the casein micelle structure, it fails to clearly describe the mechanism of casein micelle formation.

Nanocluster model was initially proposed by Holt (1992) who depicted the casein micelle as a tangled web of flexible casein molecules forming a gel-like structure (Figure 1.2b) in which microgranules (later they became to be known as nanoclusters) of CCP are an integral feature (Fox and McSweeney, 1998). According to this model, the nanoclusters would drive micelle formation by randomly binding more phosphoproteins and the network growth would continue since the α_{S}-caseins were noted to possess more than one such phosphoserine cluster (Lucey and Horne, 2018). It was suggested by Holt (1998) that the nanoclusters have radii of 2.3 nm and are surrounded approximately by 50 phosphopeptide chains. It was later found that the distance between adjacent nanoclusters were 18 nm and that this distance is so large that the bridging by individual proteins is unlikely (Dalgleish and Corredig, 2012). Later revisions of the nanocluster model suggested that the association of casein was driven by a collection of hydrophobic interactions, hydrogen bonding, ion bonding, and electrostatic Van der Waals attraction (Lucey and Horne, 2018). This model also fails to provide any substantive role for κ-casein and therefore can provide no indication of how this molecule controls micelle size or achieves a location where it can control micelle stability (Horne, 2006). Later this group disputed the nature of the hydrophobic interactions, but the key features remained the same.

The dual-binding model introduced by Horne (1998) viewed caseins as block copolymers with segments alternating between charge and hydrophobicity (Figure 1.3). Electrostatic

interactions involving calcium binding and the formation of nanoclusters were suggested to operate cooperatively with a second-binding interaction that was hydrophobic in nature. The κ-casein can associate with the other caseins via its hydrophobic N-terminal segment and since κ-casein does not contain a phosphoserine cluster and its C-terminal is hydrophilic, further growth (extension) of the casein chain is prevented. Horne thus proposed that κ-casein acts as a polymerization chain terminator, and this mechanism explained how it acquires the surface location on casein micelles (Lucey and Horne, 2018).

1.2.3 Salts

Milk contains salts, both organic and inorganic, that together represent about 0.9% of the milk, whereas the ash content of milk, the portion which remains after heating and combustion of milk approximates 0.7%. This is because the ashing of milk causes loss of organic acids including citrate and acetate (Walstra et al., 2006; Fox and McSweeney, 1998). Some of the prominent milk salts include calcium, magnesium, sodium, potassium, phosphorus, citrate, chloride, etc. Milk salts exist in soluble and insoluble (colloidal) forms. Certain milk salts, such as, chloride, sodium and potassium are sufficiently soluble to be present entirely in the dissolved phase whereas other salts, such as, calcium phosphate exists more as insoluble form because their concentrations are higher than can be maintained in solution (solubility) at normal pH of milk (Fox and McSweeney, 1988). The distribution of salts between the soluble and colloidal phase in milk is given in Table 1.2. The milk salts have important impact on the properties of milk, including the formation and stability of the casein micelles, acid/base buffering, and various colligative properties, as well as its key biological role that is to provide nutrition for the newborn. These salts also play a powerful influence on protein stability during processing including rennet coagulation, heat and alcohol

stability, the texture of various types of milk protein gels, cheese texture and functionality, and emulsion stability (Lucey and Horne, 2009).

1.2.3.1 Colloidal calcium phosphate

The undissolved salt present in, or on the casein micelles, i.e., the colloidal particles, are called CCP. The total amount of CCP is approximately 7g/100g of dry casein. CCP also includes other components, such as, K, Na, Mg, and citrate in trace amounts as shown in Table 1.2. (Walstra et al., 2006). These other ions are associated with caseins as counter-ions to the negatively charged organic phosphate and carboxylic acid groups of the protein. It has been calculated that even some of the calcium ions (approximately 30%) is directly attached to these protein groups (Fox and McSweeney, 1998). Calcium and phosphates in milk exist in dynamic equilibrium between solution and CCP, and between solution and proteins, with no true equilibrium, but some type of pseudo-equilibrium exists that is influenced by several factors including presence of caseins, pH, temperature, concentration, etc. (Lucey and Horne, 2009; Walstra et al., 2006). Some of these important effects on CCP is given below.

- ***Temperature:*** The solubility of CCP decreases at high temperatures and during heating heat-induced CCP is formed, which (mostly) re-solubilizes when milk is subsequently cooled. The generic reaction is as shown below. Thus, an increase in temperature would decrease the pH of milk due to the release of H^+ ions.

$$3Ca^{2+} + 2HPO_4^{2-} \rightarrow Ca_3(PO_4)_2 \text{ (precipitate)} + 2H^+$$

- ***pH:*** A decrease in pH solubilizes CCP. The extent of solubilization increases markedly below pH 5.6 and is complete at approximately pH 5.0. The approximate changes in CCP with pH drop are shown in Figure 1.4.

- ***Concentration:*** Concentrating milk by evaporation of water decreases the pH. The pH drops by 0.3 unit for 2:1 concentration and by about 0.5 units for a 3:1 concentration. This pH decrease is due to the formation of additional CCP, releasing H^+ ions, which causes a decrease in pH. Membrane filtration of milk using ultrafiltration or microfiltration results in retentates where CCP is a greater proportion of the total Ca content as some soluble Ca is lost in the permeate during processing. Extensive dialysis during milk protein concentrate production partially disrupts the casein micelle due to loss of some CCP as a result of soluble Ca removal during membrane filtration.

- ***Ca sequestrants or chelating agents:*** Sequestrants, such as, citrates and phosphates disrupt the casein micelles in milk by reducing the Ca^{2+} concentration/activity and the CCP content by forming soluble metal complexes with them. Figure 1.5 shows the complexing abilities of certain sequestrants. Complexing agents that are lower on the scale (e.g., EDTA) are strong calcium chelators.

1.2.4 Lactose

Lactose is the major carbohydrate in bovine milk. It is a disaccharide composed of D-glucose and D-galactose linked together through a β-1, 4-glycosidic linkage (Walstra et al., 2006). Lactose is an important constituent component when starter cultures are used during cheese manufacture. The starter cultures convert lactose to lactic acid and this lactic acid solubilizes some

of the CCP in milk that is essential to the texture of cheese (Van Slyke and Price, 1949). During whey drain step, most of the lactose is lost in the whey and hence lactose is a major component of dried whey products (Fox and McSweeney, 1998).

1.2.5 Enzymes

Enzymes in milk can be derived from various sources: leakage from blood (e.g., plasmin), leukocytes (e.g., catalase), or the MFGM (e.g., xanthine oxidase). Other enzymes in milk can be dispersed in the serum phase of milk or be associated with casein micelles (e.g., lipoprotein lipase) (Walstra et al., 2006; Lucey et al., 2017). Significant interest in milk enzymes is due to their role in causing product deterioration (e.g., lipase), as indicators of thermal processing (e.g., alkaline phosphatase), as indices of mastitic infection (e.g., catalase), or as a source of antimicrobial activity (e.g., lysozyme) (Lucey et al., 2017). In cheese, the enzyme called plasmin, an alkaline proteinase is of significant interest because it can contribute to proteolysis for certain cheese varieties. Most of the plasmin in milk is present as an inactive plasminogen and it can be activated with heat treatments, such as, pasteurization. Milk also contains an acid proteinase, e.g., cathepsin D. Cathepsin D is associated with casein micelles, has less heat resistant than plasmin and can cause some proteolysis in some types of cheese (Walstra et al., 2006).

1.2.6 Miscellaneous

Some of the miscellaneous components present in milk include organic acids (e.g., citric, lactic, pyruvic), non-protein nitrogen, vitamins (e.g., Vitamin A and most B vitamins), ribonucleic

acids, sulfuric acid esters, carbonyl compounds, gases, hormones, and somatic cells (Walstra et al., 2006).

1.3 Low-Moisture Part-Skim Mozzarella

Mozzarella is one of the several pasta filata, or stretched curd, cheeses that originated in Italy. The name pasta-filata refers to a unique plasticizing and texturing treatment of the fresh curd in hot water that imparts to the finished cheese its characteristic fibrous structure and melting properties (Kindstedt, 1993). Although Mozzarella originated in Italy, it is consumed on a global scale, largely due to the extraordinary growth of pizza. This sharp rise in production has been accompanied by large increases in plant production capacities, with many cheese plants routinely producing 100 tons or more of Mozzarella cheese per day (Kindstedt et al., 2010). In 2020, about 4.45 billion pounds of Mozzarella was produced in the United States (USDA, 2020). Cheesemaking on this scale requires precise control over all aspects of the manufacturing process. This created a pressing need for a better understanding of the scientific and technological basis of Mozzarella cheese manufacture (Kindstedt et al., 2010).

In the US, Mozzarella cheese is divided into four separate categories defined by the standards of identity on the basis of moisture content and fat-in-dry matter (FDM). Mozzarella and part-skim mozzarella are high in moisture (>52 but ≤ 60%) and resemble somewhat traditional Italian Mozzarella. They are used rarely by the food service industry as a pizza ingredient due to their poor shredding and matting properties and limited shelf-life. Low-moisture (LM) and low-moisture part-skim (LMPS) Mozzarella have a lower water moisture (typical 47 to 48%) and are used primarily as ingredients for pizza. In the US, the term ‘pizza cheese’ was used to designate low-moisture Mozzarella until 1964 when the current standards of identity were adopted

(Kindstedt, 1993), but now pizza cheese may reflect Mozzarella that does not meet the standards of identity for Mozzarella.

1.3.1 The Cheesemaking Process

Traditional manufacture of LMPS Mozzarella is somewhat similar to that of Cheddar as far as the milling stage, with some notable exceptions. Figure 1.6 shows the various steps involved in the manufacture of LMPS Mozzarella (Kindstedt, 1993). A major difference in the production of curd for LMPS Mozzarella is that the acidification by the starter occurs far more rapidly in most procedures, due to the use of thermophilic cultures. Stretching takes place after curd has developed an optimum level of acidity.

Pasteurization and standardization of cheese milk. Cheese milk is standardized to a specific protein to fat (P/F) or casein to milk fat (C/F) ratio to achieve satisfactory fat in dry matter (FDM) content in cheese. For example, standardization of the cheese milk to 2.5% fat will result in a cheese with FDM of about 41%, that is, at a high end of the composition range for LMPS, whereas standardization to 1.5% fat will result in cheese with FDM near 30%, the minimum for LMPS (Kindstedt and Fox, 1993). Mozzarella cheese milk then undergoes pasteurization, designed to destroy pathogens (Gernigon et al., 2010) and non-starter lactic acid bacteria.

Addition of starter cultures. Addition of starter cultures is done once the cheese milk is warmed in the vat to a temperature of around 32-35ºC to allow rapid growth of starters. The main purpose of adding starter culture is to achieve sufficient acidification of the milk by microorganisms in order to reach optimal pH and calcium content at the stretching step. Starter cultures ferment the lactose in milk to lactic acid dissolving some of the insoluble calcium phosphate associated with caseins (Kindstedt and Fox, 1993). The added cultures help control the development of non-starter

flora, inhibit pathogens, improve shelf-life, contribute to flavor development, proteolysis, and cheese ripening (Hong et al., 1998).

The commonly used starters for Mozzarella cheesemaking are thermophilic bacteria, such as, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* or sometimes *Lactobacillus helveticus*, the latter being used mainly for its specific ability to ferment galactose. Galactose accumulation in the cheese leads to excessive browning of the cheese during baking of the pizza due to the Maillard reaction (Gernigon et al., 2010). A mesophilic starter blend, such as, *Lactococcus lactis* ssp. *lactis* or *cremoris* may be used as well, but it has a blander flavor than the typical acetaldehyde flavor obtained when using thermophilic starter cultures (Kindstedt, 1993; Kindstedt et al., 2010). Acid production during Mozzarella manufacture depends on a non-obligatory symbiosis between *S. thermophilus* and *L. bulgaricus. S. thermophilus* is weakly proteolytic and cannot alone produce sufficient free amino acids and small peptides from casein to sustain optimum growth and acid production in milk. In contrast, *L. bulgaricus* is much more proteolytic and stimulates the growth of *S. thermophilus* through production of peptides and free amino acids from the caseins in milk. During cheese making, acid production in the early stages of manufacture (before whey drainage) is dominated by *S. thermophilus.* As lactic acid accumulates and the pH declines, *S. thermophilus* is partially inhibited and *L. bulgaricus* becomes the dominant acid producer towards the end of manufacture (Kindstedt and Fox, 1993).

Two important features that define the acidification schedule are: (a) the overall rate of acid production and (b) the amount of acid produced before whey drainage versus after whey drainage. The overall rate of acid production is important because it determines the total manufacturing time, which in turn influences the amount of syneresis during manufacture and

therefore the moisture content of the final cheese (Barbano et al., 1994). A shorter make time, achieved by faster acid production, generally results in cheese with higher moisture content (less cooking and stirring times). The amount of acid produced before, versus after, whey drainage is essentially defined by the pH at rennet addition and at draining. More than any other parameters, the pH values at rennet addition and at draining influence the calcium/protein ratio in the final cheese because most of the calcium losses to the whey occur by the time of (main) whey draining (Lucey and Fox, 1993; Kindstedt et al., 2010). For example, the combination of a very rapid rate of acidification (short make time) and low draining pH results in Mozzarella cheese with high moisture content and low calcium/protein ratio. Higher moisture contents and lower calcium/protein ratios both favor a softer unmelted texture, a less fibrous and less chewy melted consistency. Consequently, such a softer cheese will require only limited ageing to develop optimum functionality (Yun et al., 1993b, 1995; Kindstedt et al., 2010). Rapid acidification can be achieved by using very active starters, such as, one propagated under conditions of external pH control, or a large rate of inoculation, or by pre-acidifying the milk before renneting (Guinee et al., 2002). At the other extreme, the combination of a slow rate of acidification (long make time) and higher draining pH results in a low moisture and high calcium/protein ratio. This type of cheese is firm and melts to a tough, chewy consistency that requires extended ageing for optimum functionality (Kindstedt et al., 2010).

Addition of coagulant. The manufacture of all renneted cheese varieties involves this essential characteristic step. Coagulant addition aids in coagulation of the casein in milk to form a gel that entraps the fat, if present. Coagulation may be achieved by the following three ways: (1) limited proteolysis by selected proteinases (rennet), (2) acidification to pH ~4.6, and (3) acidification to pH values >4.6 (perhaps 5.2) in combination with heating (Fox, 1993). The majority of cheeses

are produced by enzymatic (rennet) coagulation. Chymosin from the stomachs of young animals (calves, kids, lambs, buffaloes) was used traditionally as rennet but limited supplies of this type of rennet with increased cheese production worldwide led to a shortage of calf rennet, and consequently to the creation of rennet substitutes (Fox, 1993). A range of coagulants are commercially used for cheese making, including calf rennet, fermentation-produced (recombinant) chymosin, and fungal proteases from *Rhizomucor pusillus* and *Cryphonectria parasitica* (Kindstedt and Fox, 1993).

The rennet coagulation of milk can be divided into three stages: (1) Primary (enzymatic hydrolysis) where κ-casein is cleaved by rennet, or rennet substitutes, at Phe_{105}-Met_{106} bond to yield two peptides, one the macropeptide moiety (residues 106 to 169) that is hydrophilic, soluble and diffuses away from the micelle after hydrolysis, the other is the para-κ-casein that is strongly hydrophobic and remains attached to the micelle; (2) secondary stage involves aggregation of rennet-altered micelles. The progressive hydrolysis of κ-casein during the primary stages alters the properties of the micelles, reducing its surface charge, thereby reducing steric repulsion between micelles and facilitating aggregation and gel formation; (3) tertiary stage of rennet coagulation involves syneresis of the gel with the expulsion of water and structural rearrangement of the gel network (Lucey and Fox, 1993).

The residual coagulant left in the cheese (much is lost at whey drainage) can cause slow, ongoing breakdown of the casein network. The activity and specificity of the particular coagulant used in cheesemaking, as well as its thermal stability and extent of heat inactivation during stretching, have important impacts on proteolysis, functional characteristics, and ageing behavior (Kindstedt et al., 2010). Different coagulants show different proteolytic profile as shown in Figure

1.7 (Kindstedt and Fox, 1993). The amount of coagulant used also influences the meltability of the cheese over time (Dave et al., 2003). In Mozzarella, the high temperature used during the stretching process can inactivate, or greatly reduce, the residual coagulant activity, the extent to which this happens is dependent upon the temperature used in the cooker/stretcher during cheese manufacture (Lucey et al., 2003). Typically, a less proteolytic coagulant is often used for LMPS Mozzarella manufacture to reduce the rate of protein breakdown during storage thereby increasing the shelf-life and functional properties of the cheese.

Cutting and cooking. Shortly after rennet gelation, the coagulum is cut to initiate syneresis. The choice of knives for cutting is very important. For a cheese like LMPS Mozzarella, a sharp knife with smaller gap (3/4″ knives) between the blades is preferred to aid in higher syneresis. The cut coagulum is allowed to heal for 5 minutes before cooking begins. The cooking temperature range is between 40-44ºC for LMPS Mozzarella. Cooking process provides a suitable temperature for growth of the starters and increases moisture expulsion from the curd (Kindstedt and Fox, 1993).

Draining. The curds and whey are essentially separated during this step. The whey is allowed to drain gradually over a period of ~15 minutes in order to prevent excessive curd losses or slowly pumped out from a large vat. The impact of the pH value at whey drainage and its effect on calcium losses during manufacture is discussed later in this chapter.

Matting. In small-scale cheesemaking**,** the curds after whey drainage are allowed to fuse before cutting into blocks and stacked one above the other in a process traditionally called cheddaring, in order to further remove or expel moisture from the curd. In large operations the curd can be either allowed to mat on belts and then milled (milled-curd) or instead is constantly stirred (stirred-curd) until a desired target pH is attained.

Milling and dry salting. The curds are milled after the desired pH is reached (pH ~5.2) and sometimes dry salted twice (5 minutes apart) for better salt absorption when a combination of dry salting and brining is utilized during Mozzarella cheese manufacture.

Stretching and molding. Milled curd undergoes a hot water stretching step (typical of a pasta-filata style cheese) in a cooker/stretcher. Stretching involves two stages: During the first stage, curd enters mixer and is quickly warmed by the hot water to a temperature range of at least 50-55ºC, which is necessary to transform the curd into a plastic and workable consistency. The temperature of the mixer water may vary widely, ranging approximately 55-85ºC, depending on the design of the equipment and the operating conditions (e.g., auger speed). In the second stage, the plastic curd is worked by the auger(s) or series of augers into a unidirectional fibrous ribbon of plastic curd. The hot plastic curd then exits the mixer and is transported by an auger to the molding machine, where it is forced under pressure into a mold which gives the cheese its final shape. The molder also serves as a pre-cooling function (due to contact with chilled water), so that the block will retain its shape when removed from the mold. Substantial moisture, protein and fat losses may occur during stretching and molding if the operating conditions in the mixer and molder are not properly controlled (Kindstedt et al., 2010).

The stretching process transforms the three-dimensional protein matrix of the cheese curd into a network of parallel-aligned protein fibers, which is important in relation to the stretching and melting properties of Mozzarella. Serum and fat droplets accumulate in the open channels that separate the bundles of protein fibers, resulting in the partial alignment of the fat and serum phases of the cheese (Kindstedt et al., 2010). Stretching temperature has implications for coagulant survival and proteolysis during aging. It also aids in the reduction of microbial starter numbers.

Plasmin, a naturally occurring protease in milk, contributes to proteolytic activity in Mozzarella during aging. Plasmin occurs primarily as plasminogen in milk, but it is activated and readily converted into plasmin during the stretching process due to the high temperature. This possibly leads to elevated plasmin levels in Mozzarella cheese (Kindstedt and Fox, 1993).

Brining/ salting. Salt is incorporated into Mozzarella cheese either by brining or by combination of direct salting and brining. Brining aids in cooling the cheese block as well as salting. In modern plants, the cheese blocks coming out of the cooker/stretcher are typically dipped in cold water for a certain period of time to reduce the curd temperature before brining thereby reducing the length of brining time required (Kindstedt and Fox, 1993). Commercial plants often utilize a combination of dry salting and brining because using only brining as a salting step produces Mozzarella cheese with an initial heterogenous chemical composition. Differences in block size and shape cause different patterns of non-homogeneity. Open brine systems can also be a source of microbial contamination of cheese by yeast, mold, and bacteria (Barbano et al., 1994). For Mozzarella cheese, the brine should be maintained at near saturation (i.e., approximately 26 g/100 mL) to maximize the rate of salt absorption while minimizing microbial growth.

Brining causes moisture loss from cheese. Hence, care has to be taken in minimizing moisture loss by maintaining constant low brine temperature combined with continuous circulation to prevent localized temperature gradients in the brine surrounding the cheese surface. Newly prepared brine that is used for the first time can detrimentally affect the quality of the Mozzarella cheese, unless the brine pH and calcium content are adjusted to prevent changes in calcium distribution and loss of calcium from the cheese during brining. This can be accomplished by acidifying the fresh brine with food grade lactic or acetic acid to the approximate pH of the cheese

and by increasing the calcium content of the brine (~0.06 g/100 g) through the addition of food grade calcium chloride (Kindstedt et al., 2010). Historically, whey was added to brine as a source of calcium, but whey could contaminate the brine with microorganisms like mold or phage.

Storage. After brining, the cheese is vacuum sealed and stored in refrigerated conditions, typically below 4ºC. These conditions allow for slower aging and increased shelf-life of cheese. Some modern technologies, such as, super-chilling (below 4ºC but above freezing) or high-pressure processing are done to extend the performance shelf-life of LMPS Mozzarella cheese in order to meet the export demands of a growing pizza market, such as, in Southeast Asia.

1.3.1.1 Optional Cheesemaking Variables

Direct acidification. This process involves directly acidifying the cold milk (approx. 4ºC) before renneting using food grade organic acids, such as, citric, acetic, lactic acid or by adding glucono-δ-lactone until a pH close to 5.6 is reached (Kindstedt, 1993; Gernigon et al., 2010). One of the key purposes of acidifying the milk prior to cheesemaking is to reduce the final calcium content of the cheese. The other advantages of PA milk includes reduced amount of rennet required for gel formation (which saves cost) and also extends the shelf- life of cheese due to lowered proteolysis. During direct acidification (DA), some of the CCP solubilizes, and some caseins partially dissociate from the micelles. The amount of proteins dissociated from the casein micelles is temperature dependent with lower levels of protein released at higher temperatures. The maximum protein dissociation happens at pH values of ~5.5 (Smith et al., 2018). The type of acid used for acidification also influences the release of calcium and this is discussed in the later part of the chapter.

The advantages of using DA includes reduced processing time, lower costs, and provides another method of standardizing the characteristics of cheese. The other important factor noted is that the Mozzarella cheese can be suitable for use on pizzas shortly after manufacture (Smith et al., 2018). This is because the right amount of calcium balance (soluble/insoluble) can be immediately attained in the Mozzarella cheese using this process whereas the traditional manufacture takes 2-3 weeks of refrigerated aging to reach the right amount of insoluble calcium before it can be best used for pizza applications.

Use of concentrated milks. In recent times, ultrafiltration (UF) has become a common practice in the dairy industry. The milk to be used for cheese making is ultrafiltered (pore size of approx. 10 kDa) using a membrane filtration process and the retentate from this process is used for standardizing the protein content of cheese milk. During the membrane filtration process, the proteins in the milk are concentrated while the low molecular components, such as, salts, water, and lactose pass through the membrane (Smith et al., 2018).

The advantages of using UF concentrated milk in cheesemaking includes increased cheese yield by 10-30%, reduced volume of milk to handle, decreased whey production volume and increased consistency in the final product because protein levels are standardized (Cheryan, 1998). UF can also be used to improve manufacturing efficiency by enhancing the casein content of milk to optimize gel formation, improve the recovery of casein and fat during cheesemaking, and to maximize plant throughput (McSweeney, 2007).

The use of UF for cheesemaking is classified into three categories: low-concentration (LC), medium-concentration (MC) and high concentration (HC) factor UF, depending on the extent of concentration. LC and MC factors are used typically in Mozzarella cheese manufacture. In LC UF,

the milk is concentrated approximately less than 2-fold and conventional cheese manufacturing techniques can be applied to manufacture cheese. The advantages of cheese-making using low-concentration factor retentates are uniformity in milk composition, production of firm coagulum, which encourages lower losses of casein in whey, increased cheese yield, improved cheesemaking efficiency in terms of higher throughput per vat, and no requirement for new cheesemaking equipment with the exception of the UF unit. In MC, a UF concentration factor of 2-6-fold is used to achieve the final solids content of the cheese. The use of MC UF has been limited for the production of LMPS Mozzarella owing to problems with flavor, texture, and functionality. Mozzarella manufactured by MC UF and only using starter cultures has poor melting properties. These problems are partly associated with changes in the buffering capacity of milk after concentration by UF, which impacts critical cheesemaking parameters. UF of milk at its normal pH of 6.7 results in an increase in buffering capacity of the retentate. The increase in buffering capacity results from the concentration of CCP, which is bound to casein micelles and is concentrated to the same extent as the caseins. Critical factors influencing cheese quality, flavor, and texture development are altered owing to the enhanced buffering capacity. These include rate and extent of acidification by the lactic acid bacteria, the rennet coagulation time, the rheological properties of the curd, the activity of ripening enzymes and the water holding capacity of the cheese. In order to avoid undesirable effects on cheese quality due to the increased buffering capacity, the mineral content of the UF retentate, or the UF standardized milk, must be lowered. In UF retentate, the mineral content, i.e., CCP can be lowered by reducing the pH of the milk prior to, or during UF so that soluble minerals pass into the permeate. During cheesemaking, the CCP in the MC UF standardized milk can be lowered by pre-acidifying it using organic acids before starter culture and rennet addition (McSweeney, 2007). Pre-acidification aids in reducing the CCP

content of the final cheeses thereby providing the desirable functional properties in Mozzarella cheese (Kindstedt and Fox, 1993).

1.3.2 Importance of Calcium Solubilization during the Cheesemaking Process

The solubilization of calcium during cheesemaking process serves two main purposes: (1) Stretching or curd plasticization during manufacture and (2) imparting good functional characteristics to cheese, such as, melt and stretch on food applications such as pizza. For both of these to be accomplished, the cheese should have the right amount of insoluble calcium (Lucey and Fox, 1993). The mineral content of cheese, i.e., the insoluble calcium phosphate, is mainly dependent on the acidity developed before the whey is drained off. This loss of CCP determines the extent to which the casein particles that were originally present in milk are disrupted and thus determines in large part the basic structure of the cheese (Lawrence et al., 1984). Many of the undesirable body or texture attributes seen in cheeses (e.g., curdy texture) can be traced back to inadequate pH development prior to rennet addition or excessive proteolysis (e.g., soft) due to prolonged aging. Cheese plants routinely monitor the acid development in their cheese making process, paying close attention to the finish pH of their cheese at the end of manufacture. The final pH of a cheese at the end of manufacture does not always provide critical information about the acid development journey (history), and this history has a major impact on the textural and performance attributes of the finished cheese (Johnson and Sommer, 2013). Young cheese varieties with the same final pH values do not always have the same body characteristics. This is because pH is a tool that is used to indirectly estimate the likely loss of calcium from casein particles, and pH loses its meaning when the cheesemaker does not consider milk composition,

concentration, and the rate and extent of acid development (as indicated by pH history) during both the cheesemaking process and the cheese during ripening (Johnson and Lucey, 2006).

1.3.2.1 Key Factors Affecting Curd Demineralization during Manufacture

Three principal factors affect curd demineralization during manufacture: (1) pre-acidification of cheese milk, (2) the pH value of whey (or curd) during drainage, and (3) the cooking temperature (Lucey and Fox, 1993).

Pre-acidification of cheese milk

Directly acidifying the cheese milk converts some of the insoluble calcium into soluble calcium, which can be removed from the curd particles during whey drainage (Lucey and Fox, 1993). The acid development prior to renneting, including the rate and extent of acid development is important during cheese manufacture. After cutting, the serum containing soluble calcium is readily expressed from the curds during syneresis. Acidification, after the majority of the serum has been expelled from the curd particles, results in an accumulation of solubilized calcium within the serum of the curd since there is less (amount of) serum loss from the curds post whey drainage. Depending on the moisture content of the curds, the calcium equilibrium might change, i.e., if the curds have lower moisture content after whey drainage, the solubilization of CCP slows down and a pseudo-equilibrium is more quickly established. This pseudo-equilibrium is highly influenced by pH, i.e., a curd cut at pH 6.1 contains less insoluble calcium than a curd cut at a pH of 6.5, even though a pH of 5.2 may eventually be attained in both types of curds (Johnson and Lucey, 2006).

Mozzarella curd will not readily stretch in hot water until sufficient CCP is solubilized from the curd through acidification. Calcium is more readily solubilized during acidification in

cheese milk (early stages) than in curds (later stages). The cheeses acidified using starter cultures and DA are ready (suitable) to stretch at different pH values, i.e., between 5.15-5.35 and 5.6-5.7, respectively. This is because the right amount of calcium balance is achieved much more easily in DA Mozzarella (typically acidified using acetic or lactic acid) due to the calcium being solubilized readily from the protein matrix in cheese milk prior to renneting. Since the calcium is more easily removed from the matrix, DA cheese will stretch well at a much higher, say pH 5.6, than in starter culture acidified Mozzarella, where the majority of the acid is produced in the curds after whey drainage. Therefore, more calcium is retained in the protein matrix and the cheesemaker needs to decrease the curd pH to a much lower level (pH 5.15-5.35) to try to achieve the necessary calcium solubilization to allow the curd to be successfully stretched (Johnson and Sommer, 2013). Guinee et al. (2002) investigated the effects of calcium content and pH value, and their interaction, on the texture and heat-induced functionality of LMPS Mozzarella cheese. It can be observed in their study that the cheese acidified using starter culture (SC) and DA using lactic acid were milled and stretched at pH 5.14 and pH 5.63, respectively. The levels of total calcium and pH values in SC and DA cheeses at day 1 were 27.7 mg/g protein (pH 5.42) and 21.8 mg/g protein (pH 5.96), respectively. It was observed (Guinee et al., 2002) that the total calcium in DA cheese was much lower than SC cheese even though the pH value was significantly higher in DA cheese, this was because more of the calcium was readily solubilized in DA cheese before renneting, compared to SC cheese where most of the calcium was solubilized after whey drainage. It is also important to note that the protein content in the DA cheese was significantly lower than the SC cheese, which can also lead to a slightly higher level of total calcium per g of protein. It is not clear whether the faster rate of solubilization of CCP in milk (or the initial curd particles) is due to physical inhibition (shielding) of the removal of calcium from the curd particles by the formation of an outside curd

membrane or if the CCP solubility is decreased when the moisture content of the curd or cheese decreases. The latter explanation may be the most likely cause, but this area needs to be investigated (Lucey et al., 2003).

The type of acid used during pre-acidification and the pH at curd formation also affects the amount of calcium dissolved from the caseins and thereby the amount of moisture retained in the cheese, its texture, and functionality. Keller et al. (1974) studied the effects of the type of acid (i.e., phosphoric acid, acetic, hydrochloric, malic, and citric acid) on calcium solubilization used during DA. As expected, the calcium retention in cheese acidified to pH 5.6 using malic, hydrochloric, acetic, and phosphoric acids was significantly higher than cheeses acidified to pH 5.2. Curds made from milk acidified to pH 5.6 yielded rubbery agglomerates that were relatively non-cohesive in whey. At pH 5.2, the curd was plastic and tacky. One of the interesting finding was that the cheese acidified to similar pH using citric acid, a calcium chelator, had significantly higher calcium losses (lower CCP associated with the caseins), and the cheese exhibited greater meltability than cheese made with a non-chelating acid.

pH of whey during drainage

Whey drainage is a critical step in cheese making because the calcium phosphate content of a cheese is largely determined at the point at which the curds and whey are separated, which in turn influences the basic structure of the cheese and its physical properties (Kindstedt and Fox, 1993). For Mozzarella, mineral retention may be very important because functional characteristics, such as, shredding, and melting are related to cheese texture.

The pH of whey is related to the pH of cheese curd, but the curd pH is usually slightly lower than the whey pH. The pH of whey is more commonly measured for routine quality control

because a representative sample of whey is easier to obtain than curd is, at this point in the cheese making process. If the draining is delayed because of the slow acid development, then the resulting cheese may be low in moisture due to increased stirring and cooking time (Yun et al., 1995).

Yun et al. (1995) studied the effects of the pH value of whey at draining on cheese composition, proteolysis, and functional properties of LMPS Mozzarella. They manufactured two vats of cheese with consistent composition, the only difference in the make process being that the whey was drained at pH 6.40 in one vat and at pH 6.15 in the other. They observed that the cheese drained at lower pH (pH 6.15) had lower total calcium as compared to the cheese drained at higher pH, i.e., 27.2 and 29.6 mg Ca/ g protein, respectively. Cheese moisture was also slightly higher for the cheese with lower draining pH (probably because of shorter manufacturing time). As the pH of curd and whey decreases prior to draining, calcium is transferred from the curd into whey. This transfer generally favors a softer cheese texture if all other manufacturing factors are kept consistent.

Variations in pH value at draining can also affect the retention of coagulant in the curd. Cheese undergoes proteolysis during refrigerated storage, which can affect the functional properties of cheese. If the retention of coagulant in the curd changes due to the variation in the draining pH, then proteolytic changes during storage could be affected, and the cheese functionality upon baking could also be affected. With a lower pH value at draining, increased amount of coagulant may be retained in the curd due to higher moisture retention and more association with the casein particles. All types of coagulants become more resistant to thermal inactivation at lower pH values. Therefore, a significantly firmer cheese texture in the case of

lower drain pH can be hard to achieve unless a less proteolytic coagulant is used during cheesemaking (Yun et al., 1995).

Cooking temperature

Cooking temperature provides a suitable environmental condition for the fermentation of lactose to lactic acid by the starter culture. In addition to that, cooking also plays a major role in syneresis and in determining the final moisture content of cheese, indirectly affecting curd demineralization (Kindstedt and Fox, 1993). The moisture loss and the rate of acidification during the cooking process is dependent on the set temperature in the vat and also the activity of the added starter cultures. For example, in general, lower cooking and matting temperatures results in less syneresis and higher moisture content in the final cheese and vice versa. For example, Yun et al. (1993a) reported that the moisture content of Mozzarella cheese increased by about 2% when cooking temperature was decreased from 44 to 38ºC. However, in this study (Yun et al., 1993a), acid production by the starter was slower at the lower temperature, thereby adding an additional 30 min to the total make time at a cooking temperature of 38ºC. Presumably, if the total make time had been held constant (e.g., by adding more starter culture to the cheese milk), the increase in cheese moisture content with decreasing cooking temperature would have been greater than 2% (Kindstedt et al., 2010). Moisture content in the cheese is an important factor because it strongly influences cheese rheological and proteolytic properties (Kindstedt and Fox, 1993).

1.3.3 Changes in Properties of Cheese during Ripening

Several biochemical events, primarily proteolysis, glycolysis, and lipolysis as well as the slow solubilization of some residual CCP occurs during cheese ripening. The shifts in both insoluble calcium, as well as ongoing proteolysis by residual coagulant and plasmin, alter cheese

functional performance as shown in Figure 1.8. It is important to note that although proteolysis had often been considered to be the key event responsible for textural changes in cheese during ripening, changes in insoluble calcium are mostly responsible for the textural shifts in young cheese (Lucey et al., 2003; Johnson and Lucey, 2006). In LMPS Mozzarella, proteolysis is very slow and may not have a significant effect on melt or stretch for several weeks of refrigerated storage.

1.3.3.1 Physicochemical properties

The physicochemical changes that take place during the first weeks after manufacture can be thought of as a gradual, partial reversal of the abrupt changes in physicochemical state to the proteins that occurred when curd the curd was plasticized and stretched. During stretching, the high curd temperatures strongly favor hydrophobic protein-to-protein interactions, which cause the para-casein matrix to aggregate and contract. This in turn triggers partial phase separation of protein and curd within the curd structure and contributes to poor water holding capacity in newly manufactured cheese. Typically, about 30% of the total moisture content of Mozzarella cheese can be expressed by centrifugation during the first few days after manufacture, but levels of expressible serum usually decrease to zero within two weeks of ageing at 4ºC due to increased water-binding capacity of the protein matrix.

During this brief initial period of aging, the composition of expressible serum changes considerably, with notable increases in calcium concentration and levels of intact casein. It appears that the intact (i.e., unhydrolyzed) caseins and mineral constituents (i.e., insoluble calcium) migrate into the serum phase as soluble species, presumably until an “equilibrium” is established (Guo and Kindstedt, 1995). Thus, a partial reversal of protein-to-protein (hydrophobic) and

protein-to-calcium interactions occur during ageing as both casein molecules and casein-associated calcium ions (i.e., insoluble calcium) dissociate from the para-casein fibers. This results in the weakening of para-casein fibers, thereby triggering the transformation to a softer and less elastic cheese that melts to a more flowable and stretchable consistency. The transformation of the cheese performance with age and insoluble calcium is shown in Figure 1.8. Young cheese is initially rubbery and curdy due to high amounts of insoluble calcium. After a few weeks of aging, the insoluble calcium solubilizes from the para-casein matrix, this leads to an acceptable performance window of cheese having good stretch and melt characteristics (Kindstedt et al., 2010). After this period, the cheese becomes soupy and does not stretch well as proteolysis overtakes the initial effects of insoluble calcium solubilization.

1.3.3.2 Proteolytic properties

The initial breakdown of caseins to large peptides (i.e., primary proteolysis) in Mozzarella cheese occurs primarily through the action of the coagulant on α_s-caseins when chymosin is the coagulant that was used in cheesemaking. Some evidence suggests that the starter culture may also hydrolyze intact β-casein to a small extent during ageing in Mozzarella cheese (Hong et al., 1998). However, the significant contribution of starter culture to casein breakdown occurs in the form of secondary proteolysis (i.e., subsequent hydrolysis of primary peptides to smaller peptides and free amino acids). There is a proteolytic synergy, or relationship, between coagulants and starter culture such as when the initial hydrolysis of caseins by coagulant fails to occur, the secondary proteolysis by starter cultures is severely restricted. When Mozzarella cheese contains active coagulant but no active starter cultures as in the case of directly acidified Mozzarella, large peptides accumulate but few small peptides and amino acids are produced (Barbano et al., 1993).

With age, proteolysis becomes the dominant factor in changing the character of cheese, both in terms of body and texture, but also in terms of increasing melt. The rates of both primary and secondary proteolysis in Mozzarella cheeses can vary greatly depending on the proteolytic activity of the coagulant, the extent to which the coagulant and starter culture are inactivated by heat during stretching, and the ripening temperature (Kindstedt et al., 2010). The rate of proteolysis can increase at lower pH values probably due to the increased activity of residual coagulant with higher acidity. The rate of proteolysis also increases with increasing moisture content in the cheese and increasing storage temperature. As proteolysis progresses, the remaining casein molecules may rearrange, form different associations, or produce non-interconnecting aggregates of casein, that may result in loss of stretch. Extensive proteolysis results in excessive melt and softening of cheese, as well as the loss of stretch quality, and this is exacerbated by the loss of insoluble calcium (Johnson, 2000; Johnson and Lucey, 2006).

Most U.S. cheese manufactures would like to be able to extend the acceptable performance period of LMPS Mozzarella as it is mostly used as an ingredient cheese. This can be achieved if the biochemical and microbiological activities are kept at a minimum and when a stable state of calcium equilibrium is achieved immediately after manufacture. A summary of various strategies for reducing changes in cheese functionality are given in Table 1.3 (Lucey, 2008).

1.3.4 Functional Characteristics of LMPS Mozzarella

LMPS Mozzarella is mostly used as an ingredient cheese in pizza and other prepared foods that contain melted cheese. Hence the functional properties of this cheese are essential determinants of the quality and acceptability (Kindstedt et al., 2004). Mozzarella has to possess certain essential functional characteristics in both unmelted and melted form, textural and melting

properties being more important than flavor as this cheese is typically combined with other more flavorful ingredients on pizza (Kindstedt, 1995).

1.3.4.1 Unmelted cheese

LMPS Mozzarella is usually produced in block form, ranging in weight from 2.3-9.5 kg and must therefore be shredded or diced before it can be used as an ingredient in prepared foods, such as, pizza (Kindstedt et al., 2004). These converted particles should be of mostly uniform size to facilitate uniform distribution and melting (Kindstedt, 1993).

Shreddability and machinability are common functionality terms used to describe the performance of unmelted LMPS Mozzarella. In industry, shreddability is used in reference to several characteristics including the ease with which the block of cheese shreds (e.g., length and thickness of cut; ragged vs clean edges); the propensity of the shredded particles to remain free flowing or to mat together after shredding; the propensity of the cheese to shatter into fines during or after shredding. If the cheese is too dry and firm like a low-fat Mozzarella, it is susceptible to excessive shattering of particles or fines, whereas on the other hand if the cheese is too soft, wet, or pasty, such as, a very young cheese (one- day old cheese) or an aged LMPS Mozzarella cheese, the shredder can become clogged with gummy cheese giving rise to shredded particles with ragged edges and excessive matting after shredding (Kindstedt, 1995). Machinability is used to describe the successful ability of the cheese to be cut/sliced/shredded by machine (e.g., wires or high speed knives) (Lucey, 2008). Machinability is influenced by cheese composition, pH, protein breakdown, and temperature of operation. Most size reduction operations are performed at room temperature although the cheese may be much colder; if cheeses are soft then they may be cooled to low temperatures to increase the cheese firmness and thereby improve their machinability

(Lucey, 2008). Shreddability is commonly measured through sensory analysis by a descriptive panel as shown in Table 1.4. Other parameters, such as, matting, fusion, opacity, adhesiveness, surface oil, etc. are also measured on shredded cheese (Table 1.5) by sensory panelists. Very few quantitative analytical methods have been attempted to quantify the unmelted functional characteristics of Mozzarella cheese (Kindstedt et al., 2004).

Hardness, or firmness, of Mozzarella is another parameter measured on unmelted cheeses and it is quantitatively measured using a texture profile analyzer. Many researchers use similar uniaxial compression tests to study the softening of cheese over time caused by proteolysis (Yun et al., 1995; Metzger et al., 2001; Kindstedt, 1995). The sample size of cheese used for measurement, the temperature of cheese, speed of compression, etc., can affect the hardness values obtained by this method. The decrease in hardness of cheese during the first 3 weeks to one month is due to a shift in calcium equilibrium after manufacture (i.e., solubilization of insoluble calcium) because the proteolytic action is very modest during this time. The cheese becomes softer as more insoluble Ca dissolves during the first month and establishes stable “equilibrium”.

1.3.4.2 Melted cheese

The end-users of Mozzarella cheese have very specific requirements for what kind of melt performance they want from their cheese. Cheese manufacturers therefore manipulate cheese performance to consistently meet these specifications (Lucey, 2008).

A few important heat-induced functional properties for LMPS Mozzarella are defined as follows: (1) ***Meltability*** is the extent to which the melted cheese flows and spreads upon heating; (2) ***Stretchability*** is the ability of the molten cheese to stretch and form strings when extended; (3) ***Elasticity*** is the ability of the cheese to resist deformation during extension, which is related to

chewiness; (4) ***Oiling-off*** is the release of free oil; (5) ***Blistering and browning*** is the formation of dark-colored patches of varying size and color intensity; (6) ***Shred identity*** refers to individual shreds still visible after baking (caused by lack of softening and especially flow); (7) ***Tenting*** refers to the bulging of the cheese that may occur over a large area during baking due to the entrapment of water vapor; (8) ***Skinning*** is the formation of a tough (dry) surface layer (Kindstedt et al., 2004; Lucey, 2008).

An ideal LMPS Mozzarella for ingredient use on pizza should, upon melting, flow readily to form a continuous melt with complete loss of shred identity, possess a stretchable and slightly to moderately elastic, chewy consistency and display limited blister formation, limited intensity of browning and a glistening, but not greasy, surface (Kindstedt et al., 2004). Melted cheese on pizzas are analyzed for sensory attributes as shown in Table 1.5.

Complex analytical methods have been used to assess the functionality of Mozzarella cheese involving complex rheological assessment of specific parameters that are not always directly related to consumer perception. However, these methods have been shown to be useful in developing an understanding of the rheological properties of cheese. Dynamic rheological test methods have allowed researchers to gain an understanding of the viscoelastic nature of Mozzarella cheese (Rowney et al., 1999). The principle and concepts for the rheological tests are discussed in detail in Section 1.4. Other analytical tests commonly reported to measure functionality of melted cheese include Schreiber test, UW Melt-Profiler to measure the meltability and softening point (temperature at which the cheese begins to flow) of cheese (Yun et al., 1993b; Wang et al., 1998); spinning test method (Cavella et al., 1992), horizontal (Ak et al., 1993) and vertical uniaxial extension (Ak and Gunasekaran, 1995) methods have been used in the past to

measure the stretchability of the cheese; differential scanning calorimetry (DSC) offers useful method of determining the melting behavior of fat in cheese (Rowney et al., 1999).

Molecular interactions involving melt and stretch in cheese

Melt is the ability of the cheese to flow and spread. During the melting of cheese, fat is the only component that truly melts. Proteins do not melt, but their interactions with each other can change to produce an effect that we call melt (Lucey et al., 2003). Stretch is the ability of casein network to maintain its integrity (not break) when a continuous stress is applied to cheese. For a cheese to stretch, casein molecules must interact with each other and release stress and become pliable but still maintain sufficient contact between them or the fibers will break.

Electrostatic and hydrophobic interactions during heating of the cheese determines its melt and stretch. As can be described by the Horne model, casein-casein bonds in a matrix are the result of a localized balance of attractive interactions (hydrophobic, CCP crosslinks and +/- charge bridges) and electrostatic repulsion (Lucey et al., 2003). Hydrophobic interactions play a major role in determining the conformation and interaction of protein molecules. Although hydrophobic interactions are stronger at higher temperatures, the net result may be a weakening of the gel due to reduction in contact area between the casein particles. Various types of electrostatic interactions including charge repulsion between similar charges on proteins, plus minus (±) charge interactions, salt bridges, and CCP bridges are important in casein interactions and thereby cheese melting. An assumption for melt to occur is that when a cheese is heated, electrostatic repulsion ≥ electrostatic interactions. A summary of casein interactions as a result of cheesemaking conditions and its impact on cheese melting is shown in Table 1.6 (Lucey et al., 2003).

1.4 Rheology

Rheology involves the deformation and flow of materials when subjected to stress or strain. The rheological properties of cheese are those that determine its response to stresses or strains (e.g., compression, shearing, or cutting) that are applied during processing (e.g., portioning, slicing, shredding, and grating) and consumption (slicing, spreading, masticating, and chewing) (Lucey, 2008). These properties include intrinsic characteristics, such as, elasticity, viscosity, and viscoelasticity that are related primarily to the composition, structure, and the strength of attractions between the structural elements of cheese. The rheological characteristics of cheese are measured instrumentally by the application of stress or strain under defined conditions and measuring the response of the cheese (Fox et al., 2016).

Any rheological measurement involves deforming a sample of a material by applying force, e.g., by compression or by shear. The displacement in response to the force at the point of application is known as deformation. The term deformation used in this sense does not imply permanent deformation but rather a change in shape (i.e., form) which may be temporary, permanent, or partly recoverable (O'Callaghan and Guinee, 2004).

Cheese is a viscoelastic material that exhibits characteristics of both an elastic solid and Newtonian fluid. The rheological properties of viscoelastic materials differ from those of perfectly elastic and viscous materials in that they are dependent on: (1) time, being a function of the time, over which fixed stress or strain is applied and (2) magnitude of the stress. However, on the application of a strain that is sufficiently small so as to not cause permanent damage or fracturing of the microstructure, viscoelastic materials behave as an elastic solid. The relationship between stress and strain are not linear except at very low strains. The strain at which the linearity between stress and strain is lost is referred to as a critical strain (i.e., linear viscoelastic region) and it is relatively low for cheese (0.02-0.05, <5%). Linear viscoelastic measurements are typically carried

out by applying low oscillating strain to the cheese using a controlled stress rheometer and the resultant strain is measured. The rheometer applies a dynamic oscillating shear deformation (γ) to the sample and the measures the resultant stress (τ). Typically, measurements involve placing a disc-shaped sample of cheese (typically 20-50 mm diameter, 2 mm height) between two parallel plates with serrated surfaces (parallel plate geometry), one of which is fixed, while the other applies a low-amplitude torsional harmonic motion. The use of serrated plates ensures that the cheese sample is gripped firmly between the plates, and thereby minimizes the risk of slippage associated with fat liquefication and leakage, especially when rheological measurements are performed at high temperatures between 20-90ºC (Fox et al., 2016).

Shear stress, τ is defined as $\tau = \frac{F}{A}$, where A is a cross-sectional area over which the shear force (F) is distributed.

Shear strain, γ is defined as $\gamma = \frac{\Delta L}{L_o}$, where L_o is the original height of the sample and ΔL is the shear (tangential) displacement on the application of shear stress τ.

At any time, t, the angle of rotation, θ, of the oscillating plate is defined by:

$$\theta = a \sin \omega t$$

where a is the maximum angle of rotation and ω is the angular velocity.

The shear applied by the plate results in a strain γ(t) at any radius r:

$$\gamma(t) = \gamma_o \sin \omega t,$$

γ_o is the amplitude of γ(t). For viscoelastic materials, such as, cheese, the resultant oscillating stress is out of phase with the applied shear by a phase angle, δ.

The total stress (τ) is given by $\tau = \tau'_o \sin \omega t + \tau''_o \cos \omega t$,

where τ'_o and τ''_o indicate the stress components that are in-phase and out-of-phase with the strain, γ, and are related by the phase angle, δ. The tangent of the phase angle,

$$\tan \delta = {}^{\tau''_o}/_{\tau'_o}$$

The storage modulus (or elastic shear modulus) G′, and loss modulus G″ (or viscous modulus), may be defined as from the relationship between τ and γ, where

$$G' = {}^{\tau'_o}/_{\gamma_o}$$

$$G'' = {}^{\tau''_o}/_{\gamma_o}$$

Therefore,

$$\tan \delta = {}^{G''}/_{G'}$$

(O'Callaghan and Guinee, 2004)

Typically, G′ and G″ values are measured using oscillatory rheology to indicate the softening of cheese, an effect which is due to:

1. Liquefaction of the fat phase, which is fully liquid at ~40ºC and to protein aggregation.
2. Contraction and shrinkage of the para-casein network (owing to a temperature-induced increase in the extent of hydrophobic interactions between the casein molecules) and a simultaneous expulsion of moisture from the casein network.

As the melted cheese becomes more liquid, the phase angle increases from ~20 to 70º, reflecting transformation from a viscoelastic solid (δ<45º) to a viscoelastic liquid (δ>45º). These measurements give an insight into the effects of different treatment parameters used in cheese manufacture (e.g., milk heat treatment, milk homogenization, make-procedure alterations to affect calcium content) and composition (e.g., levels of fat, protein, or calcium) on the cooking (melting) properties of cheese. The cooking properties are important in applications where cheese is heated to a high temperature during baking, grilling, or melting, e.g., toasted sandwiches, pizza, lasagna, and sauces. Max phase angle (δ_{max}) or the maximum loss tangent (LT_{max}) is used as an indicator of the fluidity of the melted cheese and the degree to which it flows; the cross-over temperature, where G′ equals G″, is indicative of the temperature required to transform cheese from a viscoelastic solid to viscoelastic liquid (melt point). The temperature of LT_{max} is indicative of the temperature which gives the maximum fluidity. It is frequently observed that the LT values decrease at elevated temperatures (>70ºC), probably due to an increase in hydrophobic interactions (Fox et al., 2016).

1.5 Various Methods to Quantify the Amount of Insoluble Calcium in Cheese

Over the years, various methods have been used to measure the insoluble calcium content in the cheese as it is an important parameter to predict cheese texture and functionality. It is important to note that the insoluble calcium content measured should preferably be represented in terms of the concentration of calcium per gram of protein, i.e., the calcium that is bound to casein. This information would provide much more information on what factors (such as, rate of acid development and whey drainage pH) affected the calcium losses during manufacture and give a better indication of cheese structure at that particular insoluble calcium value (Lucey and Fox,

1993) otherwise there would be confounding factors due to differences in moisture contents. The most common methods used for insoluble calcium measurement are discussed below.

1.5.1 Hydraulic Press Cheese Juice Extraction

Cheese juice extraction method has been used to study the chemical composition of the serum phase in cheese. This method involves pressing ground cheese at high pressure for a certain period of time until the required amount of serum phase, i.e., juice, from the cheese is obtained. Morris et al. (1988) was one of the first to use this method to study the mineral composition in the aqueous phase of 1 month old Cheddar cheese. They found that about 43% Ca and 12% PO_4 and all Na, K, Cl, and lactate were present in serum phase of the cheese. They also determined that the expressed aqueous solution was in near equilibrium with the cheese serum. Lucey et al. (1993b) studied the contribution of buffering of cheese juice to the buffering of cheese samples. Boutrou et al. (1999) studied the changes in the composition of juice from Camembert cheese during ripening until day 16. They found that the aqueous phase in cheese becomes less expressible as the proteolysis progressed, i.e., they were able to express about 67% of the juice at day 0 whereas only 19% of the juice at day 16. Thierry et al. (1998) also observed a decrease in the amount of expressible juice from Emmental cheese during ripening but they were able to express about 30% of the water from 60-day old cheese. Expressability of cheese juice therefore appears to be related to the cheese variety, i.e., the curd type (rennet, lactic, or mixed), the curd treatments (stirring, cooking, pressing, etc.), cheese composition (e.g., moisture content) and to the stage of ripening. As proteolysis limits the cheese juice extraction from cheese, this method can only be used to measure the insoluble calcium in fresh or young cheeses. Other drawbacks of this method are the

longer periods of time taken to express the serum phase from cheese and also larger quantities of cheese required for analysis.

1.5.2 Acid-Base Titrations

This method utilizes the chemical property of CCP causing buffering between the pH range of 6.7 to 4.0. Lucey et al. (1993a) studied the buffering curves of raw milk and CCP-free milk. They observed a hysteresis loop when the raw milk was titrated forward and backwards with acid and base, respectively, whereas no real loop was observed in the CCP-free milks (Figure 1.9). This demonstrates that the buffering curve that has a peak at pH ~5.1 during acid titration of milk is due to the solubilization of CCP and the area of this peak reflects the amount of CCP in milk. A similar buffering was also found when a dispersion of young Cheddar cheese was titrated (Lucey and Fox, 1993) as shown in Figure 1.9. The buffering peak in Cheddar cheese occurs at pH 4.8, which is slightly lower than for milk.

Lucey and Fox (1993) suggested that by comparing the area of the peaks found in milk to those in cheese one should be able to obtain a useful index of the amount of residual CCP present in cheese. Hassan et al. (2004) compared the acid-base titrations and cheese juice method to quantify the proportions of soluble and insoluble Ca in Cheddar cheese during maturation. The buffering capacity index (db/dpH) and the insoluble calcium content using acid-base titrations was calculated using the equations shown below. They found that there were no significant differences between the two methods and the proportion of insoluble calcium in cheese decreased from ~73% to ~58% between day 1 and 4 mo timepoints.

$$\frac{dB}{dpH} = \frac{ml\ of\ acid\ or\ base\ added\ \times normality\ of\ acid\ or\ base}{volume\ of\ sample\ \times pH\ change\ produced}$$

$$Insoluble\ Ca\ content\ (mg/100\ g) = \frac{mg\ insoluble\ Ca\ per\ 100\ g\ milk\ \times (A_C \times D)}{A_M}$$

where A_M and A_C are the residual areas when the back (titration with base) buffering curve is subtracted from the forward (acidification) buffering curve for milk and cheese, respectively.

1.5.3 Water-Soluble Calcium Extraction

Guo and Kindstedt (1995) developed and evaluated a centrifugation method to express aqueous liquid from Mozzarella cheese. This method involves centrifuging grated cheese at 12,500 × g for 75 minutes at 25ºC. The centrifugation resulted in an aqueous fraction (i.e., the expressible serum) similar to the cheese juice method, which can be used for soluble calcium determination. A major drawback of this method is that it can only be used for fresh cheeses like Mozzarella that have high moisture contents. An improvement to this method is to use water to homogenize the cheese, centrifuge the prepared slurry and extract the serum phase of the cheese. This method was used by Metzger et al. (2001) to study the water-soluble calcium (WSC) in various cheeses pre-acidified with different acids. One important note with this method is that the use of water to prepare the cheese slurry can dilute the cheese matrix, increase the pH of the sample, and potentially change the calcium equilibrium. Care has to be taken to ensure that the homogenization and centrifugation time are kept to a minimum in order to avoid changes in the proportion of calcium in soluble/insoluble forms. WSC method is a rapid test and therefore, we believe could be used to measure the insoluble calcium in curds during the cheese manufacturing phase. Cheese juice and acid-base titrations can take relatively longer period of time and hence, cannot be used to quickly measure the changes in insoluble calcium during cheese manufacture as the ongoing acidification by starter cultures would change the calcium equilibrium while testing. Thereby, the

calcium measured by the traditional methods would not be a true representation of the calcium balances at the time the sample was drawn.

Based on the literature review done, we have identified some of the gap areas including no data on calcium losses during cheese manufacture with the change in various manufacturing steps, lack of data on cheese made from concentrated milk (> 4% casein), and the amount of insoluble calcium in the curds during cheese manufacturing and in the cheese during storage. The following chapters in this thesis therefore focus on many of these gap areas to create and generate more data for a better understanding of the Mozzarella cheese manufacturing steps and to expand the knowledge base in this field of research.

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1.7 Tables and Figures

Table 1.1 Approximate composition of bovine milk (Walstra et al., 2006)

Component	Average content in milk (% w/w)	Range (% w/w)
Water	87.1	85.3-88.7
Solids-non-fat	8.9	7.9-10.0
Fat in dry matter	31	22-38
Lactose	4.6	3.8-5.3
Fat	4.0	2.5-5.5
Protein	3.3	2.3-4.4
Casein	2.6	1.7-3.5
Mineral substances	0.7	0.57-0.83
Organic acids	0.17	0.12-0.21
Miscellaneous	0.15	-

Table 1.2 The most important salts in milk and their distribution between serum and casein micelles (Walstra et al., 2006)

Compound	Molar Mass (Da)	Range (mmol/kg)	Average (mg/100g)	Fraction Present in Serum	Micelles (mmol/g Dry Casein)
Cations					
Na	23	17-28	48	0.95	0.04
K	39.1	31-43	143	0.94	0.08
Ca	40.1	26-32	117	0.32	0.77
Mg	24.3	4-6	11	0.66	0.06
Amines	-	~1.3	-	~1	-
Anions					
Cl	35.5	22-34	110	1	-
CO_3	60	~2	10	~1	-
SO_4	96.1	~1	10	1	-
PO_4[a]	95	19-23	203	0.53	0.39
Citrate[b]	189	7-11	175	0.92	0.03
Carboxylic acid	-	1-4	-	~1	-
Phosphoric esters[c]	-	2-4	-	1	-

[a] Inorganic only.

[b] $(CH_2\text{-}COO^-)\text{-}(COH\text{-}COO^-)\text{-}(CH_2\text{-}COO^-)$.

[c] Soluble.

Table 1.3 Summary of some strategies that have been used to extend the performance window of cheese functionality during storage (Lucey, 2008)

	Chemical/ Biological changes targeted	Methods/Treatments
Milk	Bacterial/ enzyme activity	Heat, CO_2 addition, microfiltration (MF), non-thermal processes
	Seasonal or milk supply variations Ratio of proteins/minerals	Reconstitution of cheese milk from dairy powders or the use of retentates (pre-cheese)
	Ratio of total and insoluble Ca to protein	Pre-acidification, direct acidification, addition of Ca sequestrant, altered rate of acidification
	Ratios of individual caseins	Use of cold MF of cheese milk to remove some beta-casein
	Additional protein crosslinking	Use of transglutaminase, attachment of denatured whey proteins to caseins
Curd	Ratio of total and insoluble Ca to casein	Pre-acidification, direct acidification, addition of Ca sequestrant, altered rate of acidification
	Residual rennet, other enzymes, and starter culture activities	Cooking treatment (time and temperature of curd in the cheese vat or in the mixer/molder) to denature rennet and other enzymes and reduce bacterial numbers, High salt to inhibit bacterial growth/activity
Cheese	State of Ca in cheese (e.g. preventing solubilization of insoluble Ca during ripening)	Addition of Ca sequestrant during cheese manufacture, Prevent pH changes post-manufacture by washing/diafiltration or using salt sensitive cultures High pressure processing
	Residual rennet, other enzymes, and starter culture activities	Use of very storage temperatures, frozen or individually quick frozen (IQF) cheese High pressure processing

Table 1.4 The sensory attributes of shredded cheese, using a 0-15-point scale (Chen et al, 2009)

Attribute	Definition	Scale
Straightness	The degree of curving of shreds without taking fines into account	Visual reference used
Matting	Percentage of shreds that are clumped/matted, relative to the total amount of shreds	0: 0% matted 7.5: 50% matted 15: 100% matted
Fusion	Percentage of shreds that have fused back together, relative to the total amount of shreds	0: 0% fusion 7.5: 50% fusion 15: 100% fusion, single lump
Opacity	How opaque or translucent the shreds are	2.5: Low-fat Mozzarella 7: Kraft fat-free Mozzarella 14.5: Whole milk Mozzarella
Avg. shred length	The average length of three shreds selected at random	Measure with a ruler in cm
Shred surface oil	Residual oil left after manipulating the shreds. Lightly manipulate (rub) shreds between thumb and fingers, evaluate oil left on finger	0: Kraft fat-free Mozzarella 6: Belgioioso Asiago 10: Shullsburg Muenster
Shred adhesiveness	Degree to which shreds stick to themselves and other surfaces. Lightly manipulate shreds between thumb and fingers, drop them from 20 cm height, observe degree of adhesion	1: Belgioioso Parmesan 6: Carr Valley Bread Cheese 10.5: Whole Milk Mozzarella 14.5: Shullsburg Muenster

Table 1.5 The sensory attributes of cheese on pizza, using a 0-15-point scale (Chen et al., 2009)

Attribute	Definition	Scale
Blister quantity	Amount of the surface covered by blisters	Visual reference used
Blister color	Intensity of the brown hue of the blisters	Visual reference used
Shred melt	The degree to which the cheese shred melts	3: Shreds ~75% orig. shape, 7.5: Shreds ~50% orig. shape, 12: Shreds ~25% orig. shape
Free oil release	The amount of free oil on surface of the melted cheese. This is a temperature dependent attribute conducted at 96.1ºC	2.5: Discontinuous layer, 5: Thin continuous layer, 7.5: Small pools, not numerous, 10: Pools, 12.5: Pools, oil splatter, fat leaking
Flow-off crust	The degree to which the melted cheese flows off crust	2.5: ~2 points of cheese flow, 5: ~3-4 points of cheese flow, 7.5: ~25% of perimeter melted off, 12: ~50% of perimeter melted off
Skinning	Thickness of surface layer of cheese	Visual reference used
Strand thickness	Width of strand at 3 in. of height, when strands has been pulled to 6 in.	Visual reference used
Avg. strand length	The average height of the strand when stretched three times	Visual reference used
First chew hardness	Amount of force required to completely bite through sample for the first time	1: Cream cheese, 3: Kraft singles, 5: Kraft deli deluxe, 7.5: Medium cheddar, 11: Baby carrot
Cohesiveness	Degree to which a chewed sample holds together between molars	1: Baby Carrot, 3: Mushroom, 7: Tostitos, 11: Apricot, 14: White bread
Chewiness	The total amount of energy required to masticate the sample to a state pending swallowing	1: Cream cheese, 6: Medium Cheddar, 9: Dots gum drops, 13.0: Gummy bears

Table 1.6 Summary of casein interactions involved in melting (A) and examples of how various cheese manufacturing conditions affect casein interactions and impact meltability (B) (Lucey et al., 2003)

Cheesemaking conditions/situation	Electrostatic repulsion	Attractive interactions	Net impact on melt
Acid production (pH ≥ 4.9)	Loss of CCP increases localized repulsion by exposing phosphoserine residues	Decreases due to fewer CCP crosslinks	Increases
Very low pH values (< 4.9)	Repulsion decreases as caseins approach their isoelectric point	Hydrophobic interactions increase with concomitant reduction in electrostatic repulsion, more +/- charge bridges	Decreases
Proteolysis	Decreases when charged peptides are hydrolyzed	Decreases as total number of protein-protein bonds is reduced	Increases
CCP solubilization post-manufacture	Further loss of CCP increases localized repulsion by exposing phosphoserine residues	Decreases due to less CCP crosslinks	Increases
Concentrated milk (without corrective measures)	Decreases due to higher CCP content (per g of casein)	Increases due to more CCP crosslinks (per g of casein)	Decreases
Use of emulsifying salts in processed cheese	Increases due to exposure of phosphoserine residues when CCP is partly chelated, these salts can increase pH and thereby repulsion	Decreases due to less CCP crosslinks	Increases

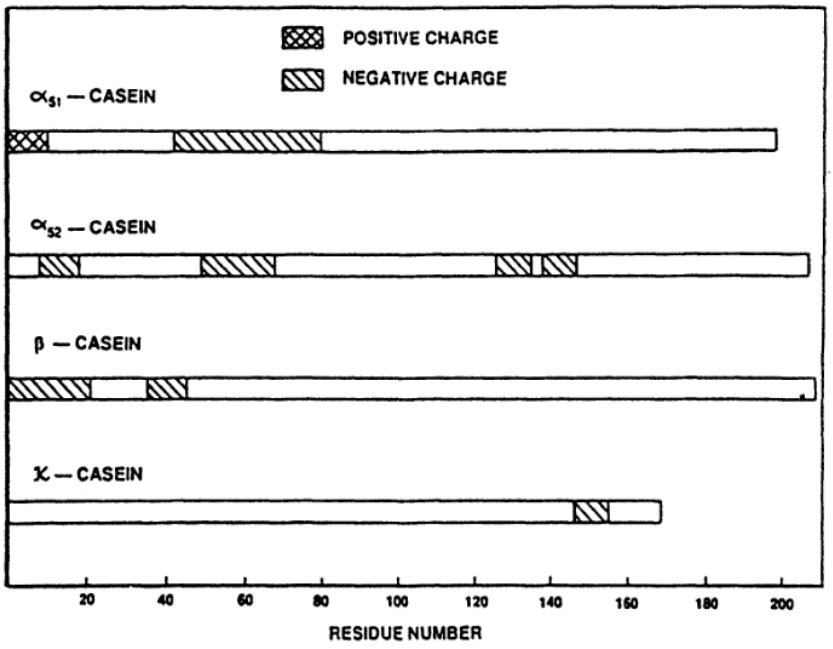
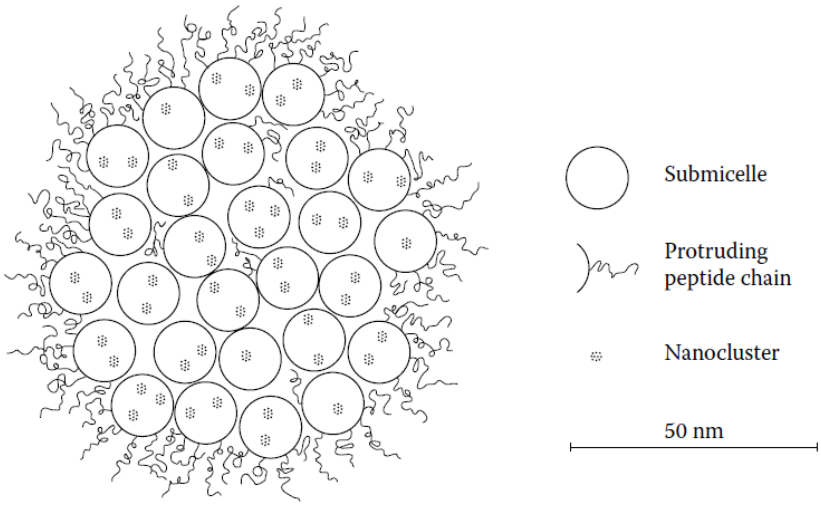


Figure 1.1 Ten residue sequences of bovine milk proteins with a charge density of 0.5 or greater at the natural pH of milk (Fox and McSweeney, 1998).

A



B

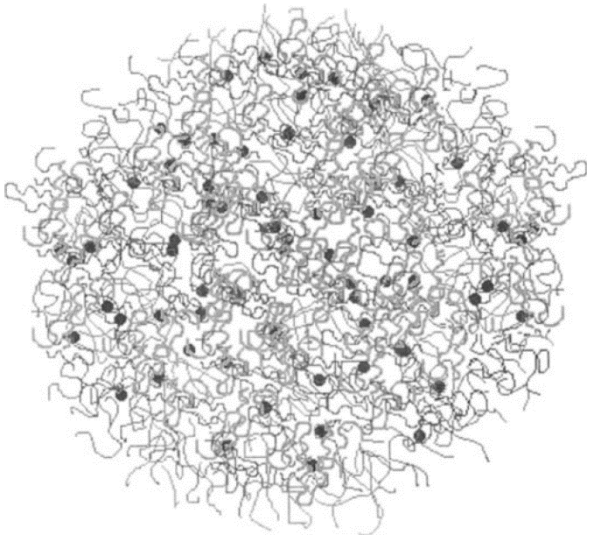


Figure 1.2 (a) Submicelle (Walstra et al., 2006) and (b) nanocluster model (Adapted from Fox and McSweeney, 2003) of the casein micelle.

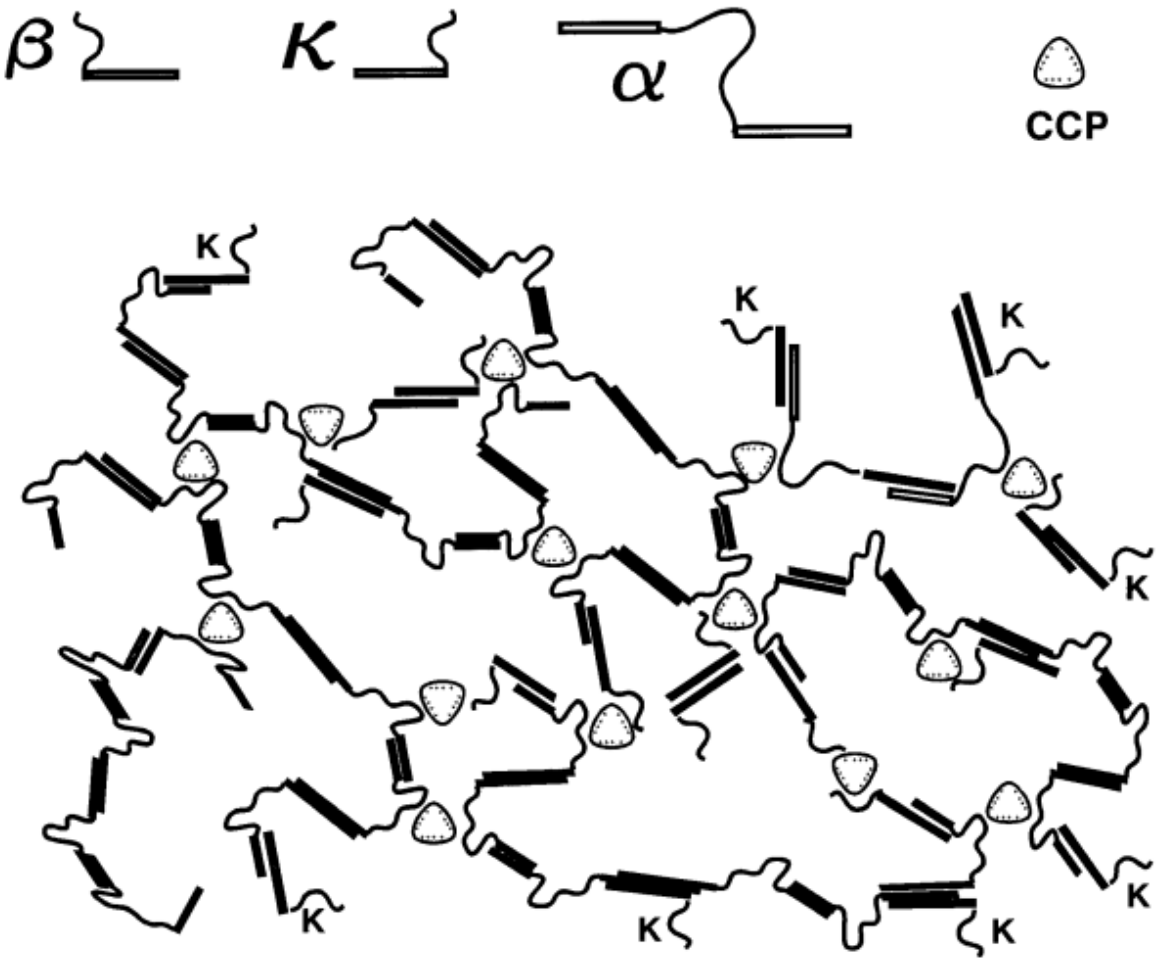


Figure 1.3 Dual binding model of the casein micelle (Horne, 1998).

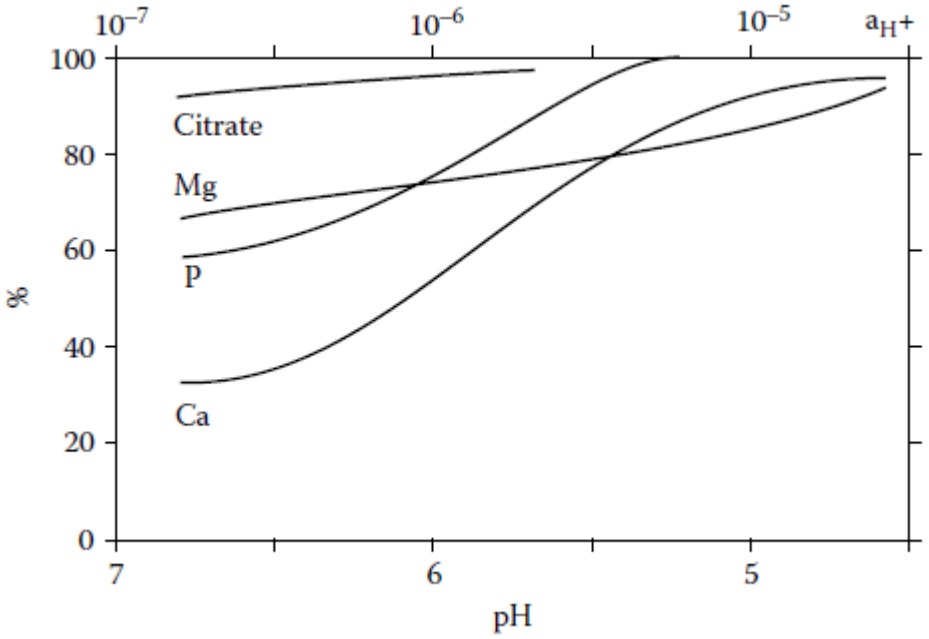


Figure 1.4 Approximate percentages of calcium, inorganic phosphate, magnesium, and citrate that are dissolved as a function of the pH of milk (Walstra et al., 2006).

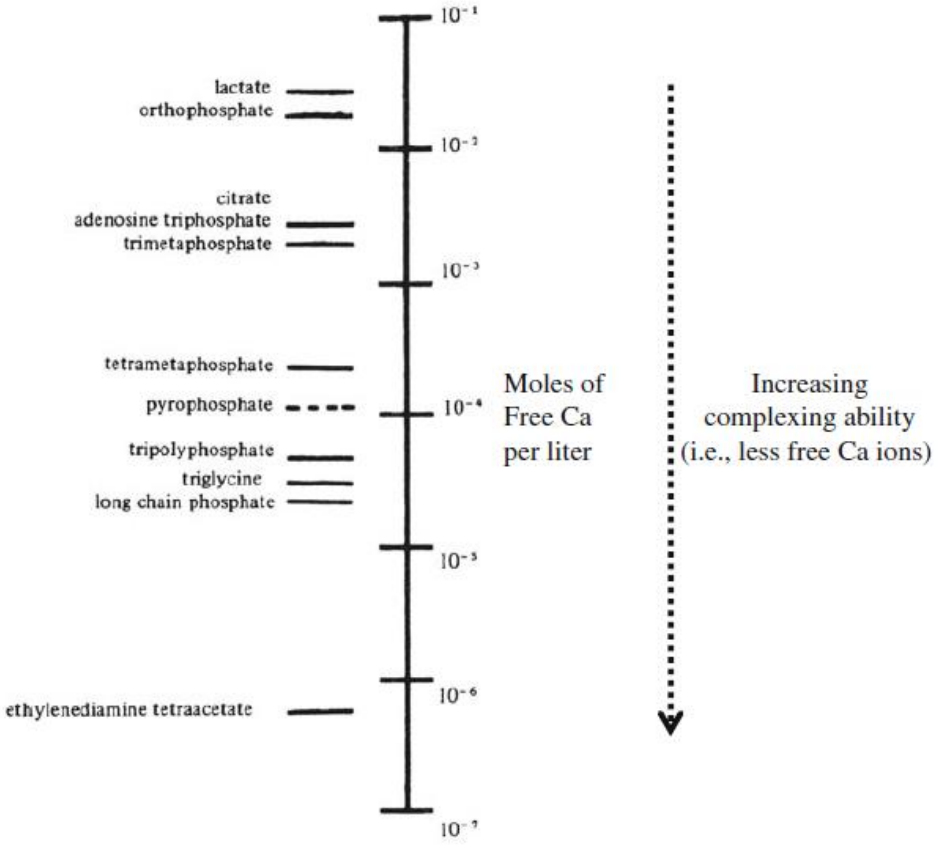


Figure 1.5 The free calcium concentration (i.e., not chelated or sequestered) for various types of complexing agents are estimated for the dissociation of a 0.01 M solution of the 1:1 Ca complex (Lucey and Horne, 2009).

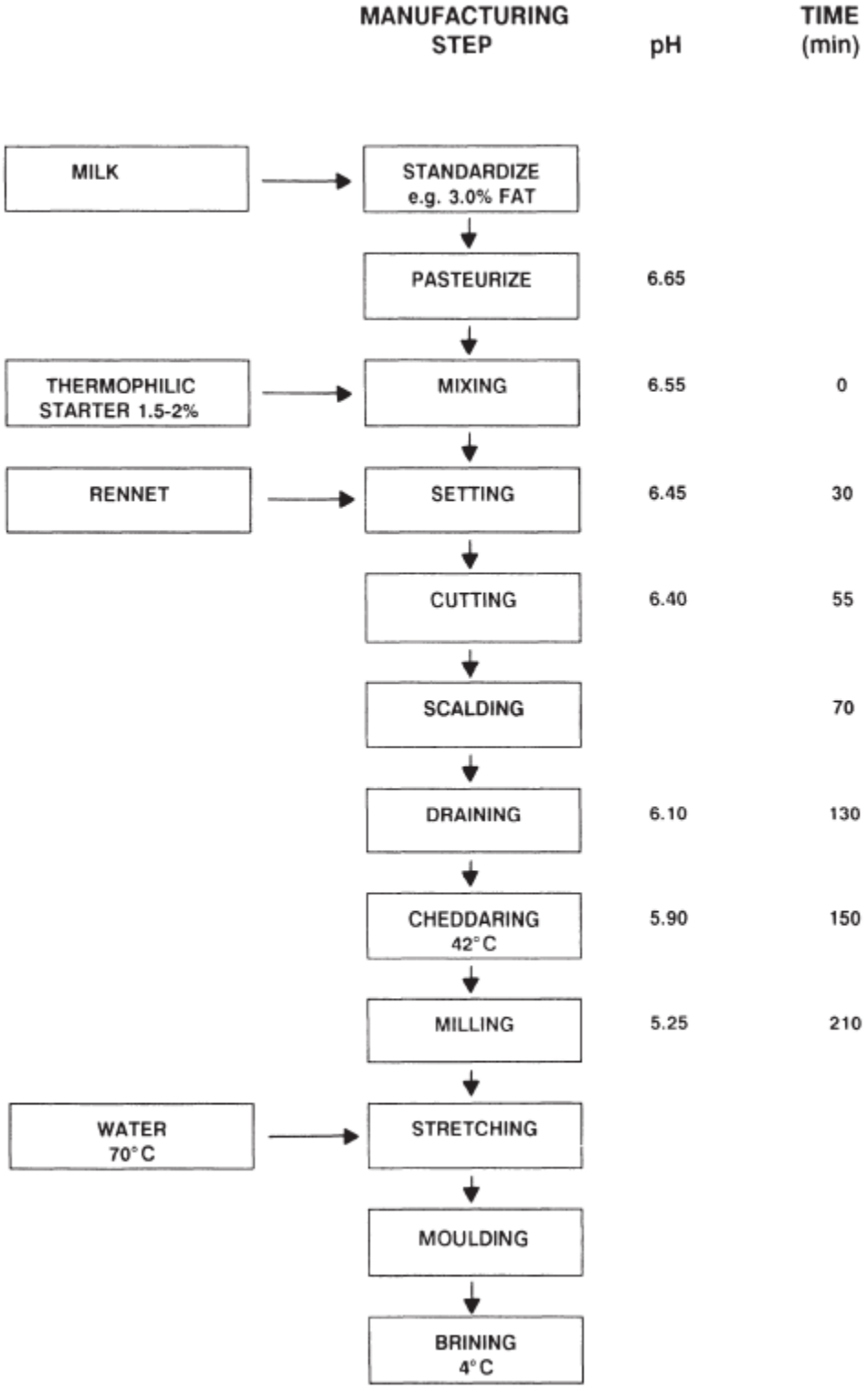


Figure 1.6 Example of a manufacturing process for LMPS Mozzarella by a traditional process (Kindstedt, 1993).

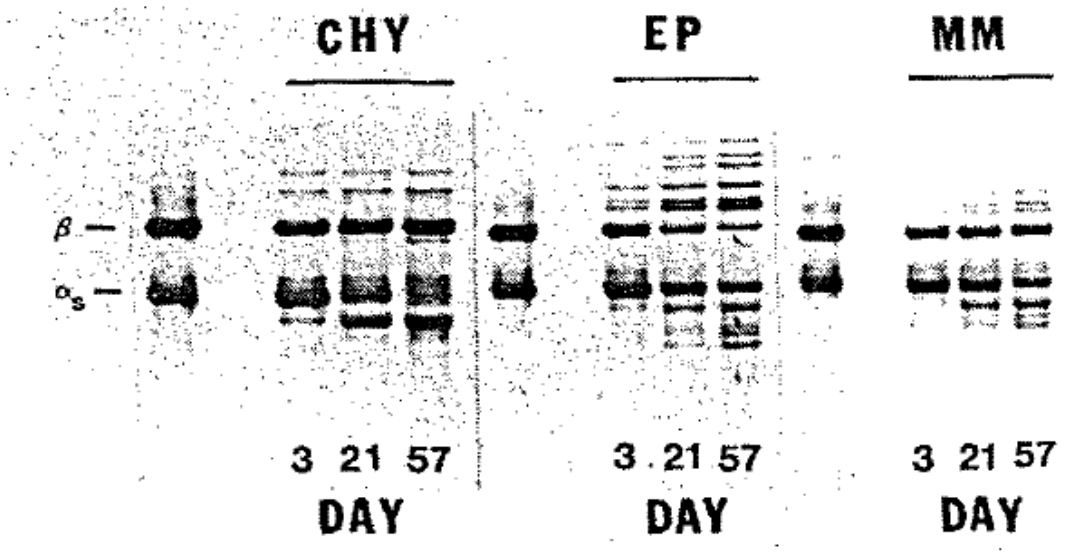


Figure 1.7 Electrophoretic patterns of LMPS Mozzarella cheeses made with three different coagulants (CHY=fermentation produced chymosin; EP=*Endothia parasitica*, MM=*Mucor miehei*) and stored at 4ºC for 57 d (Kindstedt and Fox, 1993).

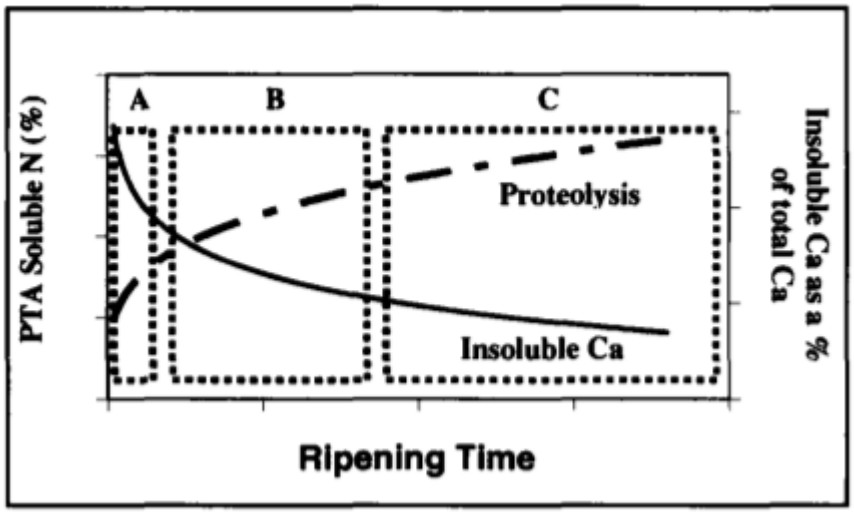


Figure 1.8 Changes in Mozzarella cheese performance during ripening as a result of calcium shifts and proteolysis. (A) young cheese is tough and has poor melt, (B) acceptable performance window- good melt, stretch and shred, (C) unacceptable performance window-cheese is too soft, soupy, and sticky (Johnson and Lucey, 2006).

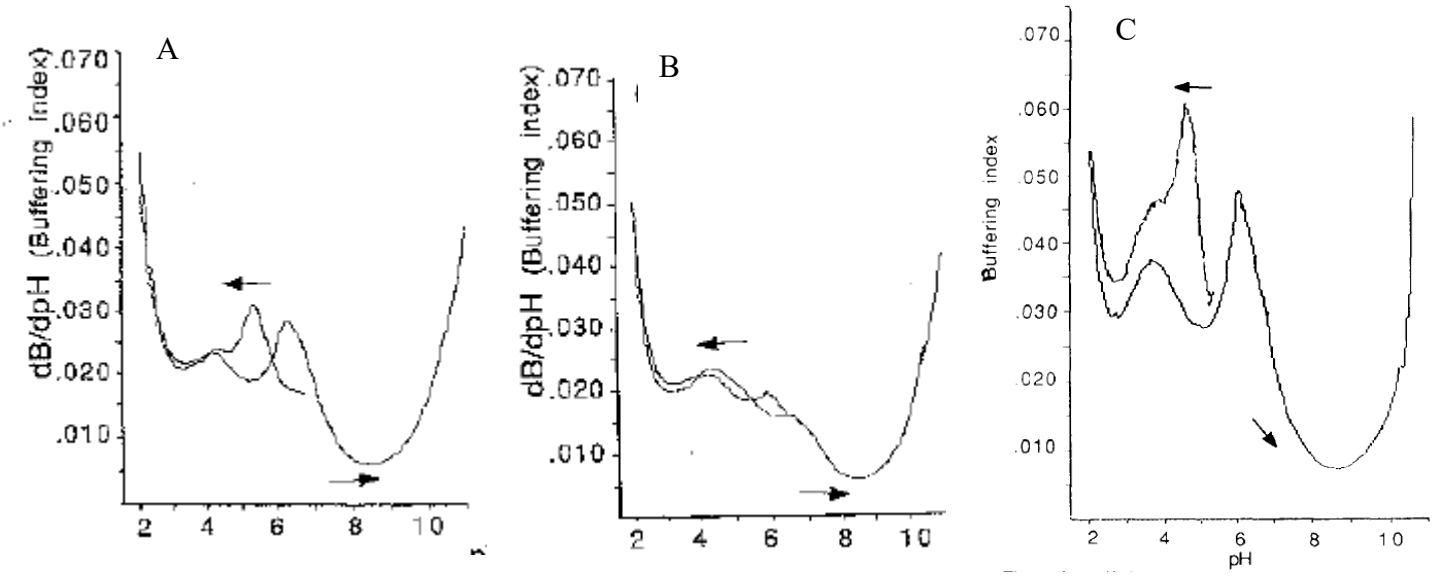


Figure 1.9 Acid-base buffering curves of (a) raw milk (b) CCP-free milk (c) one-month old Cheddar cheese (Lucey et al., 1993)

Chapter 2. Evaluation of a Water-Soluble Calcium Method for Insoluble Ca Measurements in Milk and Cheese Samples

A part of this chapter has been published in the International Journal of Dairy Technology

Swaminathan, A.V., S.K. Lillevang, and J.A. Lucey. 2023. Measuring insoluble calcium content in curds during cheesemaking. Int. J. Dairy Technol. 1–9. doi:10.1111/1471-0307.13016.

Abstract

The functionality of cheese, like low-moisture part-skim (LMPS) Mozzarella, is greatly impacted by the solubilization of insoluble calcium phosphate during cheese manufacture. Measuring insoluble calcium content in cheese at various points during the manufacturing process would help to predict its final functionality. Traditional methods to estimate insoluble calcium content, including acid-base titrations and cheese juice take several h to perform, which is an issue due to rapid pH changes during the cheesemaking process, which impact the insoluble calcium content. We evaluated a simple water-soluble calcium (WSC) extraction method as an alternative method to quickly quantify the insoluble calcium content. A comparison between traditional methods and the proposed WSC technique for direct-acid (DA) and cultured (SC) derived String and LMPS Mozzarella cheeses was done to compare their performance in measuring the insoluble calcium content. No method differences were observed for DA String cheese but there were significant differences in methods observed for SC String cheese and LMPS Mozzarella. Although the methods varied with cheese type and age, the insoluble calcium measured by these methods was comparable. Therefore, WSC can be used to indirectly measure the insoluble calcium in the curds during cheese manufacture.

2.1 Introduction

The texture and rheological properties of LMPS Mozzarella are dependent on its insoluble calcium content. Most research that was reported in the past measured only total calcium in the cheese (Yun et al., 1995; Sheehan and Guinee, 2004), but it is the level of insoluble calcium in cheese that directly affects casein interactions, and would therefore be a better indicator of cheese functionality.

Several manufacturing factors, such as, milk composition, rate and extent of acid development, whey drain pH, cooking temperature, etc., affect the amount of insoluble calcium in the cheese and thereby influence cheese functionality (Lucey et al., 2003). It was only in the 1990s that the importance of the levels of soluble to insoluble calcium in cheese and its effects on cheese functionality was clearly established (Johnson and Lucey, 2006). Since then, many researchers have regularly measured the total calcium in cheese (Yun et al., 1995; Metzger et al., 2001; Guinee et al., 2002; Sheehan and Guinee, 2004) but only very few have measured the insoluble calcium content in cheese to better describe cheese functionality (Hassan et al., 2004; Mizuno et al., 2009; Moynihan et al., 2014). Very limited research has been published to understand the effects of manufacturing factors on calcium balances (total, soluble calcium) in real-time during cheese manufacture. Although the final insoluble and total calcium content in cheese give us a partial understanding of why the cheese behaves the way it does, it is more important to understand how we ended up with a specific amount of insoluble calcium. This can be done by measuring the changes in insoluble calcium in curd samples at various stages during cheese manufacture.

Commonly used methods for measuring insoluble calcium in cheese are the cheese juice method, acid-base titrations, and water-soluble calcium method. In the past, all these methods have

been used to measure the insoluble calcium in cheeses during ripening or storage (Morris et al., 1988; Guo and Kindstedt, 1995; Metzger et al., 2001; Lucey et al., 1993). To measure the insoluble calcium changes during cheese manufacture, a quick method has to be used. This is because fermentation in curds continues as we draw samples, and this acidification likely changes the insoluble calcium level in the samples. Neither cheese juice nor acid-base titrations can therefore be used to measure the insoluble calcium changes during manufacture. This is because both of these methods take a long time for testing, i.e., about 3 h. The cheese juice method also requires a large amount of sample for analysis. The water-soluble calcium (WSC) method requires small amount of sample and considerably less time, i.e., 10 min, to prepare the samples for insoluble calcium measurement. Many variations of WSC have been used in the past with different procedures for homogenizing the samples, different centrifugation speeds and water temperatures used during sample preparation. These parameters need to be evaluated to determine if they have any effects on the results of WSC from the curd samples.

A concern with using WSC is the dilution step involved during sample preparation. Diluting the samples with water can cause an increase in pH, possibly shifting the calcium balance in the samples (Hassan et al., 2004). It was therefore important to compare the insoluble calcium content obtained from the WSC method with traditional methods, such as, cheese juice and acid-base titrations to make sure that the values were similar to each other. Hassan et al. (2004) found no significant differences between the cheese juice and acid-base titrations method, but the method comparison in their study was performed on Cheddar cheese during ripening. There might be differences with insoluble calcium methods due to the structure or type of the cheese. Our research study was focused on LMPS Mozzarella and hence, a method comparison should to be done using

LMPS Mozzarella to find out if the WSC method is acceptable to measure the insoluble calcium content in curd and LMPS Mozzarella cheese samples.

Research Hypotheses

- The soluble calcium content in the aqueous phase of the milk samples measured using the rennet-gel method (WSC) and ultrafiltration permeate will be similar
- The dilution step in the WSC method could slightly affect the insoluble calcium content in cheese but the values obtained from the WSC method will still be comparable to the cheese juice and acid-base titrations method, if the sample preparation time and dilution factor are kept to a minimum in the WSC method.

2.2 Materials and Methods

2.2.1 Materials

Milk

Milk experiments were conducted on four different types of store-brought milk samples, i.e., whole milk, 2% reduced fat (gallon), 2% reduced fat (quart bottle), and 1% low-fat milk. They were all stored under refrigeration conditions until experiments were performed.

String cheese

Direct acid (DA) String cheese and starter culture (SC) String cheese were procured from VV Supremo (Chicago, IL) and Baker's cheese (St. Cloud, WI), respectively, on the same day that they were made. These cheeses were stored at 4ºC and analyzed at 2 d, 2 w, 2, 4, and 6 m.

Low-moisture part-skim Mozzarella

Ten vats of milled curd LMPS Mozzarella were manufactured at the University of Wisconsin-Madison dairy plant over 2 d. One block of cheese (block 8) from each vat was procured for our experiment, i.e., 4 blocks from day 1 and 6 blocks from day 2. All the cheeses were manufactured using a standard make procedure with target whey drain and milling pH values of 5.90 and 5.20, respectively. The curds were pre-salted, stretched in a mixer molder, and brined for a few h. The cheese blocks received from the dairy plant were vacuum sealed and stored at 4ºC.

2.2.2 Methods for Measuring Insoluble Calcium in Milk

2.2.2.1 Rennet Gel Method

Milk samples were weighed into centrifuge tubes and warmed to 32ºC using a water bath. Fermentation-produced calf chymosin (Chymax®, Chr. Hansen, Milwaukee, WI) (~55 µL/ 100 g sample) was added to the milk and incubated for 2 h or until the samples gelled. The gelled sample is called rennet gel and was used for one of the experiments in this chapter to study the parameters for the WSC method. For measuring the soluble calcium content, the rennet gel was centrifuged (Beckman Coulter, Avanti J-E centrifuge, Brea, CA) at 2000 × g for 10 min. The supernatant was filtered through a Whatman #1 (GE Healthcare Life Sciences, USA) filter paper. The total calcium in the milk and the filtrate were measured using an inductively-coupled plasma optical emission spectrophotometer (ICP-OES) (Agilent 5100, Agilent Technologies, Santa Clara, CA) (Park, 2000). Insoluble calcium in the milk sample was calculated by subtracting the total calcium in the filtrate, i.e., soluble calcium, from the total calcium in the milk sample.

2.2.2.2 Ultrafiltration Permeate

Bench-top Amicon stirred cell (Series 8000, EMD Millipore Corporation, Billerica, MA) with Ultracel® 10kDa membrane and 100 kDa membrane (EMD Millipore Corporation, Billerica, MA) was used for ultra-filtering milk samples. Approximately 100 mL of milk sample was added to the stirred cell with an ultrafiltration (UF) membrane. The unit was pressurized to optimum operating pressure using a nitrogen gas tank while the sample was being stirred. UF was continued until enough permeate was obtained for total calcium measurement using ICP-OES (Agilent 5100, Agilent Technologies, Santa Clara, CA) (Park, 2000).

2.2.3 Methods for Measuring Insoluble Calcium in Cheese

2.2.3.1 Acid-Base Titrations

The acid-base titrations were performed on both the milk and cheese using the method described by Hassan et al. (2004). Samples were prepared by mixing 8 g of grated cheese in 40 g Milli-Q water at 55ºC for 4 min using an Ultra-Turrax T-25 Basic Homogenizer (IKA Works Inc., Willmington, NC). The heterogeneous mixture was cooled to room temperature (25ºC) and titrated using an automatic titration system (Mettler Toledo DL50 Auto titrator, Schwerzenbach, Switzerland). Milk was weighed out (40 g) into titrator cups and allowed to reach room temperature (~20ºC) before analysis. The samples were titrated from the initial pH of the sample to pH 3.0 with 0.5 N hydrochloric acid (HCl) and then back titrated from pH 3.0 to pH 7.0 with 0.5 N sodium hydroxide (NaOH). 0.1 mL of acid or base was added every 30 sec. The buffering curves were created by plotting the buffering index as a function of pH (dB/dpH) as shown in the equation below. The area between the forward and back curve is the buffering capacity of insoluble calcium or colloidal calcium phosphate (CCP) and it was calculated from pH 4.0 for String cheese (pH 4.2 for LMPS Mozzarella) and the initial pH for cheese, and from pH 4.1 to pH 5.8 for milk.

The insoluble Ca was calculated by using the equation below. Milk titrations were done on the cheesemaking day and cheese titrations were done at the respective storage time points.

$$\frac{dB}{dpH} = \frac{ml\ of\ acid\ or\ base\ added\ \times normality\ of\ acid\ or\ base}{volume\ of\ sample\ \times pH\ change\ produced}$$

$$Insoluble\ Ca\ content\ (mg/100\ g) = \frac{mg\ insoluble\ Ca\ per\ 100\ g\ milk\ \times (A_C \times D)}{A_M}$$

where A_M and A_C are the residual areas when the back (titration with base) buffering curve is subtracted from the forward (acidification) buffering curve for milk and cheese, respectively. D is the dilution factor, which was 6 as 8 g of cheese was mixed with 40 mL of water. The insoluble calcium in milk was calculated by subtracting the soluble calcium in milk (rennet whey) from total calcium as measured using ICP-OES (Agilent 5100, Agilent Technologies, Santa Clara, CA) (Park, 2000). The soluble calcium in milk was multiplied using a correction factor for whey (Davies and White, 1960).

2.2.3.2 Hydraulic Press Cheese Juice Extraction

The cheese juice procedure was based on the method developed by Morris et al. (1988) and Lucey et al. (1993). Freshly grated cheese (~800 g) was thoroughly mixed with 1000 g of washed sea sand (Fisher Scientific) and placed in the stainless-steel mold lined with cheesecloth (Pyrex Heavy Duty Cheesecloth, Robinson Knife Company, Buffalo, NY). The cheese-sand mixture was subjected to high pressure using a hydraulic press (Fred S. Carver, Inc., Summit, NJ) at room temperature. The pressure was increased gradually over 1 h up to a maximum of approx. 8 MPa, and liquid fat and juice were collected in a graduated cylinder until all flow of liquid

stopped, which was approx. 3 h. The juice in the graduated cylinder was stored in the refrigerator overnight at 4ºC to allow the liquid fat on the top to solidify. A hole was made in the solid fat layer using a spatula, and the juice was decanted through the opening. The juice was then centrifuged at ~2000 × g for 10 min at 4ºC to remove any remaining fat and curd particles.

The cheese, juice, and milk were analyzed for Ca content using ICP-OES (Agilent 5100, Agilent Technologies, Santa Clara, CA) (Park, 2000) and the moisture content was also measured. The percentage of insoluble Ca in cheese was determined as described by Morris et al. (1988).

$$\%\ Insoluble\ Ca = 100\left(\frac{m_x}{H} - \frac{m'_x}{H'}\right)\frac{H}{m_x}$$

Where m_x and m'_x are the concentrations of Ca in cheese and juice represented as mg/100g sample, respectively, and H and H' are the numbers of kilograms of H_2O per kilogram of cheese and juice, respectively.

2.2.3.3 Water Soluble Calcium Extraction

WSC from the cheese samples was extracted by mixing 4 g of cheese in 40 g Milli-Q water at 55ºC using an Ultra-Turrax T-25 Basic Homogenizer (IKA Works Inc., Willmington, NC). The heterogeneous mixture was centrifuged at 10,000 × g for 7 min at 22ºC. The supernatant was filtered through a Whatman #1 filter paper (GE Healthcare Life Sciences, USA), and the filtrate was measured for total calcium using ICP-OES (Agilent 5100, Agilent Technologies, Santa Clara, CA) to obtain the WSC in cheese samples. Insoluble calcium (mg/g protein) was calculated using the formula below.

$$Insoluble\ Ca\ \left({}^{mg}/_{g\ of\ protein}\right) = \frac{Total\ calcium - Soluble\ calcium\ \left({}^{mg}/_{100g\ of\ cheese}\right)}{\%\ Protein}$$

2.2.4 Compositional Analysis

Milk samples were analyzed for total solids (Marshall, 1992), fat by Mojonnier (AOAC International, 2000) protein (total percentage N × 6.38) by Kjeldahl (AOAC International, 2000), and casein (AOAC International, 2000), and non-protein nitrogen (AOAC International, 2000). Total calcium in milk, rennet whey, and cheese was analyzed using ICP-OES (Agilent 5100, Agilent Technologies, Santa Clara, CA). Total calcium in rennet whey, which was corrected for the volume of precipitate, was considered the soluble calcium in milk. The cheeses were analyzed for composition at the 2-wk time point. Total solids, fat, and crude protein was measured using Food Scan™ Lab (Foss), and the pH was measured at 2 d, 2 wk, and 1 mo timepoint by using a spear tip pH probe (accuCap Capillary Junction pH combination electrode; Fisher Scientific, Itasca, IL). The salt content in cheeses was measured using a Chloride Analyzer Model 926 (Corning Glass Works, Medfield, MA).

2.2.5 Statistical Analysis

Statistical analyses were done using SAS (Version 9.4, SAS Institute Inc., Cary, NC). A randomized complete block design (RCBD) was used for method comparison in milk samples and LMPS Mozzarella. RCBD was also used to compare the differences in parameters for the WSC method in a rennet-gel system. A split-plot design was used for DA and SC String cheese to compare methods along with age effects where the trial cheese making days were blocked.

Analysis of variance (ANOVA) was carried out using the PROC GLM procedure for SAS. The level of significance was $P<0.05$ and Tukey's multiple comparison test was done.

2.3 Results and Discussion

2.3.1 Comparison of Various Methods for Insoluble Calcium Measurement in Milk Samples

The insoluble calcium content in four different milk samples measured using UF-10, UF-100, and rennet gel method is shown in Figure 2.1. There were no significant differences between the insoluble calcium values measured using UF-10 and UF-100 ($P=1.00$) but they were significantly different from the rennet gel method ($P<0.0001$) in all four milk samples as seen in Figure 2.1. The rennet gel method consistently measured lower amounts of insoluble calcium as compared to the UF method, approx. 1 ± 0.3 mg/g Ca, i.e., the insoluble calcium measured by UF was always slightly higher. Similar observations were recorded by Davies and White (1960). They observed a sieving effect with the UF membrane since some of the calcium is present as calcium citrate and it may not pass through the membrane easily because of the large size of the molecules. They also found that the temperature affected the amount of soluble calcium diffusing through the UF membrane, i.e., lower temperatures of 4ºC allowed more calcium to pass through than at 20ºC. Our experiments were performed at a consistent temperature of 20ºC.

A further investigation into the soluble calcium retention in the UF retentate from the milk samples was estimated by preparing a 5% calcium chloride solution and filtering it through the 10 kDa UF membrane. It was found that the UF membrane had a retention coefficient of 0.04, i.e., the UF membrane retained about 4% of the total soluble calcium from the samples. This retention of soluble calcium could also be due to the fact the UF employed in this study was a dead-end filtration system. A correction factor of 0.96 was therefore applied to the insoluble calcium values

obtained from the UF method. These corrected UF values when compared with the rennet gel method had no significant differences (P=0.82). Thus, the rennet gel method was comparable to the traditional UF method used for measuring insoluble calcium content in milk samples.

2.3.2 Impact of Centrifugation Speed and Water Temperature on the Insoluble Calcium Measurement in the Rennet Gel System

The insoluble calcium values of a cheese curd system, i.e., rennet gel were used to investigate the impact of operational parameters of the WSC method for curd including centrifugation speed and water temperature used for homogenization of the sample. This experiment was done to see if the method parameters (conditions) would alter the calcium balance in the curd sample, i.e., shifting the calcium from soluble to insoluble calcium form or vice versa. The results of this experiment can be seen in Table 2.1. Neither the water temperature nor the centrifugation speed significantly ($P > 0.05$) affected the insoluble calcium values. Koutina *et al*. (2014) observed similar trends in their skim milk samples when they studied the effects of pH and temperature (4-40ºC) on calcium equilibria. It is important to note that the calcium shifts observed at higher temperatures (20, 30, 40ºC) by Koutina et al. (2014) was because their milk samples were stored for a long time (1 to 7 d) at these temperatures. The kinetics of calcium solubilization is a slow process and we believe that large shifts in the calcium equilibria can be mostly avoided if the sample preparation time is kept to a minimum (e.g., 10 min in our WSC method). Based on these results, the final parameters chosen for the WSC method for cheese curds was 55ºC water temperature and 10,000 × g centrifugation speed; a high water temperature ensures that the curd samples are readily homogenized for soluble Ca extraction, and the centrifugation speed chosen was high enough to obtain separation of supernatant and sediment in a relatively short (10 min) time period.

2.3.3 Comparison of Various Methods for Insoluble Calcium Measurement in String Cheese

Composition of cheese

The composition of the DA and SC String cheeses is shown in Table 2.2. There were no significant differences in moisture or salt content between the two cheeses. SC cheese had a lower amount of fat and higher protein content as compared to DA cheese likely due to the differences in the composition of the initial standardizated milk. The compositional differences between these two cheeses are due to the differences in their manufacturing process. The SC cheese has significantly high amounts of total calcium as compared to the DA cheese as seen in Table 2.2. This was because the amount of acid produced before and after the whey drain step defines the final calcium content in cheese (Lucey and Fox, 1993; Kindstedt et al., 2010). Since DA cheese was acidified directly with an acid to reach a lower pH, more CCP would have been dissolved and lost into the whey as compared to SC cheeses where the acidification was solely due to fermentation of lactose to lactic acid by the starter culture. The rate of acidification in the SC cheese was probably gradual over time and hence not as much CCP would have been dissolved by the whey drain step. Since these two cheeses were commercially obtained, details about pH at rennet addition, whey drainage pH, etc. were unknown. These parameters could also have affected the final total calcium content in the cheeses. DA cheese had a higher pH at the 2 d time point compared to the SC cheese. Similar observations were noticed by Guinee et al. (2002) in their study. Typically, DA cheeses are acidified to higher pH values due to the greater loss of insoluble Ca through direct acidification of milk using organic acids. Therefore the target final pH values of curds are 5.5-5.6 whereas cultured cheeses are acidified until the curds reach pH values between 5.10-5.30.

Method comparison

The insoluble calcium content as measured in the DA and SC String cheese using acid-base titrations and WSC is shown in Figure 2.2. Since there were differences in the protein contents between the two types of String cheese, all the insoluble calcium results were normalized and presented as mg/g protein. As expected, there were significant differences in the insoluble calcium values between the two types of cheeses. DA cheese had lower amounts of insoluble calcium as compared to SC cheese as seen in Figure 2.2. There were no significant differences in test methods for insoluble Ca in the DA cheese but the titrations and WSC methods gave significantly different results for the SC cheese (Table 2.3). In additional testing, when SC String cheese samples were homogenized with water for 2 min and allowed to equilibrate for 3 h at room temperature before centrifugation, i.e., allowing longer extraction time, the equilibrated cheese samples had slightly lower insoluble calcium content (about 0.4-0.6 mg Ca/g protein) as compared to the samples with no equilibration, i.e., more soluble calcium were extracted from the serum phase of the cheese with longer equilibration. This suggests that the dilution of curds involved in the WSC method could shift or alter the calcium balance if the added water was left long enough to dissolve insoluble calcium. The insoluble calcium content was significantly impacted by storage time due to ongoing solubilization during ripening; there was no interaction between age and treatment in the DA cheese but there was a significant interaction between age and treatment in SC cheese as seen in Table 2.3 and Fig 2.2, with titrations measuring significantly lower amounts of insoluble calcium with age.

2.3.4 Comparison of Various Methods for Insoluble Calcium Measurement in LMPS Mozzarella

Composition of cheeses

The composition of the ten different LMPS Mozzarella obtained was similar (results not shown). The moisture, protein, fat, and salt content of these cheeses were approximately 45%, 26%, 23.5%, and 1.7%, respectively.

Method comparison

The insoluble calcium content in ten different LMPS Mozzarella as measured by the different methods, i.e., titrations, cheese juice, and WSC method is shown in Figure 2.3. There were significant differences in insoluble calcium values as measured using the three methods ($P < 0.0001$). WSC gave the highest insoluble calcium values followed by titrations and cheese juice method. Hassan *et al*. (2004) found no significant differences between cheese juice and the titration method in Cheddar cheese but their insoluble calcium values had large variability (error bars) compared to our results. There is no actual reference or standard method for determining insoluble calcium content in cheese. There could be differences in results with these insoluble calcium methods due to differences in mechanism involved in the measurement, but the WSC method gave values of insoluble calcium that were very close to cheese juice and the differences were small and consistent, around 1.5-2 mg Ca/g protein, as shown in Figure 2.3. The cheese juice method does not require buffers nor dilutions and is probably the closest technique to a reference method for insoluble calcium. Overall, the trends for insoluble calcium content in the LMPS Mozzarella cheeses were generally similar, and there was a small but significant impact of the insoluble calcium methods used.

2.4 Conclusions

In this study, we found that the insoluble calcium values obtained from the WSC method were comparable to the traditional methods, i.e., titrations and cheese juice method in String cheese and LMPS Mozzarella samples. In milk samples, the rennet-gel method was similar to the ultrafiltration permeate method in determining the insoluble calcium content. There were no significant differences in the insoluble calcium methods in DA String cheese but the different methods gave slightly different results in SC String cheese. In LMPS Mozzarella, the WSC method gave insoluble calcium values with similar trends as the cheese juice method even though the exact values were slightly different. We found that age could also play a role in the extraction of WSC from the cheeses. The absolute values of the insoluble calcium content in the samples cannot be confirmed using any of these methods since there is no reference method. The WSC method was intended to be a rapid test to measure the calcium balances in cheese during cheese manufacturing process. Therefore, since the WSC method was comparable to traditional method (cheese juice) for LMPS Mozzarella cheese, this WSC method will be used to measure the insoluble calcium values in cheese curds and cheese samples for future experiments in this thesis.

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2.6 Tables and Figures

Table 2.1 Insoluble calcium content (mg/g protein) of rennet curds measured by water-soluble calcium method by varying the water temperature and centrifugation speed (n=2)

Centrifugation speed (× g)	**Temperature (ºC)**		
	20	**40**	**55**
4,000	$31.75^{a,A}$	$31.78^{a,A}$	$31.70^{a,A}$
15,000	$31.79^{a,A}$	$31.85^{a,A}$	$31.79^{a,A}$
30,000	$31.85^{a,A}$	$31.57^{a,A}$	$31.82^{a,A}$

[a]Means within the same row not sharing a common superscript differ ($P<0.05$)

[A]Means within the same column not sharing a common superscript differ ($P<0.05$)

Components	DA Cheese	SC Cheese
Moisture, %	49.96[a]	48.08[a]
Fat, %	23.23[a]	21.05[b]
Salt (Chloride), %	1.90[a]	2.09[a]
Protein (N x 6.31), %	22.11[b]	25.68[a]
MNFS, %	65.07[a]	60.89[b]
FDM, %	46.43[a]	40.54[b]
S/M, %	3.81[a]	4.35[a]
pH @2d	5.46[a]	5.31[b]
Total Ca (mg/g protein)	16.3[b]	27.6[a]

Table 2.2 Composition of direct acid and starter culture String cheese (n=3)

[a,b] Means within the same row not sharing common superscript differ ($P<0.05$)

Table 2.3. Probabilities and R^2 values for comparison of methods used for measuring insoluble calcium content in DA and SC String cheese

Cheese sample	Factor[1]	df	Method
	Whole plot		
DA String cheese	Treatment (T)	1	0.12
	Error	2	0.02
	Split plot		
	Age (A)	4	<0.0001
	A × T	4	0.0625
	R^2	0.94	
	Whole plot		
SC String cheese	Treatment (T)	1	0.01
	Error	2	0.04

Split plot		
Age (A)	4	<0.0001
A × T	4	<0.01
R^2	0.96	

[1]Split-plot design with 2 treatments (insoluble calcium methods) and replicates were blocked (2 × 3). The subplot included the effect of aging of cheese (A) and the interactive term, age × treatment as variables

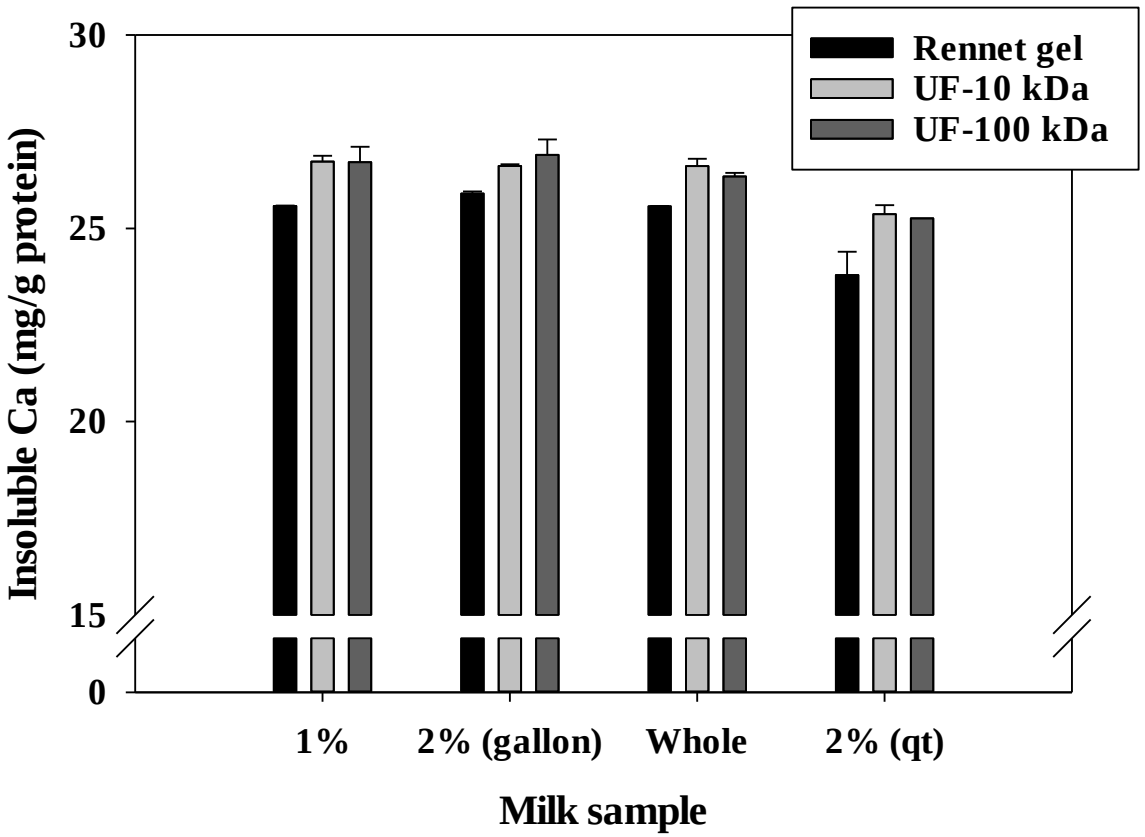

Figure 2.1 Insoluble calcium content in different milk samples with different fat content measured using the rennet-gel method and two different ultrafiltration membranes with a pore sizes of 10 and 100 kDa. Vertical bars represents standard deviations (n=3)

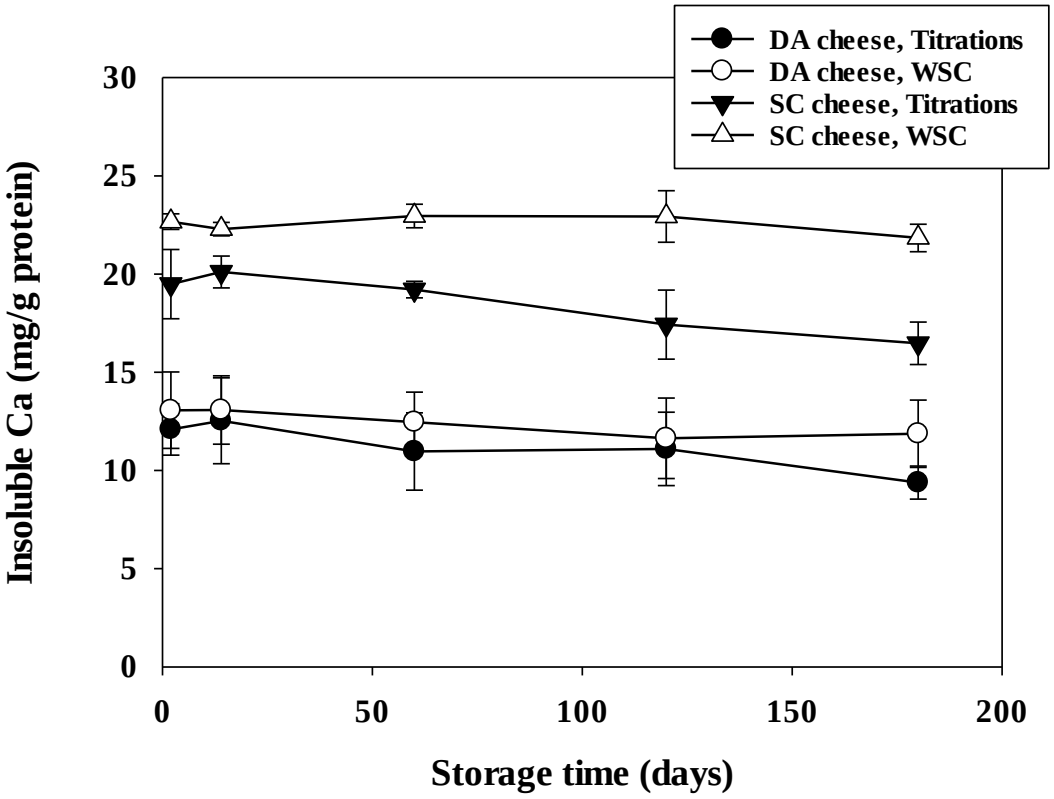

Figure 2.2 Insoluble calcium content (mg/g protein) in direct acid and starter culture String cheeses measured using WSC and titrations. Vertical bars represents the standard deviation (n=3).

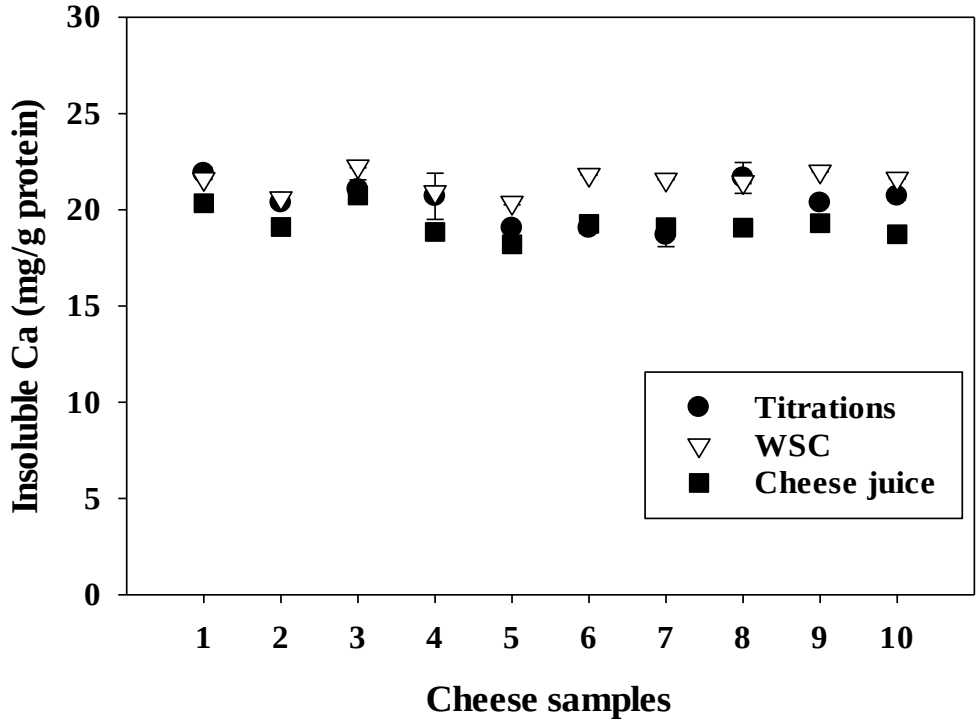

Figure 2.3 Insoluble calcium content (mg/g protein) in ten different LMPS Mozzarella cheeses measured using titrations, cheese juice, and WSC. Insoluble calcium content was measured in duplicate for titrations and cheese juice and in triplicate for WSC. Vertical bars represents the standard deviation (n=3).

Chapter 3. Impact of Rate and Extent of Pre-Acidification and pH at Whey Drainage on Calcium Balances in Milk and Cheese Samples

A part of this chapter has been published in the International Journal of Dairy Technology

Swaminathan, A.V., S.K. Lillevang, and J.A. Lucey. 2023. Measuring insoluble calcium content in curds during cheesemaking. Int. J. Dairy Technol. 1–9. doi:10.1111/1471-0307.13016.

Abstract

The effects of various manufacturing factors, including rates of pre-acidification, pH values at whey drainage and extent of pre-acidification, were studied in model milk and cheese systems. Bench-scale experiments were performed to study the impact of varying the rates of pre-acidification in a milk-like system using glucono-delta-lactone (GDL). This bench-top work showed that the insoluble calcium content in the milk dispersion was higher when faster rates of acidification was used, probably due to insufficient time for complete solubilization of the insoluble calcium phosphate. Pilot-scale experiments were performed on low-moisture part-skim (LMPS) Mozzarella to study the impact of varying the pH values at whey drainage (pH 6.30, 6.10, and 5.90) and extent of pre-acidification (control, pH 6.40, and 6.20) on total and insoluble calcium changes during cheese manufacture. The changes in pH, insoluble calcium content and rheological properties of these cheeses were studied during storage. The pH at whey drainage had a significant effect on the total and insoluble calcium content in curds during cheese manufacture and therefore impacted the final insoluble calcium content in cheeses, even though the milling pH was the same in all the cheeses. Cheeses that had a lower pH value at whey drainage had the lowest insoluble calcium content compared to cheeses that had a higher pH value at whey drainage and this difference was observed throughout the storage period. These differences in the insoluble calcium

content between cheeses also affected its rheological properties. Varying the extent of pre-acidification of milk (using lactic acid) slightly affected the total calcium and insoluble calcium losses from the curds during cheese manufacture even though the cheeses were manufactured using the same pH values at whey drainage and milling step. During storage, there were no significant differences in insoluble calcium, pH, or the rheological properties of these pre-acidified cheeses probably due to these cheeses having the same whey drain and milling pH values as well as same pH values during storage.

3.1 Introduction

Low-moisture part-skim (LMPS) Mozzarella is mostly used an ingredient cheese on pizza and related foods due to the cheese having a long shelf life, firm body, and good shredding properties (Kindstedt, 1993). The textural and functional performance of cheese is influenced by casein interactions (between and within casein molecules) and the amount of calcium associated with the micelles (Lucey et al., 2003). The insoluble calcium content in cheese is more important indicator of cheese functionality than the total calcium content (Lucey and Fox, 1993). If the insoluble calcium content in the cheese is too high or too low, the functional performance of cheese (e.g., on pizza) will be affected, i.e., the cheese won't melt or stretch as much as desired.

During cheesemaking, due to acid production and the decrease in pH, the colloidal (insoluble) calcium phosphate (CCP) solubilizes from caseins. This soluble calcium, now in the aqueous phase of milk/ curd, can be lost from cheese via the whey during the manufacturing process, thereby influencing the total (final) and insoluble calcium content in cheese. The amount of soluble calcium generated from the solubilization of insoluble calcium in milk and curds is dependent on how the cheese is manufactured. If there is not enough acid development in the curd

during cheese manufacture, most of the CCP remains intact. High insoluble calcium levels results in excessive crosslinking between molecules, which limits movement or rearrangement between casein molecules in the curd (Johnson and Lucey, 2006) and the resulting cheese has a curdy/grainy texture. Various manufacturing factors affect the final amount of insoluble calcium in cheese, with the most important parameters being the rate and extent of acidification, pH at whey drainage, and pre-acidification pH values for milk, i.e., the pH at renneting (Lucey and Fox, 1993; Lucey et al., 2003).

The pre-acidification process involves acidifying the cold milk (usually ~ 4ºC) before renneting using food grade organic acids, such as, citric, acetic, lactic acid, etc. This is usually done to: (1) reduce the total cheese manufacturing time rather than relying completely on starter cultures for acidification and (2) to dissolve some CCP as calcium is also more easily dissolved in milk than from the coagulum/curd environment (Johnson and Sommer, 2013). The amount of insoluble calcium solubilization is also dependent on the overall rate and extent of acid production (from starter culture addition to milling) and the amount of acid produced before and after the whey drainage step. The overall rate of acid production is important because it determines the total manufacturing time, which in turn influences the amount of syneresis during manufacture and therefore impacts the moisture content of the final cheese (Barbano et al., 1994). The rate of pre-acidification (time taken to pre-acidify the cheese milk) also influences how much insoluble calcium is dissolved from the cheese milk. If the cheese milk is quickly acidified to a target pH value, say within 5 min, the pH can buffer up and insufficient insoluble calcium may be dissolved. This is one of the topics of interest in this chapter where we want to study the impact of varying the rates of pre-acidification on the amount of insoluble calcium solubilization during cheese making.

The pH value at whey drainage is a very critical step in the manufacturing process. Making cheese is partly a concentration process, and the whey drainage step is where most of the moisture is separated from the curds (Lucey and Fox, 1993), approx. 35-45% depending on the pH value at whey drainage and cooking temperature employed during cheese manufacture. Therefore, the whey drainage step is where most of the calcium that is in the soluble phase is removed from the curds. Thereafter, the curd only slowly synereses and the total calcium content in the curds can only slightly decrease. But the calcium balance, i.e., the ratio of soluble to insoluble calcium, can still change after the whey drainage step depending on the moisture lost after whey drainage step and the pH of the curd. The pH at the whey drainage step is therefore important and it likely influences the total and insoluble calcium contents in the final cheese.

Yun et al. (1995) studied the effects of various pH values at whey drainage on the properties of LMPS Mozzarella cheese, and they showed that the cheese manufactured with lower pH values at whey drainage had lower amounts of total calcium (per g of protein). This, in turn, influenced cheese hardness where cheese that had a lower pH value at whey drainage was softer than cheese that had a higher pH value at whey drainage. The limitations of this study are: (1) they only measured total calcium but not the insoluble calcium after manufacture, and (2) the amount of insoluble calcium dissolved as a result of acid production before whey drainage, and subsequently the amount of soluble calcium lost from whey during cheese manufacture, was unknown. We therefore wanted to study the total and insoluble calcium changes before and after whey drainage step (during the cheese manufacturing process). This would help us understand the impact of varying the pH at whey drainage on the final total and insoluble content in the cheese, as well as its effects on the rheological properties of the cheese.

Metzger et al. (2001b) studied the effects of pre-acidification of milk on the functionality of fat-free Mozzarella. They pre-acidified the cheese milk to pH 6.0 and 5.8 with acetic acid before the addition of starters and rennet. They found that the cheeses that were pre-acidified to a lower pH (i.e., 5.8) had a significantly lower amount of total calcium as compared to the cheese that was pre-acidified to pH 6.0. It should be noted that these two cheeses also had different pH values at whey drainage, i.e., 5.90 and 5.72, respectively, even though the final milling pH was the same (pH 5.30). Similar experiments were performed by Mizuno et al. (2009). Mizuno et al. (2009) pre-acidified the milks to different pH values, control (no pre-acidification), pH 6.46, 6.25, and 6.05 using citric acid. Each of these cheeses also had different pH values at whey drainage, i.e., 6.13, 6.07, 5.92, and 5.81, respectively, with all cheeses having the same milling pH (pH 5.30). They found in their study that the insoluble calcium content in the cheeses decreased significantly as the pre-acidification pH values of the cheese milks were reduced. In both these studies, as the pre-acidification pH value was lowered, the pH at whey drainage also was lower. Therefore, it is difficult to separate the effects of pre-acidification from the impact of the pH values at whey drainage on the calcium solubilization in these studies. In our experiment, we want to separately study the effects of extent of pre-acidification (varying pre-acidification pH values) on calcium changes during cheese manufacture, while keeping the pH at whey drainage and milling pH values constant during cheese manufacture. The insoluble calcium content changes and the rheological properties of manufactured cheese were measured during storage to understand the calcium effects on the functional performance of cheese.

Research Hypotheses

- Milks pre-acidified with a slower rate of acidification will dissolve a lot more insoluble calcium than milks acidified at a faster rate due to the increased time allowed to dissolve insoluble calcium phosphate.

- While it is known that the pH value at whey drainage has a significant effect on the total loss of calcium in cheese but exactly how much calcium is lost in whey at whey drain and how much more is lost after whey drain step in cheeses made with differing pH values at whey drainage is still unknown.

    - Cheeses manufactured using a lower pH value at whey drainage lose a lot more calcium in the whey during the drainage step as compared to cheeses, where whey is drained at a higher pH value. This is due to the progressive increase in calcium solubilization with a decrease in pH, thereby resulting in a cheese with lower amounts of total (final) and insoluble calcium, even when the final milling pH of the cheeses is the same.

- It is known that the extent of pre-acidification can have a significant effect on the total (final) and insoluble calcium in cheese due to the easier solubilization of calcium phosphate in milk compared to a curd environment. The amount of solubilization of insoluble calcium due to differing pre-acidification pH values, in cheese made with constant values for the pH at whey drainage and pH at milling, is still unknown. Past research studied the effect of the extent of pre-acidification, but they also had different pH values at whey drainage, therefore the effects of pre-acidification and pH values at whey drainage were confounded.

- Cheeses manufactured with lower pre-acidification pH values will have slightly lower amount of total (final) and insoluble calcium due to the increased solubilization of insoluble calcium in a milk system, resulting in more soluble calcium lost to whey compared to cheeses manufactured using a higher pre-acidification pH, even though the pH at whey drainage and milling are the same.

3.2 Materials and Methods

3.2.1 Bench-Scale Experiment

Non-fat dry milk (NFDM) powder (low heat) containing 35% protein (db) was procured from the Center for Dairy Research (CDR), UW-Madison. The NFDM solution was prepared by dissolving appropriate quantities of NFDM powder in Milli-Q water. These solutions were stirred at room temperature using a magnetic stirrer until completely dissolved and refrigerated until utilized.

Varying rates of acidification in NFDM solution using glucono-delta-lactone

Various concentrations of glucono-delta-lactone (GDL) including 0.05, 0.075, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5, and 1% were added to 10% (w/v) rehydrated NFDM to determine the GDL concentrations required to reach pH values of 6.30 and 6.10. GDL concentrations of 0.1% and 0.2% were chosen to adjust the pH of the solutions to pH 6.30 and 6.10, respectively. The milk dispersions were brought to a temperature of 40ºC in a water bath before the GDL was added. The initial pH of the solution was approx. 6.70. The change in pH of the dispersions over time was measured by using a spear tip pH probe (accuCap Capillary Junction pH combination electrode; Fisher Scientific, Itasca, IL) until the pH change plateaued and did not change further (the pH of the dispersions plateaued around 60-100 min for GDL concentrations below 0.2%, whereas for the

dispersions with ≥ 0.2% GDL it took about 120-180 min to plateau). The rate of acidification (the time taken to reach the desired pH values of 6.30 and 6.10) for a particular target pH value was varied by altering the temperature of acidification, i.e., 40, 44, and 48ºC and it took around 20, 40, and 60 min to reach the pHs at the respective temperatures mentioned for both pH values.

3.2.2 Pilot-Scale Experiment

3.2.2.1 Milk Processing and Standardization

Milk for cheese manufacturing was standardized to 2.55% fat with a casein-to-fat ratio of 1.0. The milk was then subjected to pasteurization at 74ºC for 19 seconds.

3.2.2.2 Cheese Manufacturing

pH at whey drainage experiment

LMPS Mozzarella was manufactured from standardized pasteurized milk (2.55% milk fat) obtained from the University of Wisconsin-Madison Dairy Plant on three separate days. Three 21-L mini-cheese vats equipped with an automatic variable speed agitator were used to make cheeses in this study. 20 kg of milk was transferred into each vat and the milk was warmed to 34ºC before frozen direct vat-set *Streptococcus thermophilus* (Delvo CP® Cheese-121; DSM, Waukesha, WI) was added. The starter cultures were given a ripening time of 30 min at 34ºC. Fermentation-produced calf chymosin (Maxiren® XDS, DSM, Waukesha, WI) was used as a coagulant. Once a gel was formed and the desirable firmness was attained, the coagulum was cut with 0.63-cm knives and allowed to heal for 5 min. The curds were cooked to a temperature of 41ºC. The whey was drained when the curds reached the pH values of 6.30, 6.10, and 5.90, respectively. The curds were milled at pH 5.20 and pre-salted in two additions, five min apart. The curds were then hand-stretched at a water temperature of 55-60ºC, molded into blocks, cooled below 38ºC by immersing

in cold water, brined for 20 min in a 20% (w/w) salt solution held at 4ºC, after which the cheeses were refrigerated until the selected analysis timepoint.

Pre-acidification of milk experiments

LMPS Mozzarella was manufactured using the same cheesemaking equipment as described above. 20 kg of standardized milk with 2.55% milk fat was transferred into each vat. The control milk was immediately warmed to 34ºC and direct vat-set *Streptococcus thermophilus* (Delvo CP® Cheese-121; DSM, Waukesha, WI) was added. 5 min after starter culture addition, 1.3 g of fermentation-produced calf chymosin (Maxiren® XDS, DSM, Waukesha, WI) was added to the milk. Two other vats were pre-acidified to pH 6.40 and 6.20, respectively, and the pre-acidification was performed at 6ºC by using diluted lactic acid (1 part of 88% (w/w) lactic acid diluted in 4 parts of water), which was added before starter culture addition. Since the milk in one of the vats was not pre-acidified and the other two vats were pre-acidified to two different pH values, the pH at renneting was different for all 3 cheeses: pH 6.70 (control), pH 6.40 and pH 6.20. Once the coagulant was added and a gel with desirable firmness was attained, the coagulum was cut with 0.63-cm knives and allowed to heal for 5 min. The curds were cooked to a temperature of 41ºC and held until the curds reached the target pH of whey at drainage of 5.90. The curds were all milled at pH 5.20 and pre-salted in two additions, five min apart. The curds were then hand-stretched, cooled, brined and the cheeses were refrigerated as described in the previous cheese make above.

During the manufacture of the above two types of experimental cheeses, the following samples were obtained for determining the total and insoluble calcium in the curds during cheese

manufacture: initial milk, curds after whey drain, milled curds (before pre-salting), stretched curd and brined cheese. After brining, all the cheeses were stored at 4ºC and analyzed at 2 and 4 weeks.

3.2.3 Methods for Measuring Insoluble Calcium

Water soluble calcium extraction from rehydrated NFDM dispersions

Amicon® Ultra-4 centrifugal filters with built-in Ultracel® 10kDa regenerated cellulose membrane (Merck Millipore Ltd, Tullagreen, Carrigtwohill, Co. Cork, IRL) were utilized to collect permeate from the rehydrated milk samples for soluble calcium measurement. Different centrifugation speeds and times were explored to generate various levels of permeate from the sample. It was observed that a combination of 15,000 × g for 10 min worked the best to generate the largest amount of permeate necessary for analysis.

Water soluble calcium extraction from curds and cheese

During cheese manufacture, curd samples obtained for WSC measurement were sheared for 30 sec using a food processor (Hamilton Beach®, Model 70730, Glen Allen, VA) before subjecting the mixture to water soluble calcium (WSC) extraction. The WSC from the curd/cheese samples was extracted by blending 4 g of ground curds/cheese in 40 g Milli-Q water at 55ºC using an Ultra-Turrax T-25 Basic Homogenizer (IKA Works Inc., Willmington, NC) at a speed of 7,000 rpm. The heterogeneous mixture was centrifuged at 10,000 × g for 10 min at 22ºC (selected from earlier model rennet gel preliminary work). The supernatant was filtered through a Whatman #1 filter paper (GE Healthcare Life Sciences, USA), and the filtrate was stored for subsequent analysis of soluble calcium. Curd samples drawn for WSC measurement during cheese manufacture was also measured for moisture and protein content as this is required for calculating the insoluble calcium content and for standardizing results in terms of mg Ca per gram protein. If the insoluble

calcium content was not represented as per g protein, comparison of insoluble calcium results between curd samples drawn at various points in the process (for example, curds after whey drain and milled curds) would be difficult due to large differences in moisture contents between these samples. It was important that the WSC slurry be immediately centrifuged after homogenization in order to avoid any potential shifts in calcium balances because the changes in the moisture and pH in the curd samples could also affect the amount of calcium extracted.

The WSC in curds/cheese samples was calculated as shown in the equations below; the insoluble calcium content (mg/g protein) was calculated by subtracting the soluble calcium from total calcium.

$$Soluble\ Ca\ (mg/100g) = \frac{Ca\ in\ WSC\ extract\ (mg)}{Weight\ of\ sample\ (g)} \times Moisture\ in\ cheese\ (\%)\ \times D \qquad (1)$$

$$Insoluble\ Ca\ (mg/g\ of\ protein) = \frac{Total\ calcium - Soluble\ calcium\ (mg/100g)}{\%\ Protein} \qquad (2)$$

Where, D is the dilution factor, which was 11.

3.2.4 Compositional Analysis

Milk samples were analyzed for total solids (Green and Park, 1980), fat by Mojonnier (AOAC International, 2000), protein (total percentage N × 6.38) by Kjeldahl (AOAC International, 2000), and casein (AOAC International, 2000), and non-protein nitrogen (AOAC International, 2000). Total calcium in milk, rennet whey, and cheese was analyzed using ICP-OES (Agilent 5100, Agilent Technologies, Santa Clara, CA) (Park, 2000). Total calcium in rennet whey, which was corrected for the volume of precipitate, was considered the soluble calcium in milk. The cheeses were analyzed for composition at the 2-week time point. Total solids, fat, and crude protein was

measured using Food Scan^{TM} Lab (Hillerød, Denmark), and the pH was measured at 2 d, 2 wk, and 1 mo timepoint by using a spear tip pH probe (accuCap Capillary Junction pH combination electrode; Fisher Scientific, Itasca, IL). The salt content in cheeses was measured using a Chloride Analyzer Model 926 (Corning Glass Works, Medfield, MA) (Johnson and Olson, 1985).

3.2.5 Rheological Properties

The rheological properties of LMPS Mozzarella cheeses were measured using a dynamic low-amplitude oscillatory rheology with an Anton Paar Rheometer (MCR 302; Physica Messtechnik, Stuttgart, Germany) basically as described by Lucey et al. (2005). A 25 mm diameter serrated plated probe was used. The LMPS Mozzarella cheese samples were cut using a borer and a Hobart Slicer into disks with a diameter of 25 mm and a thickness of 3 mm. The samples were held at 4ºC overnight before testing at each of the time points: 2 and 4 wk. The discs were placed on the rheometer and heated from 5 to 85ºC at a rate of 1ºC per min. Vegetable oil was placed around the sample before measurements to prevent moisture evaporation. The storage modulus (G′), loss modulus (G″), and loss tangent (LT) were measured using a frequency of 0.08 Hz and a strain of 0.5% (Govindasamy-Lucey et al., 2005). The maximum LT (LT_{max}) was used as a measure of cheese meltability, with a higher value indicating a more meltable (fluid) cheese (Lucey et al., 2003). The rheological tests were performed in duplicate or until the rheograms were closely replicated.

3.2.6 Statistical Analysis

Statistical analysis was performed using SAS (Version 9.4, SAS Institute Inc., Cary, NC). A randomized complete block design with 3 treatments and three replicates was used for analysis of the bench-scale rate of acidification experiment and to evaluate the composition of cheese. A

split-plot design was utilized for pilot-scale cheese experiments to evaluate the effects of either: (1) differing pH values at whey drainage or (2) pre-acidification pH values on differences in total and insoluble calcium in samples during cheese manufacture, and ripening times and their interactions on pH and insoluble calcium during storage. A randomized complete block design was utilized to evaluate the differences in rheological properties of cheese samples during storage. An analysis of variance (ANOVA) was carried out using the PROC GLM procedure for SAS. The cheesemaking days of the trials were blocked. Tukey's multiple comparison test was done to evaluate differences in treatments; differences between means were considered significant at $P < 0.05$.

3.3 Results and Discussion

3.3.1 Impact of the Rate of Acidification (time to reach the target pH value) on Insoluble Calcium Content in Milk Systems using Glucono-Delta-Lactone

The effect of rate of acidification on the insoluble calcium content in 10% NFDM solutions is shown in Figure 3.1. As expected, dispersions that were acidified to a lower pH, i.e., pH 6.10, had significantly lower insoluble calcium contents as compared to the dispersions that were acidified to a higher pH value of 6.30 ($P<0.0001$) (Figure 3.1). The insoluble calcium contents of the dispersions at both pH values were also significantly ($P<0.0001$) impacted by the varying time used for acidification (rates of acidification). A longer time allowed for acidification resulted in significantly lower insoluble calcium levels (Figure 3.1), i.e., a slower rate of acidification allowed more time to solubilize the insoluble calcium phosphate in the NFDM dispersions. Although the insoluble calcium differences between the samples that were acidified with varying rates were relatively small, they were still significantly different. Similar trends were observed by Peng et al.

(2009). In their yogurt experiments, the dispersions were pre-acidified with GDL to pH values of 6.55, 6.42, 6.10, 5.78, and 5.65 and the soluble calcium concentrations at these pH values were measured. A statistically significant increase in soluble calcium, i.e., decrease in insoluble calcium, with a decrease in the pH of pre-acidification was observed (Peng et al., 2009). When milk samples were pre-acidified to same pH of pre-acidification (pH 6.10) and they varied the yogurt fermentation times from 250, 375, and 500 min to reach the target pH value for their yogurt (pH 5.0), they found that the samples had a significant difference in insoluble calcium contents i.e., 42.8, 40.3, and 21.0 mg/100 g insoluble calcium, respectively.

In terms of cheesemaking approaches, the results from these experiments suggested that a slower rate of acidification should be used when pre-acidifying the cheese milks, if the goal is to dissolve more insoluble calcium before starter culture and rennet addition (especially during pre-acidification of concentrated milks, which is a topic of interest in the following chapters of this thesis).

3.3.2 Impact of Curd pH at Whey Drainage on Calcium Balances and Rheological Properties of LMPS Mozzarella Cheese

3.3.2.1 Time Taken for Cheese Manufacture

During cheese making, the total manufacturing time from starter addition to milling was slightly longer in cheese that had a pH of 6.30 at whey drainage, i.e., 205 min ± 1 compared to cheeses that had a pH of 6.10 and 5.90, which were 193 ± 2 and 193 ± 8 min, respectively. Although the time taken to reach different pH values at whey drainage was slightly different, the total manufacturing times were similar because it was compensated by the time taken to reach the milling pH. Similar observations have been made by Yun et al. (1995), who manufactured cheeses

with two different pH values at whey drainage, i.e., pH 6.40 and 6.15, and were able to obtain similar total manufacturing times between the two types of cheeses.

3.3.2.2 Composition of Cheese

The composition of LMPS Mozzarella cheeses manufactured using the three different pH values at whey drainage values is shown in Table 3.1. All the cheeses had moisture and fat levels that met the code of federal regulations (CFR) requirements (21CFR133.158) for LMPS Mozzarella. There were no significant differences ($P < 0.05$) in the moisture, protein, fat, and salt levels between the cheeses. The total calcium content was significantly lower in cheese that had a pH value of 5.90 at whey drainage. Although, the total calcium content was slightly lower in cheese that had a pH value of 6.10 compared to the cheese that had a pH value of 6.30 at whey drainage, they were not significantly different. The rate of solubilization of CCP is highly pH dependent and we observed a significant difference in total calcium value only when the pH was lowered below pH 6.0 in our study. Similar total calcium values in cheese have been reported by Yun et al. (1995). The pH values at whey drainage used by Yun et al. (1995) was 6.40 and 6.15 and the total calcium values in their cheeses were 29.6 and 27.2 mg/g of protein, respectively, which were similar to our results (Table 3.1).

3.3.2.3 Changes in Total and Insoluble Ca at Various Steps during Cheese Manufacture

The changes in total calcium content during cheese manufacture are shown in Figure 3.2. The total calcium in the initial milk sample was around 40 mg/g of protein. A large change in total calcium happened during the whey drain step, i.e., ≥ 10 mg Ca/g of protein was lost in the whey (depending on the pH value of the curd at drainage). There was only a smaller change in calcium

(< 2 mg Ca/g protein) after the whey drain step as the syneresis from the curd was lower after the drainage step. Even though all the cheeses were milled at the same pH, i.e., pH 5.20, there were still significant differences in the amounts of total calcium content in the manufactured cheeses at milling, stretching, and brining (P=0.0108). It is well known that as the pH of milk decreases, the CCP is solubilized from the casein micelles (Lucey and Fox 1993). As the pH of the curd at whey drainage in cheese decreased, more CCP was solubilized and more soluble calcium was lost in the whey in the whey fraction at the drainage step. Kiely et al. (1992) made similar observations for the total calcium content during the manufacture of LMPS Mozzarella with various pH values of whey at drainage, such as, pH 6.40, 6.15, and 5.90. The total calcium content range between their final cheese samples (24.5 – 30 mg Ca/g protein) was higher than ours, likely because in their study the whey was drained based on whey pH values whereas in our study the whey was drained based on curd pH values. The difference range in curd pH values in their study was approx. 0.3 units, which was larger than the difference in our study i.e., 0.2 units between the various treatments.

The insoluble calcium content (mg Ca/ g protein) changes during LMPS Mozzarella cheese manufacture are shown in Figure 3.3. The initial cheese milk had an insoluble calcium content of 28 mg/g of protein. There was a significant difference in the insoluble calcium contents between the cheeses at the whey drainage step ($P < 0.05$). The insoluble calcium content also decreased significantly during the remainder of the cheese making process (Figure 3.3). The curds after whey drain made with a pH of whey drainage of 6.30 had insoluble calcium content similar to the initial milk (28 mg Ca/g of protein), suggesting that little solubilization of CCP had yet occurred. A significant reduction in insoluble calcium amounts between the milk and the curd samples at the whey drainage step was observed only at pH of whey drainage values of ≤ 6.10. This can be

explained by the fact that the CCP solubilization progressively increases at pH values ≤ 6.0 (Lucey et al., 2003). The insoluble calcium content in the curds continued to decrease further between the whey drain step and milling step where the pH value was approx. 5.20. This was because as the pH of the curds decreased, ongoing CCP solubilization occurred. The insoluble calcium contents in all three cheeses only slightly changed after milling, stretching, and brining step, probably due to the lack of a significant further pH change as the heating during stretching step likely inactivated most of the starter culture and arrested further acid development.

3.3.2.4 pH and Insoluble Calcium Changes in Cheeses during Storage

The pH changes during storage for all cheeses can be seen in Figure 3.4. There were significant differences in pH values between the cheeses manufactured using different pH values at whey drainage as shown in Table 3.2. Even though all the cheeses had the same final pH at milling (pH 5.20), the pH of all cheeses had increased by the 2-d timepoint. The cheeses that were drained at pH 6.30 had the largest increase in their pH values between milling and the 2-d timepoint compared to the other cheeses. This could be related to more buffering (Hassan et al., 2004) since this cheese had the highest amount of total calcium (Figure 3.2) and insoluble calcium at the end of brining (Figure 3.3). The pH values were also significantly different between cheeses at all storage time points (Table 3.2) and the pH slowly increased in all of the cheeses between the 2 d and 1 mo timepoint (Figure 3.3). This pH increase was likely due to slow ongoing slight solubilization of insoluble calcium as seen in Figure 3.5. The insoluble calcium content was significantly different between cheeses at all storage time points (Table 3.2). The insoluble calcium content decreased between the 2 d and 2 wk timepoints but did not exhibit much further change. As calcium dissolves from CCP, the phosphate ions are released allowing them to interact with H^+ ions (buffering), thus decreasing the free H^+ ion concentration and causing an increase in the pH

of cheese (Hassan et al., 2004). Similar trends for pH and insoluble calcium changes during storage have been observed by Moynihan et al. (2014). They also observed a slight increase in pH concomitant with a reduction of insoluble Ca during the 30-d refrigerated storage period for LMPS Mozzarella.

3.3.2.5 Rheological Properties

The LT_{max} value was significantly ($P < 0.05$) impacted by the pH at whey drainage and with ripening time as shown in Figure 3.6. The LT_{max} value has been used as an indicator of cheese meltability with higher values corresponding to a more meltable cheese (Lucey et al., 2003). The LT_{max} value at 14 d of storage was lowest in the cheeses made with pH value of 6.30 at whey drainage (Figure 3.6), which indicates that this cheese did not melt as much as the cheeses made with lower pH values at whey drainage. There were no significant differences in LT_{max} values between the cheeses made with pH of whey drainage of 6.10 and 5.90. An increase in LT_{max} value was observed with an increase in cheese age, i.e., the cheese became more meltable (Figure 3.6). The crossover temperature was also significantly ($P < 0.05$) affected by pH at whey drainage and age of cheese. The crossover temperature (LT=1) slightly decreased with a reduction in the pH at whey drainage and also slightly decreased with storage time (Figure 3.7). The rheological properties of natural cheese can be influenced by both the insoluble calcium amounts in cheese and the extent of proteolysis (Lucey et al., 2003). The initial changes in rheological properties for a LMPS Mozzarella type cheese during storage (approximately 3 wks to 1 mo) can be mostly attributed to changes in insoluble calcium and related pH changes before proteolysis becomes important during extended storage (Johnson and Lucey, 2006). Since the insoluble calcium content was lowest in the cheese that had a pH of 5.90 at whey drainage, this cheese sample melted more readily than the cheeses that had a higher pH value at whey drainage.

3.3.3 Impact of Pre-acidification pH on Calcium Balances and Rheological properties of LMPS Mozzarella

3.3.3.1 Acid-Base Titrations Curves of Control and Pre-acidified Milks

Figure 3.8 shows the buffering curves of the initial milk (Figure 3.8a) and milk pre-acidified to pH 6.40 (Figure 3.8 b) and 6.20 (Figure 3.8 c) using lactic acid. The buffering areas related to CCP solubilization (Lucey et al., 1993) of these milk samples were 0.0121, 0.0120, and 0.0117, respectively. A slight reduction in the CCP buffering area was observed with the lowest pre-acidification pH. Buffering hysteresis in milk samples between pH 6.7 to 4 is mainly caused by CCP solubilization (Lucey et al., 1993). As the milk is acidified, the CCP dissolves and the phosphate ions (dissociated from CCP) combine with H^+ ions to form HPO_4^{2-} and $H_2PO_4^-$ (Lucey et al., 1993). When the milk was pre-acidified with lactic acid to lower pH values, more CCP was dissolved in this process, and this lowered the amount of residual CCP in milk and thereby lowered the buffering area measured in pre-acidified milk. An increase in buffering can be observed between pH 3.8 to 4.0 as the milk was pre-acidified to a lower pH value. The pKa of lactic acid is approx. pH 3.9, thus the increase in buffering around this pH range is likely due to the buffering of lactic acid itself added to the milk.

3.3.3.2 Time Taken for Cheese Manufacture

The total time taken for manufacturing the control, and LMPS Mozzarella cheeses pre-acidified to pH 6.40 and 6.20 were 197 ± 6, 190 ± 14, and 192 ± 17 min, respectively. The time taken by all three cheeses to reach the milling pH from the starter addition was similar. Significant

differences in coagulation times were observed between the cheeses. The control cheese took 45 ± 2 min to reach a suitable gel firmness ready for cut (as determined by a licensed cheesemaker), whereas the cheeses pre-acidified 6.40 and 6.20 took a shorter time to reach the required gel firmness, i.e., 21 ± 2 and 14 ± 5 min, respectively. The rennet coagulation properties can be affected by the pH and the insoluble calcium contents in the sample due to changes in the electrostatic and hydrophobic interactions; the reduced coagulation time for pre-acidified cheeses was likely due to the reduction in charge on the casein micelles as the pH was lowered, therefore reducing electrostatic repulsion between the casein micelles, which accelerates rennet gel formation (Choi et al., 2007). The higher concentration of ionic calcium (due to calcium solubilization from CCP) as cheese milk is pre-acidified to lower pH and an increase in rennet activity with a decrease in pH have also been shown to reduce the rennet coagulation time of milk with a reduction in pH (Fox et al., 2017). Similar observations have been found by others in their pre-acidified cheese experiments (Choi et al., 2008; Sheehan and Guinee, 2004; Guinee et al., 2002; Metzger et al., 2000).

3.3.3.3 Composition of Cheese

The composition of the cheeses made from pre-acidified milk is shown in Table 3.3. There was no significant difference in the salt and protein content between the cheeses. There was a minor difference in the moisture content in the cheese pre-acidified to pH 6.20. Cheese pre-acidified to pH 6.20 had the lowest moisture content compared to the other two cheeses. This was in contrast to a previous study by Sheehan and Guinee (2004), who reported that the pre-acidified cheeses in their study had a higher moisture content than the control cheese made with only with starter culture. It is important to note that the set pH (pH at rennet addition) and whey pH at drainage were pH 5.60 and pH 5.54 in the Sheehan and Guinee (2004) study, which was much

lower than the set pH (pH 6.60) and pH at whey drainage (pH 5.90) in our study. Their cheeses (Sheehan and Guinee, 2004) were pre-acidified to very low pH values, pH 5.60, using lactic acid, which should have caused a significant decrease in total calcium compared to the control cheese (total calcium in their pre-acidified cheeses were 10 mg/g protein lower than the control cheese). This could in turn cause a significant increase in casein hydration. Rennet coagulation at pH 5.60 would also make the gel structure firmer and could help retain more moisture in the curds (Fox et al., 2017). The slightly lower moisture content in our pre-acidified cheeses might be due to pre-acidified cheeses taking a longer time to reach the milling pH of 5.20 after whey was drained compared to the control cheese, i.e., 40 min compared to control cheese which took only 20 min. Longer cooking/stirring times during our cheese making process could have caused a reduction in curd moisture. The time taken to reach milling pH after whey drain can be reduced (adjusted) by giving some additional ripening time for the starters before coagulant addition.

There was a minor difference in fat content and a significant differences in total calcium between the cheeses. The slight differences in fat content could be due to the impact of the moisture differences between the cheeses. Control cheese had slightly higher total calcium content compared to cheese pre-acidified to pH 6.20 due to high set/ renneting pH (pH 6.60 in control cheese), i.e., less calcium was dissolved in the control milk as compared to pre-acidified milks before the milk was converted to a gel, which could restrict further calcium solubilization.

3.3.3.4 Changes in Total and Insoluble Ca at Various Steps during Cheese Manufacture

The total calcium contents in all three cheeses during cheese manufacture are shown in Figure 3.9. The total calcium in the initial cheese milk sample was around 46 mg/g of casein. The total calcium content in the cheese samples were significantly ($P < 0.05$) affected by the pre-

acidification pH value used during manufacture (Table 3.3). Pre-acidification to pH 6.20 resulted in lowered total calcium content at all stages of cheese manufacture (Figure 3.9). Even though all these cheeses had the same pH at whey drainage, the renneting pH where the milk was converted into a gel had a significant effect on the solubilization of calcium; since the control cheese had to dissolve more calcium from the renneting step to the whey drain step, the dissolved calcium in the serum phase of the curds would have become more saturated in the control cheese as compared to the pre-acidified cheeses reducing the driving force for further calcium solubilization, even though the control cheese had slightly higher moisture (2-4% higher moisture in the gel when cook temperature was reached and approx. 1-2 % higher in the curds after the whey drain step). From the whey drain step (pH ~ 5.90), the total calcium content decreased very slightly (~0.5-1 mg Ca) (Figure 3.9). The control and the pH 6.40 pre-acidified cheeses seemed to have lost slightly more total calcium from the curds during the stretching process as compared to the pH 6.20 pre-acidified cheese. The total calcium in the cheese samples of control, pre-acidified to pH 6.40 and 6.20 was 27.9, 27.1, and 25.9 mg Ca/g protein, respectively.

The insoluble calcium changes during cheese manufacture can be seen in Figure 3.10. The initial milk sample had an insoluble calcium content of 31.4 mg Ca/ g protein. The insoluble calcium content significantly decreased in all of the cheeses as cheese-making progressed. There was a trend of lower insoluble calcium levels with a decrease in the pre-acidification pH value of milk (Figure 3.10). However, there were no significant differences in insoluble calcium content between the cheeses ($P > 0.05$) probably due to large variability between trials as seen from the error bars in Figure 3.10. At the whey drain and the milling step, even though the curd pHs were similar in all cheeses, the pre-acidified cheeses had lower insoluble calcium as compared to the control cheese. As more total calcium was lost from the curds in the control and the pH 6.40 pre-

acidified cheese at the stretching step, more insoluble calcium was also dissolved as some of the soluble calcium was removed from the saturated serum phase allowing the calcium equilibrium to shift towards solubilization of CCP in these cheeses. By the brining step, the insoluble calcium content varied by approx. 2 mg Ca/g protein between the control and the pH 6.20 pre-acidified cheese.

3.3.3.5 pH and Insoluble Calcium Changes in Cheeses during Storage

The pH and insoluble calcium changes in the cheese during storage can be seen in Figures 3.11 and 3.12, respectively. Even though all three cheeses had the same pH at milling during manufacture, the pH slightly increased approx. 0.05 pH units by 2 d of refrigerated storage due to buffering (Figure 3.11). This was similar to the trends observed by Sheehan and Guinee (2004). There were no significant differences in pH values between treatments or with age (Table 3.4). This result however contrasts with the observations made by Metzger et al. (2001a) and Sheehan and Guinee (2004) in their reduced fat Mozzarella cheeses. These authors found that the control cheese increased in pH whereas the pre-acidified cheeses stayed the same or decreased over the storage period. It is important to note that the moisture content in their cheeses was dramatically higher, i.e., approx. 55% whereas the moisture content in our study was more typical of LMPS Mozzarella at approx. 45%. They also had a much lower whey drain pH values in their pre-acidified cheeses, which could cause differences in buffering content and thereby impact subsequent cheese pH changes during storage.

Insoluble calcium content during ripening was only slightly but not significantly different ($P = 0.06$) between the cheeses (Table 3.4). Cheese made from milk pre-acidified to pH 6.20 had the lowest amount of insoluble calcium as compared to cheese made from milk pre-acidified to

6.40 and control cheese as shown in Figure 3.12. The insoluble calcium content of the cheeses did not change during the 30 d storage period (Table 3.4). Metzger et al. (2001b) reported a decrease in insoluble calcium with age in their pre-acidified cheeses. This could be because they pre-acidified the cheeses in their study to pH 6.0 and 5.8 and hence the resulting pH of cheeses during storage were approx. 5.15, about 0.1-0.15 pH units lower than in our study. The moisture content in their cheeses were also significantly higher and this could have provided more quantity of serum phase available in their cheeses to hold higher amounts of solubilized calcium. In contrast, the cheeses in our study had relatively lower moisture contents, which could have limited the amount of calcium solubilization allowed before the saturation limit was reached (less serum phase to dissolve into) and reduced the concomitant buffering.

3.3.3.6 Rheological properties of cheese

The LT_{max} values and cross-over temperature measured during heating of the LMPS Mozzarella cheeses as a function of storage time is shown in Figures 3.13 and 3.14, respectively. During storage, there were no significant ($P > 0.05$) differences in either the LT_{max} values and crossover temperature between the experimental cheeses. This could be because in these cheeses there were no significant differences in insoluble calcium content nor any difference in pH during storage (Table 3.4). LT_{max} values seemed to slightly increase with cheese age but it was not statistically significant (Figure 3.13). The crossover point also did not significantly change over storage time (Figure 3.14). Similar observations was found by Moynihan et al. (2016). They did not find an increase in LT_{max} or crossover temperature with storage time since the insoluble calcium content did not change much during storage and proteolysis is limited in LMPS Mozzarella during the first 30 d.

3.4 Conclusions

Rates of pre-acidification of milk were varied using GDL, which significantly impacted the solubilization of insoluble calcium in NFDM dispersions. During cheese manufacture, the pH of curd at whey drainage impacted the amount of soluble calcium lost to whey thereby causing a significant difference in the amount of the residual insoluble calcium in the final cheese sample. These differences in the insoluble calcium between cheeses had a significant impact on the rheological properties of the cheese. Use of different pre-acidification pH values for milk during cheese manufacture affected the amount of total and insoluble calcium lost to whey. The difference in the total and the insoluble calcium content between the control and the cheese made from milk pre-acidified to pH 6.20 was higher as compared to control and cheese made from milk pre-acidified to pH 6.40 (although they were not significantly different due to large variability between trials in our study). These results suggest that the renneting pH has an impact on the calcium solubilization during cheese manufacture even though the pH at whey drainage and milling pH were kept constant. Pre-acidification of milk had only a small impact on rheological properties if the cheeses had similar pH values at whey drainage, milling and during storage.

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3.6 Tables and Figures

Table 3.1. Composition of LMPS Mozzarella cheeses manufactured using different pH values at whey drainage but same milling pH values (n=3)

Composition	Drain Whey pH			SEM
	6.30	**6.10**	**5.90**	
Moisture, %	46.0[a]	46.0[a]	45.2[a]	0.266
Fat, %	23.4[a]	23.8[a]	24.2[a]	0.153
Fat in dry matter, %	43.3[a]	44.0[a]	44.3[a]	0.297
Protein[1], %	26.5[a]	26.3[a]	26.3[a]	0.224
Salt/NaCl[2], %	1.5[a]	1.1[a]	1.2[a]	0.121
Salt in moisture, %	3.2[a]	2.3[a]	2.6[a]	0.273
Total Ca[3] (mg/g protein)	28.5[a]	27.6[a]	26.3[b]	0.175

[a,b,c] Means within a row not sharing common superscript differ ($P< 0.05$)

[1]Total % N × 6.38

[2]Determined using chloride analyzer

[3]Measured by ICP-OES

Table 3.2. Probabilities and R^2 values for pH and insoluble calcium content for LMPS cheese during 30 days of refrigerated storage made using different pH values at whey drainage but same milling pH values (n=3)

Factor[1]	df	Insoluble Ca	pH
Whole plot			
Treatment (T)	2	<0.0001	0.0007
Error	4	0.12	0.10
Split plot			
Age (A)	2	<0.0001	<0.0001
A × T	4	0.0151	0.79
R^2		0.99	0.98

[1]Split-plot design with 3 treatments analyzed as discontinuous variables and cheesemaking day was blocked (3 × 3). The subplot included the effect of aging of cheese (A) and age × treatment as variables

Table 3.3. Composition of LMPS Mozzarella cheeses manufactured using milk with different pre-acidification pH values and similar pH at whey drainage and milling pH values (n=3)

Composition	Pre-acidification pH			SEM
	Control (6.70)	6.40	6.20	
Moisture, %	45.8[a]	44.9[a]	44.3[b]	0.246
Fat, %	24.1[b]	25.1[ab]	25.5[a]	0.199
Fat in dry matter, %	44.4[b]	45.5[ab]	45.7[a]	0.170
Protein[1], %	26.1[a]	26.1[a]	26.3[a]	0.208
Salt/NaCl[2], %	1.3[a]	1.3[a]	1.3[a]	0.117
Salt in moisture, %	2.7[a]	2.9[a]	2.9[a]	0.248
Total Ca[3] (mg/g protein)	27.9[a]	27.1[ab]	25.9[b]	0.336

[a,b,c] Means within the same row not sharing common superscript differ ($P < 0.05$)

[1]Total % N × 6.38

[2]Determined using chloride analyzer

[3]Measured by ICP-OES

Table 3.4. Probabilities and R^2 values for insoluble calcium content for LMPS cheese during 30 days of storage manufactured using different pre-acidification pH values but similar pH at whey drainage and milling pH values (n=3)

Factor[1]	df	pH	Insoluble Ca
Whole plot			
Treatment (T)	2	0.69	0.06
Error	4	0.68	0.025
Split plot			
Age (A)	2	0.22	0.13
A × T	4	0.77	0.89
R^2		0.46	0.96

[1]Split-plot design with 3 treatments analyzed as discontinuous variables and cheesemaking day was blocked (3 × 3). The subplot included the effect of aging of cheese (A) and age × treatment as variables

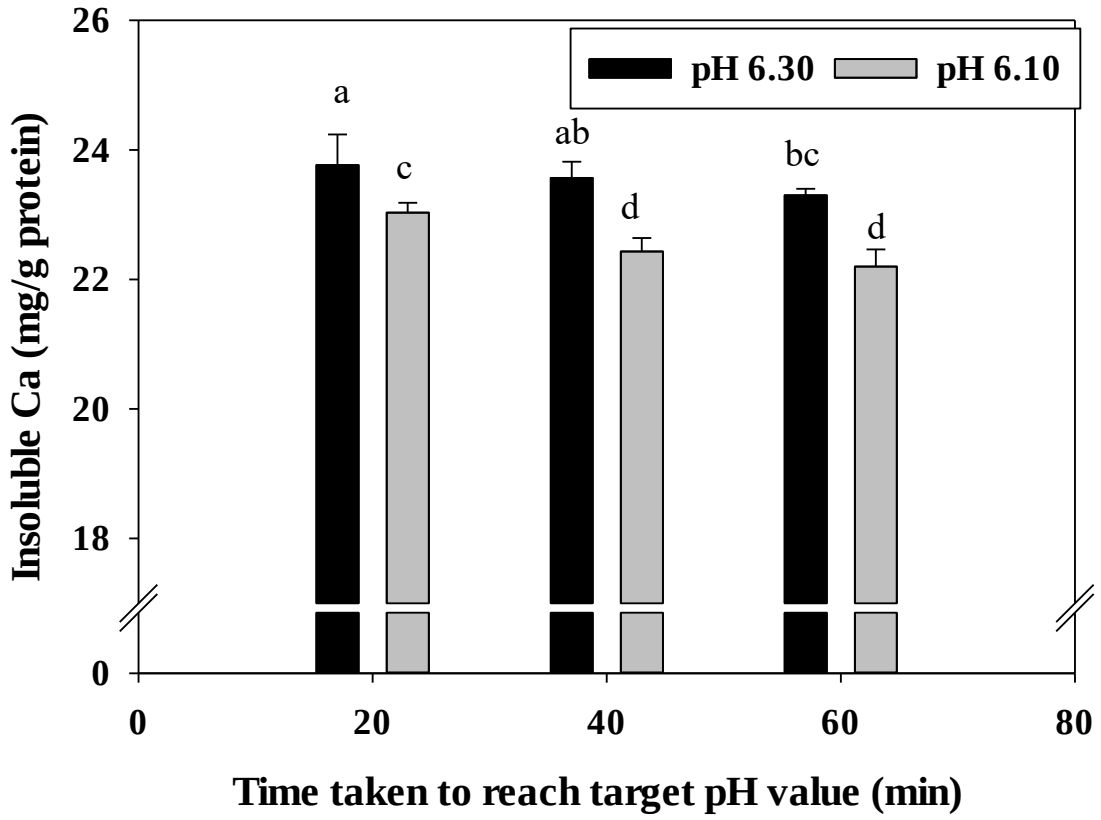

Figure 3.1. Insoluble calcium content of 10% rehydrated NFDM dispersions after acidification to target pH values 6.30 and 6.10 using GDL concentrations of 0.1 and 0.2% (w/w), respectively. The time taken to reach the target pH values (rates of acidification), i.e., 20, 40, and 60 min were varied by altering the temperature of the solutions to 40, 44, and 48ºC, respectively. Vertical bars represent standard deviations (n=3). Columns not sharing a common letter (a-d) differ (P <0.05).

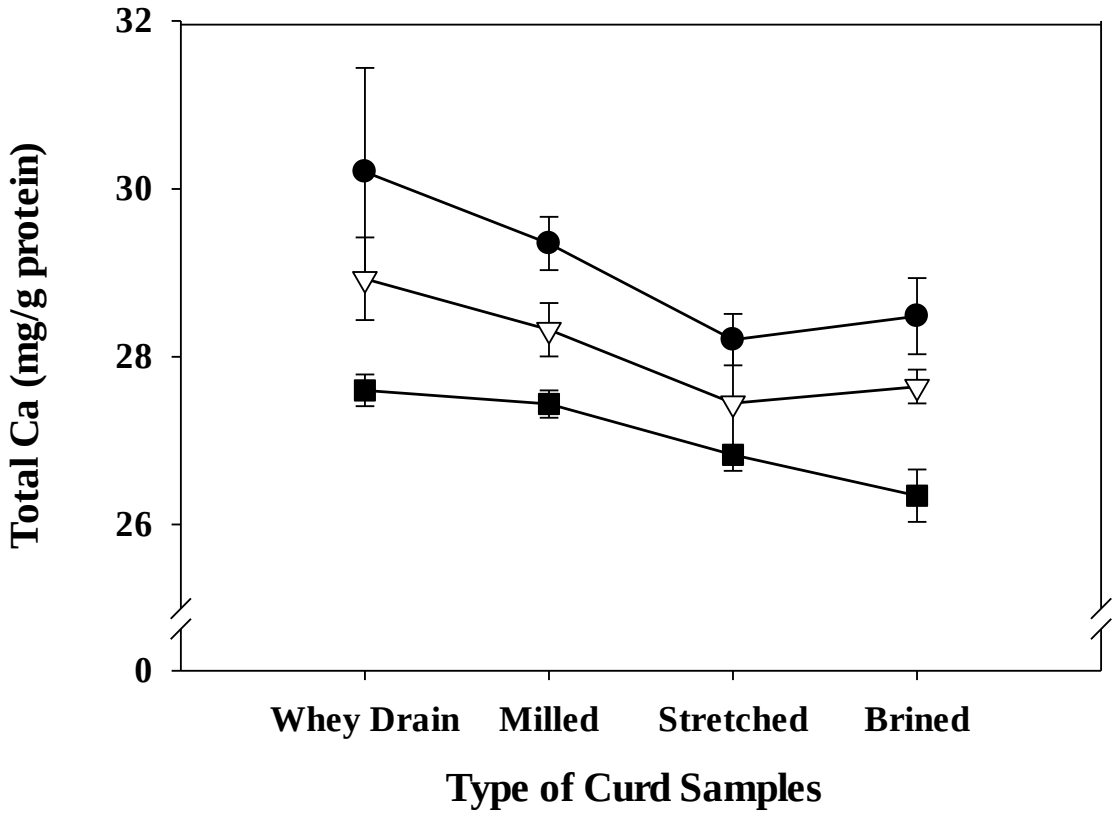

Figure 3.2. Total calcium content (mg/g protein) of curd samples measured during LMPS Mozzarella manufacture, cheeses made using pH values at whey drainage of 6.30 (●), 6.10 (▽), and 5.90 (■). Vertical bars represent standard deviations (n=3)

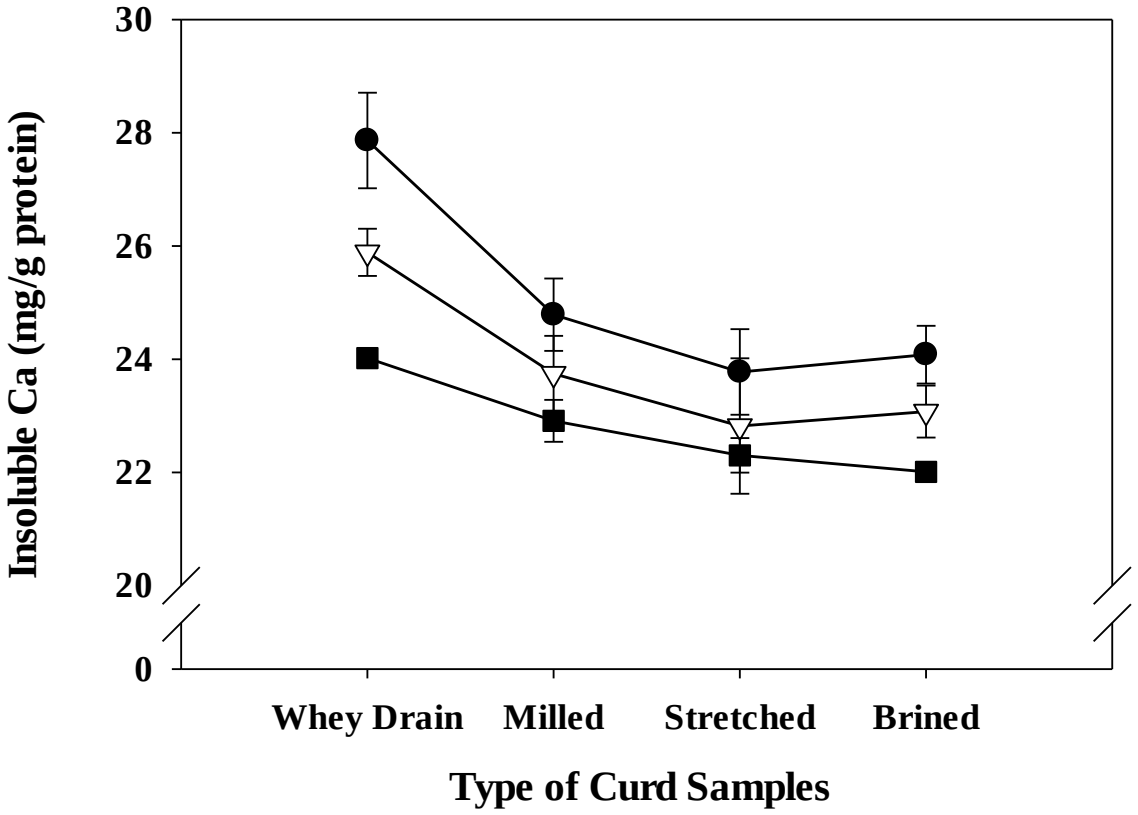

Figure 3.3. Insoluble calcium content of curd samples measured during LMPS Mozzarella manufacture, cheese made using pH values at whey drainage of 6.30 (●), 6.10 (▽), and 5.90 (■). Vertical bars represent standard deviations (n=3)

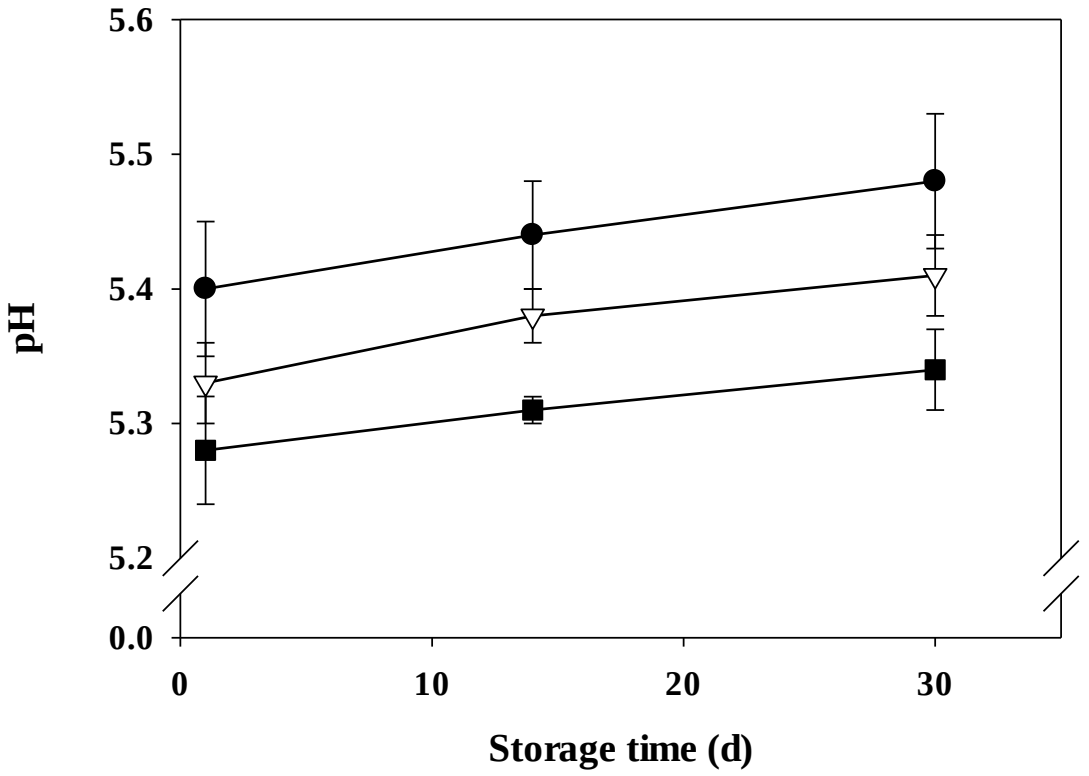

Figure 3.4. pH values for LMPS Mozzarella cheese during storage at 4ºC, cheeses manufactured using pH values at whey drainage of 6.30 (●), 6.10 (▽), and 5.90 (■). Vertical bars represent standard deviations (n=3)

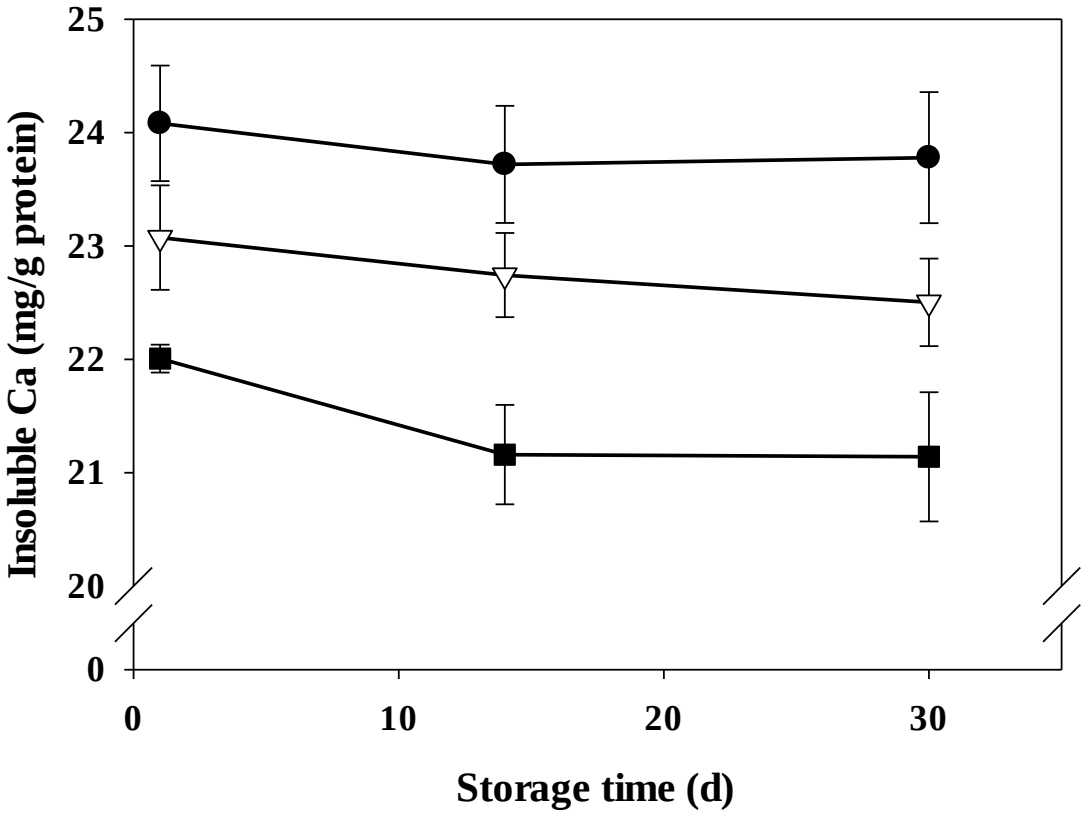

Figure 3.5. Insoluble calcium content (mg/g protein) during storage at 4ºC for LMPS Mozzarella cheese manufactured using pH values at whey drainage of 6.30 (●), 6.10 (▽), and 5.90 (■). Vertical bars represent standard deviations (n=3)

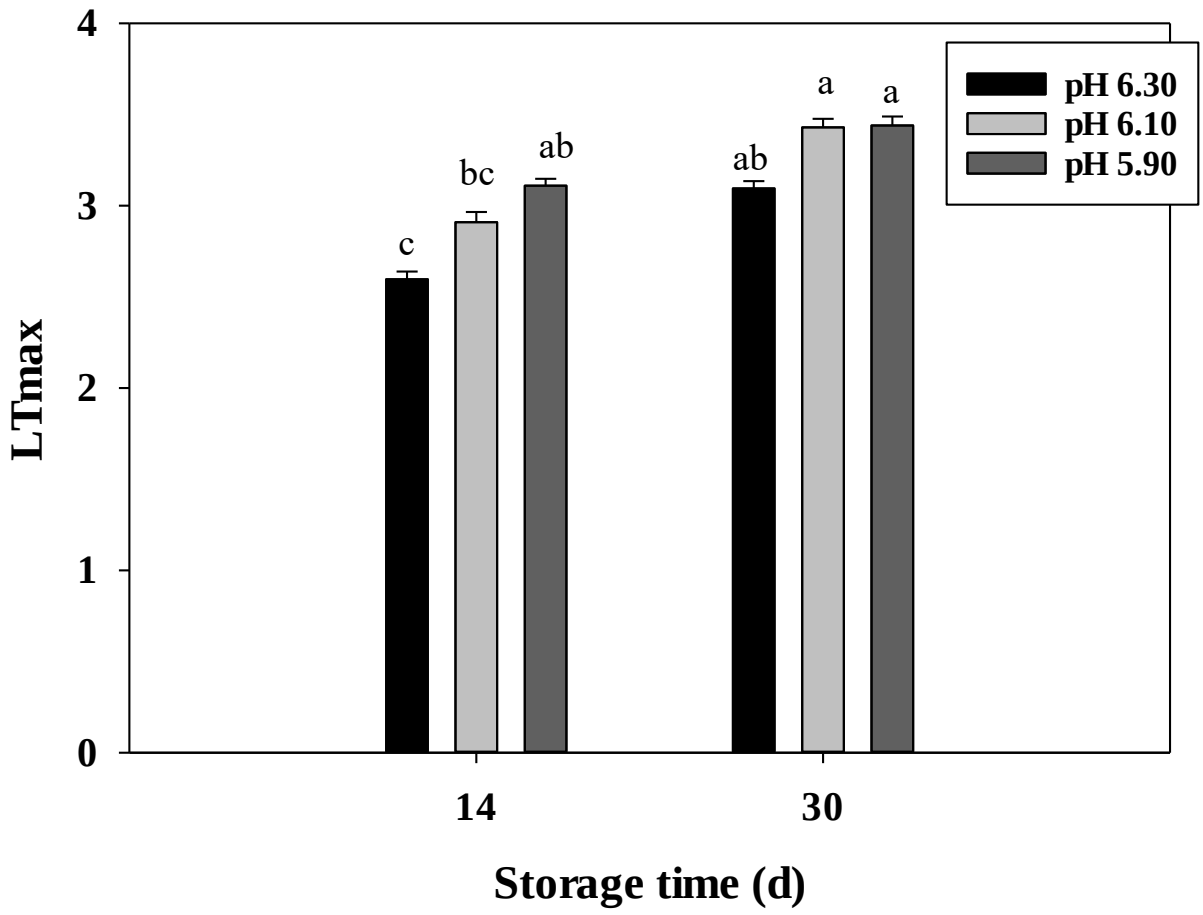

Figure 3.6. Maximum loss tangent (LT_{max}) values from rheological testing during heating of cheeses from 5 to 85ºC. The LT_{max} values were for LMPS Mozzarella cheeses during storage. Cheeses were manufactured using pH at whey drainage values of 6.30, 6.10, and 5.90. Vertical bars represent standard deviations (n=3). Bars not sharing a common letter (a-c) differ (P <0.05).

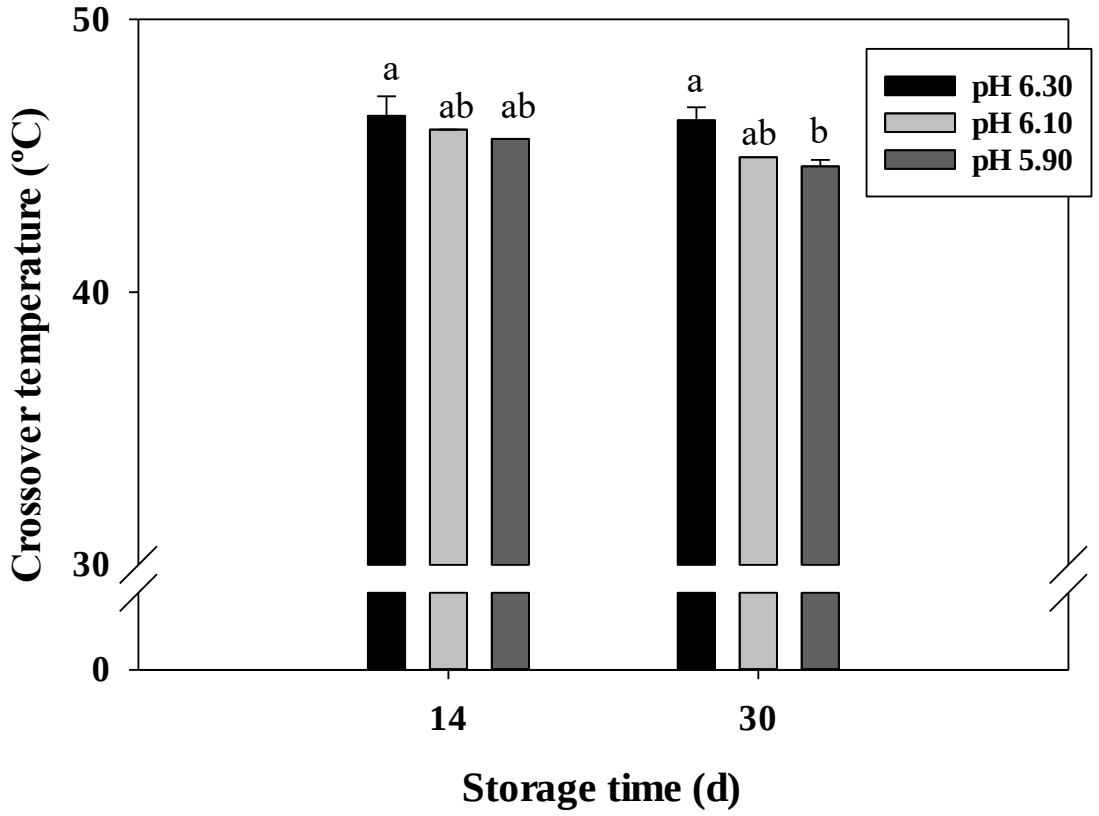

Figure 3.7. Temperature of the crossover point (where LT=1) for LMPS Mozzarella cheeses during refrigerated storage, cheeses manufactured using pH at whey drainage values of 6.30, 6.10, and 5.90. Vertical bars represent standard deviations (n=3). Bars not sharing a common letter (a-b) differ (P <0.05).

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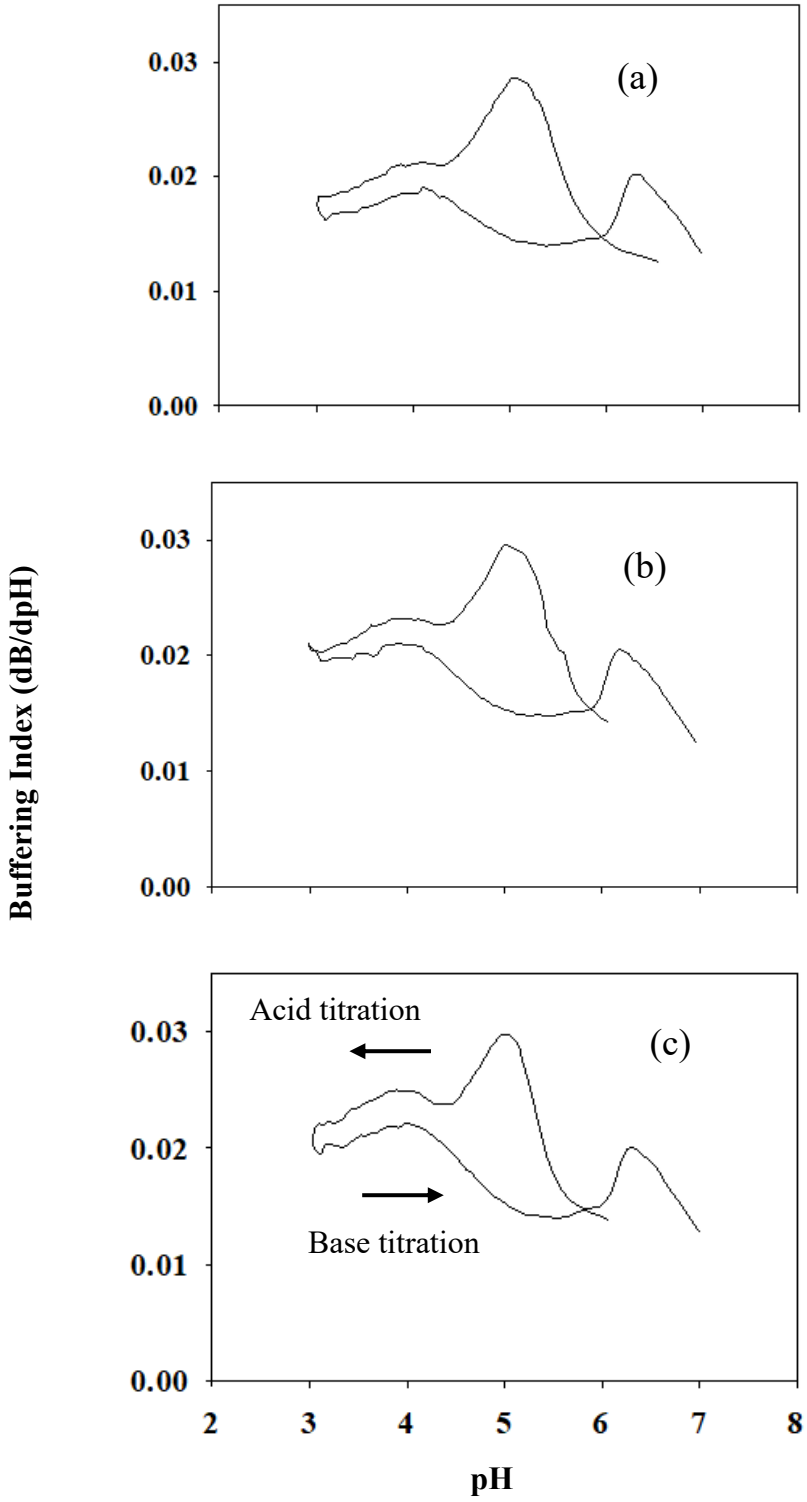

Figure 3.8. Acid-base buffering curves of cheese milk samples with different milk pre-acidification pH values using lactic acid: (a) control, (b) pH 6.40, (c) pH 6.20. For these buffering curves samples were acidified to pH 3.0 with 0.5 N HCl (Acid titration) and then with 0.5 N NaOH to pH 7.0 (base titration).

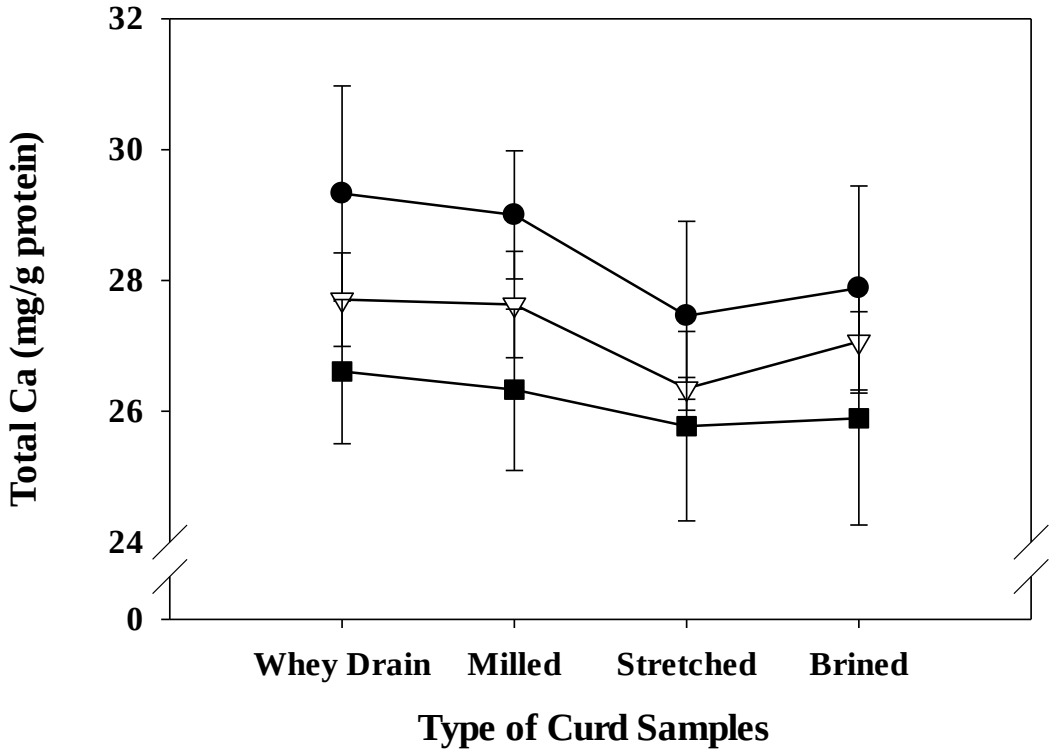

Figure 3.9. Total calcium content of curd samples measured during LMPS Mozzarella manufacture, samples made without pre-acidification: control (●), and pre-acidified to pH 6.40 (▽), and pH 6.20 (■) with lactic acid. Renneting pH in these samples were similar to pre-acidification pH, the control sample had a renneting pH of 6.70. Samples had similar pH at whey drainage (5.90) and milling pH values (5.20). Vertical bars represent standard deviations (n=3)

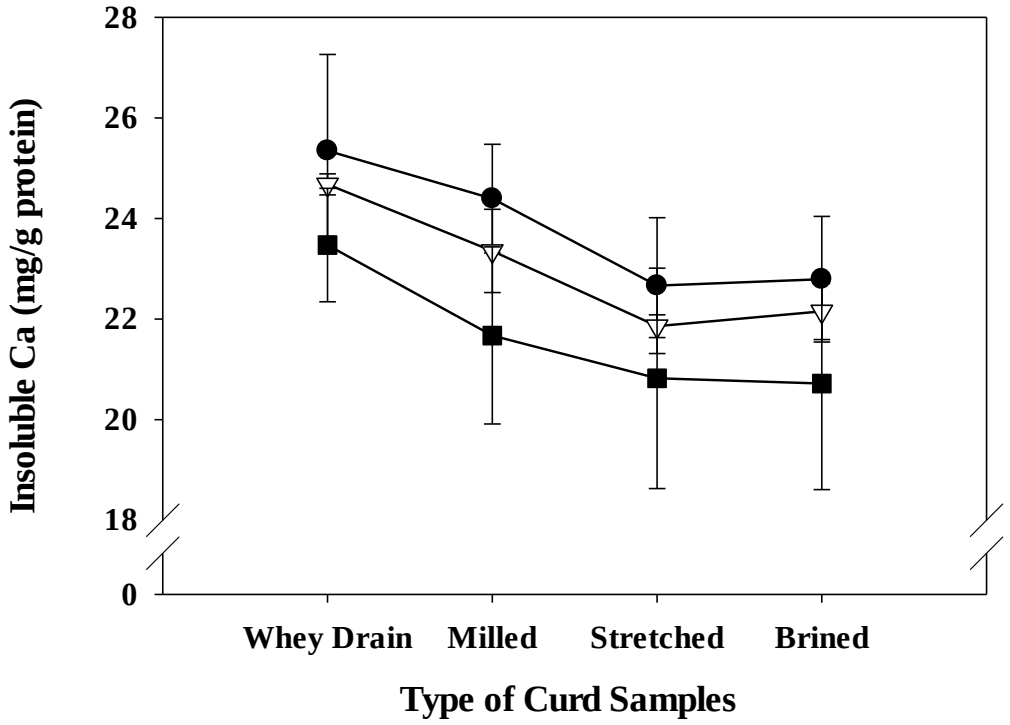

Figure 3.10. Insoluble calcium content of curd samples measured during LMPS Mozzarella manufacture, samples made without pre-acidification - control (●), and pre-acidified to pH 6.40 (▽), and pH 6.20 (■) with lactic acid. Renneting pH in these samples were similar to pre-acidification pH, the control sample had a renneting pH of 6.70. Samples had similar pH at whey drainage (5.90) and milling pH values (5.20). Vertical bars represent standard deviations (n=3)

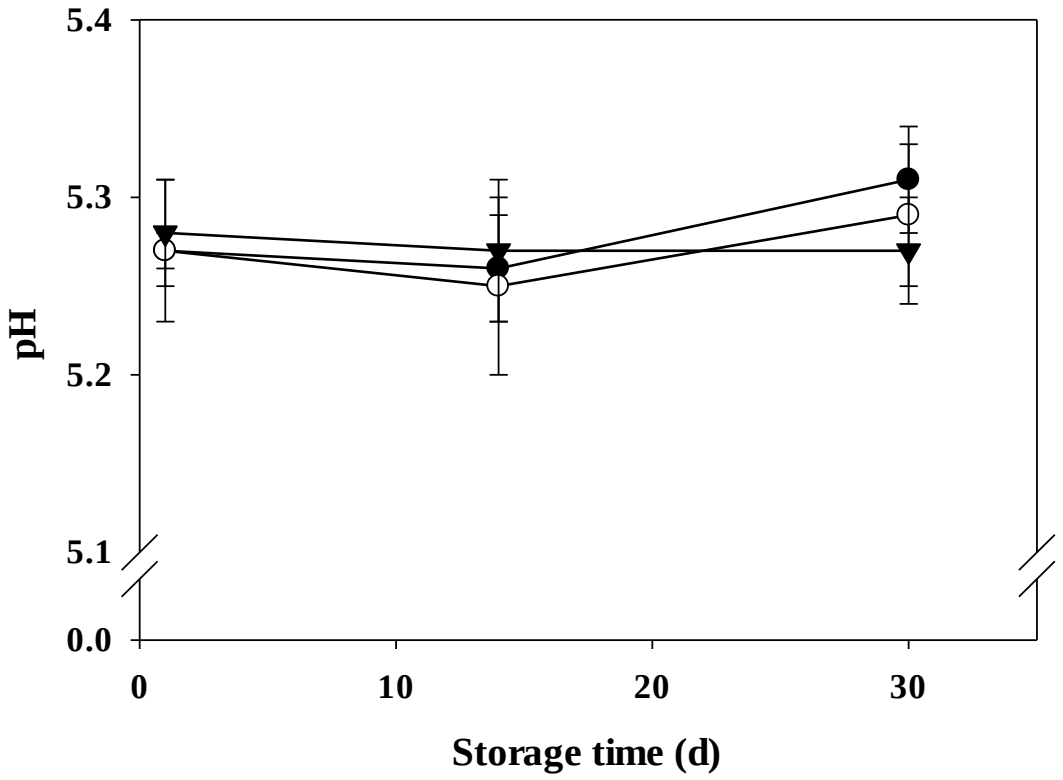

Figure 3.11. pH values during refrigerated storage at 4ºC for LMPS Mozzarella made without pre-acidification - control (●), and milk pre-acidified to pH 6.40 (∇), and pH 6.20 (■) with lactic acid. Samples had similar pH values at whey drainage (5.90) and milling pH (5.20) values. Vertical bars represent standard deviations (n=3)

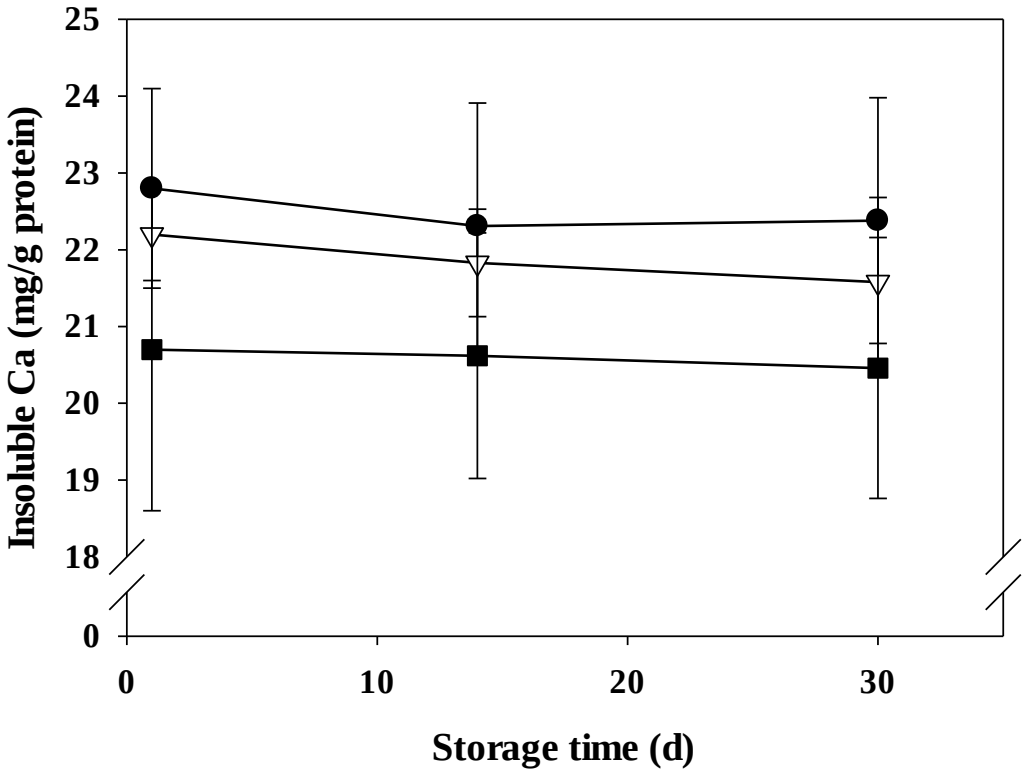

Figure 3.12. Insoluble calcium content (mg/g protein) during storage at 4ºC for LMPS Mozzarella made without pre-acidification - control (●), and milk pre-acidified to pH 6.40 (∇), and pH 6.20 (■) with lactic acid. Samples had similar pH at whey drainage (5.90) and milling pH (5.20) values. Vertical bars represent standard deviations (n=3)

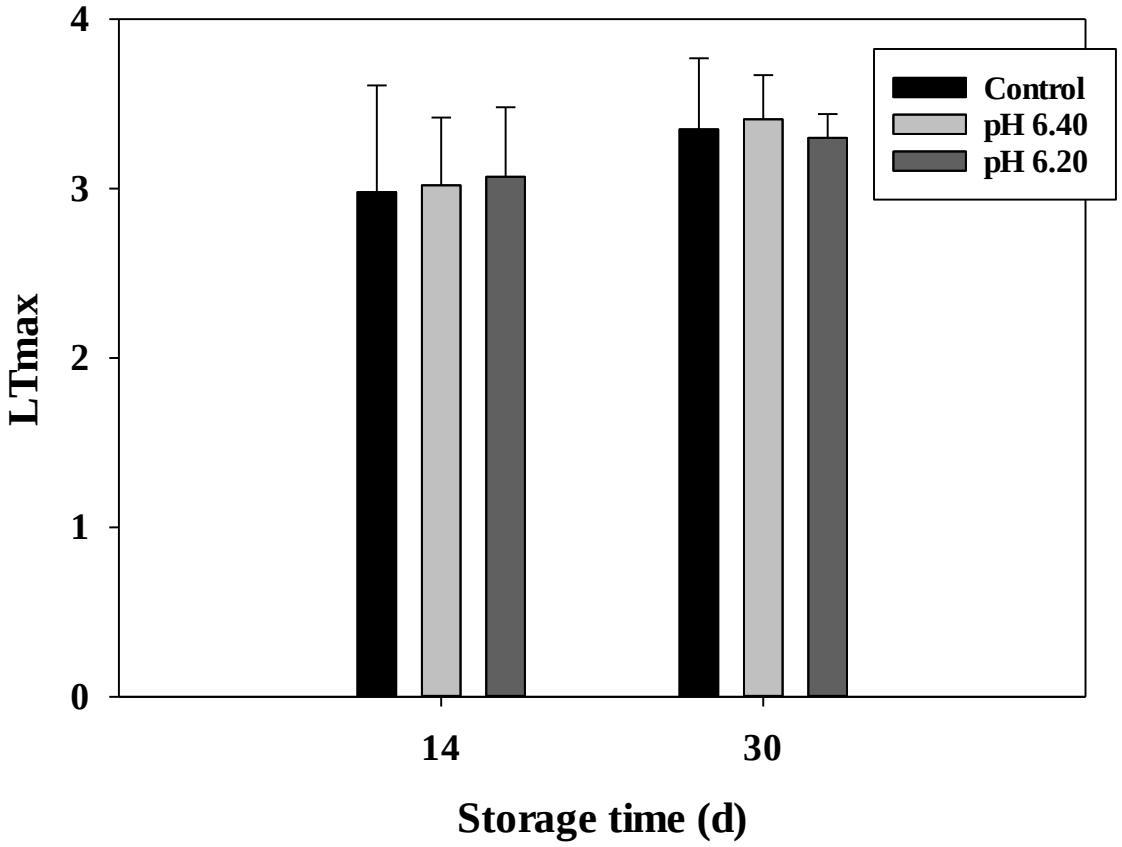

Figure 3.13. Maximum loss tangent values (LT_{max}) for LMPS Mozzarella cheeses during refrigerated storage, cheeses manufactured from milk without pre-acidification - control, and milk pre-acidified to pH 6.40, and pH 6.20 with lactic acid. Samples had similar pH at whey drainage (5.90) and milling pH (5.20) values. Vertical bars represent standard deviations (n=3)

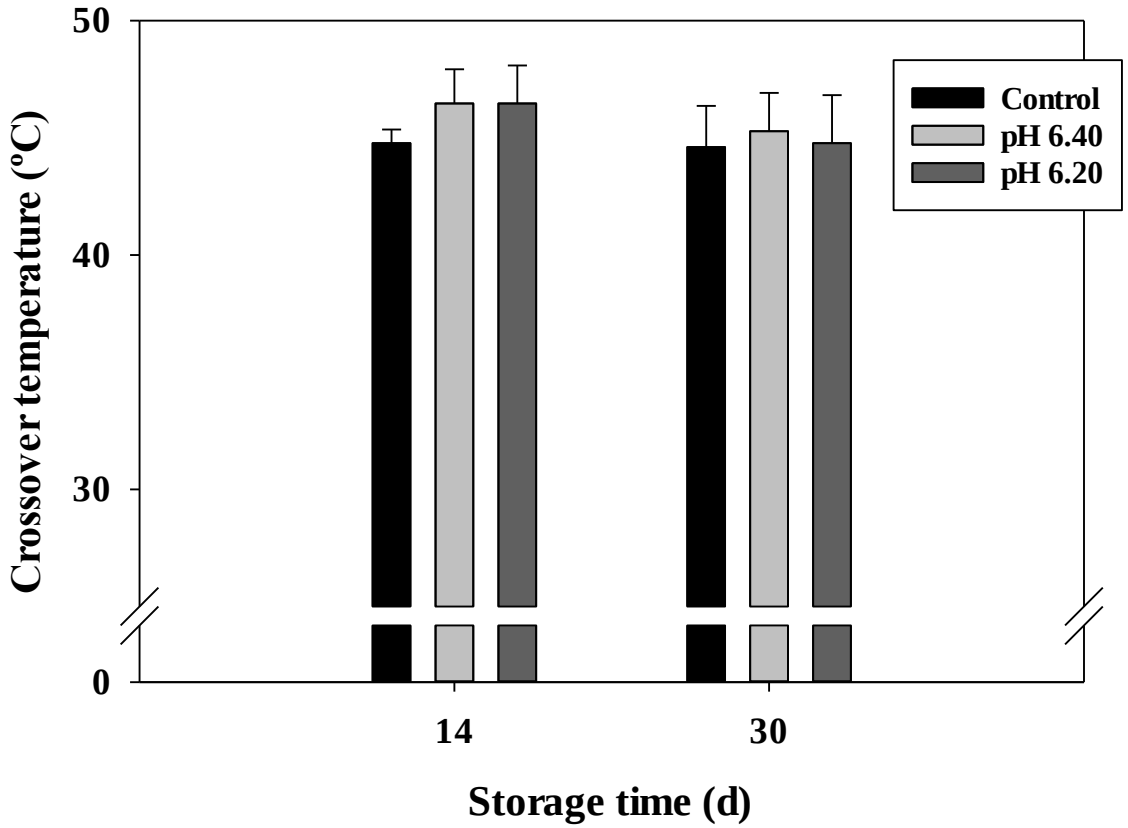

Figure 3.14. Temperature of the crossover point (where LT=1) for LMPS Mozzarella cheeses during refrigerated storage, cheeses manufactured from milk without pre-acidification - control, and milk pre-acidified to pH 6.40, and pH 6.20 with lactic acid. Samples had similar pH at whey drainage (5.90) and milling pH (5.20) values. Vertical bars represent standard deviations (n=3)

Chapter 4. Impact of Extent of Pre-acidification and Types of Acid on Calcium Balances and Functionality of LMPS Mozzarella

Abstract

Pre-acidification (PA) of cheese milk is commonly used during low-moisture part-skim (LMPS) Mozzarella manufacture, especially when using high casein (CN) milk. PA helps to dissolve excessive colloidal calcium phosphate (CCP) associated with CN. The amount of CCP dissolved and lost from curds at various steps during cheese making is not precisely known, and the extent of PA, as well as different types of acids used for PA, can influence total (final) and insoluble calcium content in cheese. Eight vats of LMPS Mozzarella, with control (no PA) and the others PA to pH 6.40 or pH 6.00, with acetic, citric, or carbonic acids, were manufactured from 4% CN milk (n=4). All cheeses had similar composition except for the cheese PA with carbonic acid, which had a lower fat-in-dry matter ($P < 0.05$) (probably due to foaming observed in this sample). The cheese PA with citric acid to pH 6.00 had the lowest amount of total and insoluble calcium ($P < 0.05$) compared to other cheeses at all steps during cheese manufacture, likely due to the calcium chelating ability of citric acid. The cheese that was PA with citric acid to pH 6.40 had a trend of slightly lower (not significant) total and insoluble calcium during cheese manufacture compared to other cheeses that were PA to pH 6.40. The pH and the insoluble calcium content in all the cheeses remained the same throughout 12 wk of storage, where the cheese that was PA with citric acid to pH 6.00 had the lowest values for both pH and insoluble calcium. There were no significant differences in the proteolysis or hardness (measured using a texture profile analyzer) between cheeses. The rheological parameters measured were different for the cheese that was PA with citric acid to pH 6.00 as compared to other cheeses, possibly due to the lower pH value of this cheese. The performance of cheeses on pizza was assessed using Sensory Spectrum® method

and quantitative descriptive analysis. At 1 and 3 mo of storage, the cheese PA with citric acid to pH 6.00 had lower first chew hardness, lower chewiness, and lower strand thickness values compared to other cheeses. Varying the extent of PA did not seem to significantly affect the total and insoluble calcium content in the cheeses when the pH at whey drainage and milling pH were kept consistent, except when citric acid was used for PA.

4.1 Introduction

Low-moisture part-skim (LMPS) Mozzarella is the most consumed cheese in the United States with a per capita consumption of 12.29 pounds (USDA, 2021). The increasing popularity of this cheese is because of its application on pizzas. As the pizza market expands and grows, the demand for LMPS Mozzarella also increases. Therefore, it is important to explore efficient ways to manufacture this cheese with more consistent and improved functionalities. One such approach is to manufacture this cheese using concentrated milk that has higher casein levels, which has become a common practice in the cheese industry due to various advantages, such as, increased cheese yield, increased plant throughput with similar cheese make time, and improved manufacturing efficiency with a consistent final product (Cheryan, 1998; McSweeney, 2007). Currently, the cheese industry mostly makes cheese using unconcentrated milk that has about 3-3.5% protein, i.e., 2.5-3% casein. There is the potential for the industry to shift towards the use of higher casein milk (≥ 4% casein), but this has been limited for LMPS Mozzarella manufacture due to concerns about the impact that the increased colloidal calcium phosphate (CCP) content would have on cheese quality, and texture development.

Ultrafiltration is the most common membrane system used for concentration of cheese milk in the dairy industry. One of its most popular applications is cheese milk standardization to avoid

seasonal variations that affect the final cheese composition. During ultrafiltration of milk, the soluble components present in the milk including soluble proteins (mainly, whey proteins), lactose, and salts (like soluble calcium) pass into the permeate, while the caseins and the insoluble calcium associated with the caseins are retained and concentrated. The protein concentration is dependent on the concentration factor employed during ultrafiltration of milk (Soodam and Guinee, 2018). As the milk becomes more concentrated, the insoluble calcium content in the milk, along with the casein, increases, which changes the calcium balance (amount of soluble to insoluble calcium) in the milk before the cheese-making process starts. This increased casein and insoluble calcium content in the milk changes the calcium solubilization dynamics during cheese manufacture which results in a cheese with rubbery and tough texture unless certain adjustments are made to the cheese-making process. One such change is to pre-acidify the milk (acidify the cheese milk using organic acids, such as, acetic, citric, etc.) before adding starter cultures to reduce insoluble calcium upfront before rennet coagulation. Pre-acidification helps with the rapid reduction of CCP in milk, with more calcium losses in whey, thereby providing the desirable functional properties in the final manufactured cheese (Kindstedt and Fox, 1993). Extensive research has been done to study the effects of direct acidification, or pre-acidification, on the final total calcium content and functionality of Mozzarella cheese made from unconcentrated milk, i.e., approx. 2.5-3 % casein (Guinee et al., 2002; Metzger et al., 2001a; Joshi et al., 2003; Choi et al., 2008, Mizuno et al., 2009; To et al., 2022; McMahon et al., 2005) but none appears to have been reported on cheese made from concentrated milk. The amount of calcium solubilization due to pre-acidification in unconcentrated milk was quantified in the previous chapter. Our objective was to determine the calcium losses in concentrated milk during LMPS Mozzarella cheese manufacture.

The type of acid used for pre-acidification may also affect the amount of calcium lost during cheese manufacture (Keller et al., 1974; Metzger et al., 2001a). Carbonic acid (injection of CO_2 into milk) is increasingly used in the dairy industry due to its extra benefit of potentially reducing microbial numbers in raw milk (Hotchkiss et al., 2006) as well as the ease of handling of whey during the production of various whey-based products as a result of lower acid produced during cheese manufacture. To et al. (2022) studied the effects of milk pre-acidification (pH 6.20) with CO_2 and lactic acid in comparison with milk that was not pre-acidified for LMPS Mozzarella that was manufactured from 3% casein milk, but we wanted to explore this aspect as well as other acids, such as, acetic and citric, in a milk with a higher casein content (>3%).

Our study was therefore focused on studying (a) the impact of the extent of pre-acidification and (b) the type of acid used for pre-acidification on the composition, textural, rheological, and functional properties of LMPS Mozzarella cheese made from concentrated milk (approx. 4% casein). The exact amount of calcium lost as a result of pre-acidification from concentrated milk has not been yet quantified or studied. Therefore, we also measured the changes in total and insoluble calcium content in milk/curd samples at various steps during cheese manufacture, while keeping other key factors constant that could affect the calcium balance, i.e., rate of pre-acidification, overall rate of acidification, pH at whey drainage, and milling pH.

Research Hypotheses

- Cheeses manufactured using a higher pH at rennet addition will have slightly higher total and insoluble calcium as compared to cheeses manufactured using lower pH at rennet addition due to the lower amount of calcium dissolved as a result of higher pH, and consequently lower moisture in the curds (serum from the curds is separated after cutting

of gel) at a much higher pH, which may restrict calcium solubilization, even though the rate of pre-acidification, pH at whey drainage and milling pH values are similar between the cheeses during cheese making process.

- Cheese PA with citric acid will have the lowest amount of total and insoluble calcium as compared to other cheeses due to the ability of this acid to chelate calcium. This extra calcium losses will affect the functional properties of this cheese more than cheese PA using non-chelating acids like acetic or carbonic acid.

4.2 Materials and Methods

4.2.1 Milk Processing and Standardization

Part-skimmed milk was concentrated using ultrafiltration (UF) to a total solids content of approx. 15%. UF was carried out using a 3.8" spiral wound polymeric 10 kDa membrane at 4ºC. The cheese milk was then standardized to approx. 4.2 % casein with a casein-to-fat ratio of 1.0, by the addition of liquid milk permeate and cream sample to UF milk retentate. The milk was then high-temperature, short-time (HTST) pasteurized at 74°C for 19 seconds.

4.2.2. Cheese Manufacturing

Seven vats of LMPS Mozzarella cheeses were made by licensed Wisconsin cheesemakers at the Center for Dairy Research dairy plant on 4 separate days. The PA pH value (pH 6.40 and 6.00) and type of acid (acetic, citric, carbonic) used for PA was varied in each vat except one of the vats where control cheese (no PA) was manufactured. 170 kg of standardized pasteurized milk was transferred into each vat. The milk in the control vat was warmed to 34°C before frozen direct vat-set *Streptococcus thermophilus* (Delvo CP® Cheese-201; DSM, Waukesha, WI) was added. The milk in the remaining vats were PA to either pH 6.40 or 6.00 by slowly adding acids in three

additions over a 45-min period at 4ºC. In the vats where carbon dioxide (CO_2) was used for PA, a CO_2 tank connected to a sparger was connected to a tee-intersection as the milk was recirculated to PA the milk. The pressure on the CO_2 tank was adjusted to 40-50 kPa for pre-acidification to pH 6.40 and to 50-60 kPa for pre-acidification to pH 6.00. The flow of CO_2 was shut off once the target pH value was achieved. Since different amounts of acids were required for acidification in each of the vats, extra water was added to two of the other vats to provide similar dilution among the three vats; no water was added to the milk pre-acidified with CO_2 since it was acidified using a gas. Once the cheese milk was pre-acidified, starter cultures were added as described above for the control vat. A ripening time of 60 min was given for control vat and in the vats where cheese milk was pre-acidified to pH 6.40 whereas a ripening time of 75 min was given to the vats that were pre-acidified to pH 6.00 before the addition of fermentation-produced calf chymosin with activity of ≥620 IMCU per g (Maxiren® XDS, DSM, Waukesha, WI). These differences in ripening time were done to achieve similar rates of acidification in all the vats and to compensate for the shorter gelation time in vats pre-acidified to pH 6.00. Once the cheese milk was clotted and the desirable firmness was attained, as evaluated by a licensed cheese maker, the coagulum was cut using 2.54 cm knives, and the gel was allowed to heal for 15 minutes. The curds were later cooked for 50 min at approx. 38°C after which the whey was allowed to drain slowly (over 30 min). Once the whey was completely drained, the curds were cut into 4 slabs and stacked 2 high or 1 high depending on the moisture content in the curds (since we wanted to achieve similar moisture in all cheeses) until the target milling pH value of 5.20 was achieved. Milled curds were stretched at a water temperature of 75°C with the curd exit temperature measuring approx. 60°C. All cheeses were extruded into molds, dipped in cold water baths for 60 min (to bring the curd temperature below 38°C), and later brined for 4 h in a 20 % (w/w) salt solution held at 4ºC. The

cheeses were vacuum sealed the next day and refrigerated until the analysis timepoints of 2, 7, 14, 30, and 90 d of storage.

4.2.3 Compositional Analysis

The milk and cheese samples were analyzed for total solids (Marshall, 1992), fat by Mojonnier (AOAC International 2000), protein (total percentage N × 6.38) by Kjeldahl (AOAC International 2000), and casein (AOAC International 2000), and non-protein nitrogen (AOAC International 2000). The total calcium in milk, curds, and cheese were analyzed using inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Agilent 5100, Agilent Technologies, Santa Clara, CA) (Park, 2000). The pH was measured using a spear tip pH probe (accuCap Capillary Junction pH combination electrode; Fisher Scientific, Itasca, IL). The salt content in all the cheese samples was measured using a Chloride Analyzer Model 926 (Corning Glass Works, Medfield, MA). The lactose, galactose, and lactic acid in cheeses during storage were measured by high-performance liquid chromatography as described by Zeppa et al. (2001).

4.2.4 Water-Soluble Calcium Method

During cheese manufacture, curd samples obtained for water-soluble calcium (WSC) measurement were ground using a food processor (Hamilton Beach®, Model 70730, Glen Allen, VA) for 30 sec before subjecting the mixture to WSC extraction. The WSC from the curd/cheese samples was extracted by blending 4 g of ground curds/cheese in 40 g Milli-Q water at 55ºC using an Ultra-Turrax T-25 Basic Homogenizer (IKA Works Inc., Willmington, NC) at a speed of 7,000 rpm. The heterogeneous mixture was centrifuged at 10,000 × g for 10 min at 22ºC (Selected from model rennet gel preliminary work; Swaminathan et al., 2023). The supernatant was filtered

through a Whatman #1 filter paper (GE Healthcare Life Sciences, USA), and the filtrate was stored for subsequent analysis of soluble calcium. Curd samples drawn for WSC measurement during cheese manufacture were also measured for moisture and protein content as this was required for the calculation of the insoluble calcium content and for standardizing results in terms of mg Ca per gram protein. If the insoluble calcium content was not represented as mg Ca per g protein, comparison of results between curd samples drawn at various points in the process (for example, curds after whey drain and milled curds) would be difficult due to large differences in moisture contents between these samples. The WSC slurry was immediately centrifuged after homogenization to avoid any potential shifts in calcium balances because moisture and pH differences in the curd samples could also affect the amount of calcium extracted.

The WSC in curds/cheese samples was calculated as shown in equations 4.1 and 4.2; the insoluble calcium content (mg/g protein) was calculated by subtracting the soluble calcium from the total calcium.

$$Soluble\ Ca\ (mg/100g) = \frac{Ca\ in\ WSC\ extract\ (mg)}{Weight\ of\ sample\ (g)} \times Moisture\ in\ cheese\ (\%)\ \times D \quad (4.1)$$

$$Insoluble\ Ca\ (mg/g\ of\ protein) = \frac{Total\ calcium - Soluble\ calcium\ (mg/100g)}{\%\ Protein} \quad (4.2)$$

Where, D is the dilution factor, which was 11.

4.2.5 Rheological Analysis

The rheological properties of LMPS Mozzarella cheeses were measured using a dynamic low-amplitude oscillatory rheology with an Anton Paar Rheometer (MCR 302; Physica Messtechnik, Stuttgart, Germany) as described by Moynihan et al. (2014). The parameters

measured during this test were storage modulus (G′), loss modulus (G″), and loss tangent (LT, which is G″/G′). The temperature at the crossover point, where G′= G″, was calculated to determine when the cheese transitioned from a solid to a liquid-like system. The maximum LT (LT_{max}) was used as a measure of cheese meltability, with a higher value indicating a more meltable (fluid) cheese (Lucey et al., 2003). The G′ value at 70ºC was used as a measure to evaluate the amount of bonds (mostly, electrostatic interactions and calcium crosslinking) left in the hot (melted) cheese system. This analysis was performed in duplicates or until the rheograms were closely replicated.

4.2.6 Texture Profile Analysis

The hardness of the cheese was evaluated using a TA-XT2 Texture Analyzer (Texture Technologies Corp., Scarsdale, NY). The samples were cut to a diameter of 16 mm and a height of 17.5 mm and refrigerated overnight before analysis. A double compression test where the sample was compressed to 70 % of the original height was done at the speed of 0.8 mm/s. Hardness was defined as the peak force achieved during the first compression. The test was replicated 10 times for each sample.

4.2.7 Proteolysis

Proteolysis was evaluated by measuring the pH 4.6 soluble nitrogen (Kuchroo and Fox, 1982).

4.2.8 Descriptive Sensory Analysis

Sensory texture and flavor properties of unmelted cheese cubes and shreds in both unmelted and melted form (on pizza) were evaluated as described by Moynihan et al. (2014). The evaluation was done by trained sensory panelists (40 hours of training) ($n \geq 8$) using a mixture of Sensory Spectrum® and quantitative descriptive analysis. The references used corresponding to

the range on the QDA scale for various attributes measured are shown in Chapter 1 (Table 1.4 and 1.5).

The size/dimensions of the cubes prepared for evaluation was 2 × 2 × 2 cm. The cheeses were shredded using a pilot scale shredder (Urschel CC-D, Urschel Laboratories Inc., Chesterton, IN). 100 g of shreds were used for evaluating the unmelted visual (straightness, matting, fusion, opacity) and surface (length, surface oil, surface moisture, adhesiveness) properties. Pizza was prepared by spreading 2 tablespoons of tomato sauce on 30 cm parbaked pizza base and 300 g of shredded Mozzarella cheese. The pizzas were baked in a forced-air commercial oven (2500 series Lincoln Impinger Oven, Fort Wayne, IN) at 260ºC for 5 minutes. The various parameters of melted cheese on the pizza were evaluated at different temperatures as the cheese cooled down as described by Moynihan et al. (2014). All sensory panels were conducted in duplicate.

4.2.9 Confocal Laser Scanning Microscopy

The cheese samples were prepared for confocal laser scanning by taking samples from the center of the cheese block. A very thin slice of cheese was cut perpendicular to the direction of the parallel fibers using a Cool-cut Microtome (ThermoFisher Scientific, Waltham, MA) to visualize the para-casein fibers in the cheese. The sliced cheese was then placed on a microscope slide and dual stained with Nile Red (0.1 mg/mL) and Fast Green FCF (1 mg/mL) (Sigma-Aldrich, St. Louis, MO) to stain fat and proteins, respectively. Nile Red was applied first to the cheese and allowed to stain for 1 min. The excess stain on the cheese was dabbed using Kimwipes (Kimberly-Clark professional, Roswell, GA) before Fast Green FCF was applied. A cover slide was placed on top of the cheese. The prepared slides (2 slides for each sample) were immediately evaluated for cheese

microstructure using a Nikon A1R+ confocal microscope (Nikon Instruments Inc., Tokyo, Japan). Images were obtained using a 20× objective as described by Swaminathan et al. (2021).

4.2.10 Experimental Design and Statistical Analysis

Statistical analysis was performed using SAS (Version 9.4, SAS Institute Inc., Cary, NC). A randomized complete block design with 7 treatments and four replicates was used to evaluate differences in the composition of the cheeses. A split-plot design was used to evaluate the effect of treatment and storage time as well as their interactions on pH, insoluble calcium, proteolysis, textural, rheological, and functional properties. In the whole plot factor, the cheese-making days were blocked, and the treatment was analyzed as a discontinuous variable. For the subplot factor, storage time and the interaction of storage time and treatment were treated as variables. The interaction of treatment and cheese-making days was treated as an error term for the treatment effect. An ANOVA was carried out using the PROC GLM procedure of SAS and Tukey's multiple comparison test was used to evaluate differences in the treatments at a significance level of $P < 0.05$.

4.3 Results and Discussion

4.3.1 Composition of Milk and Cheese Samples

The total solids, total protein, casein, fat, and lactose content in the cheese milk samples were 14.9 ± 0.2, 5.3 ± 0.0, 4.2 ± 0.0, 3.9 ± 0.0, 4.6 ± 0.1%, respectively. The average composition of the cheeses is shown in Table 4.1. There were no significant differences ($P > 0.05$) in moisture or salt content between the cheeses. The protein, fat, and fat in dry matter (FDM) was similar for all cheeses except for the cheese PA with carbonic acid to pH 6.00, which had a lower fat and FDM, and higher protein content than other cheeses, probably due to the extensive foaming that

was observed during CO_2 incorporation when the milk samples were pre-acidified to pH 6.00. The foam could have trapped some of the fat, which was eventually lost in the whey during the drainage step resulting in a lower fat and fat in dry matter; this loss of fat resulted in a cheese with higher protein content. Similarly, a lower FDM was observed by Nelson et al. (2004) in their Cheddar cheese that was PA with CO_2 to pH 5.90. To et al. (2022) did not observe a decrease in FDM when they PA their milk sample to pH 6.20 using CO_2 during their Mozzarella cheese production; likely their CO_2 incorporation system was more effective, and they did not observe any foaming problems.

A significant difference was observed in pH and the total calcium content between the cheeses (Table 4.1). The control cheese had the highest amount of total calcium content as compared to PA cheeses; a significant difference was observed when the cheeses were PA to pH 6.00. Pre-acidifying the cheeses to different pH values using the same acid did not cause a significant difference in pH at 2 d timepoint or total calcium content in the final cheeses, except for cheese PA with citric acid (Table 4.1). The cheeses that were PA with acetic and carbonic acid to pH 6.00 had slightly lower amounts of total calcium as compared to cheeses PA to pH 6.40 using the same acid, but this was not significant. Pre-acidifying milk with citric acid to pH 6.00 significantly lowered the pH and total calcium content. Metzger et al. (2001a) observed a similar reduction in the total calcium in their low-fat Mozzarella cheese made by PA with citric acid as compared to cheese PA using acetic acid. Metzger et al. (2001a) also observed a difference in total calcium content between their cheeses PA to different pH values (pH 6.0 and pH 5.8) with acetic acid, which was in contrast to our cheeses PA with acetic acid to different pH values. The observed differences in their study between their cheeses PA to different pH values could be due to lower PA pH employed or higher moisture content in their cheeses as compared to the cheeses in our

study. We also manufactured our cheeses from milk with higher casein content; it is possible that the slightly lower moisture content in the curds in the cheese PA to pH 6.00 (approx. 2-3% lower moisture) earlier in the process during cheese manufacture, as compared to cheeses PA to pH 6.40, could have restricted calcium solubilization causing only a slight difference in total calcium content between the cheeses PA to different extent using acetic acid.

The lactose and galactose contents were significantly affected by treatment and storage time as shown in Table 4.2. The cheeses that were PA to pH 6.00 had a significantly higher lactose content (Figure 4.1b) and lower galactose content (Figure 4.2b) as compared to control and cheeses that were PA to pH 6.40 (Figure 4.1a and 4.2a). This was because, in the control and the cheeses PA to pH 6.40, the starter cultures were mostly responsible for fermenting the lactose to reduce the pH of milk from pH 6.60 or pH 6.40 to the final cheese pH of 5.20, whereas, in the PA cheeses, less growth or acidification was required by the starter cultures as they only had to reduce the pH from pH 6.00 to final pH of 5.20. The cheeses that fermented more lactose tended to have higher galactose content. This was because during fermentation, the lactose was metabolized into glucose and galactose and the *Streptococcus thermophilus* strain used in our study can only utilize glucose to produce lactic acid while excreting the galactose (Johnson and Olson, 1985). Amongst the cheeses that were PA to pH 6.00, the use of carbonic acid resulted in the cheese having a significantly lower lactose content. This was because the pH values after PA to 6.00 slightly increased by 0.1-0.15 pH units as a result of injected carbonic acid getting converted to CO_2 gas, which then escaped from the milk sample. A similar observation was made by To et al. (2022). Since the pH slightly increased after PA in the carbonic acid cheese, the starter cultures needed to ferment additional lactose to get to the final milling pH compared to the cheese PA with citric or acetic acid. As more lactose is fermented, more lactic acid is produced. For this reason, the control

and the cheeses PA to pH 6.40 seemed to have a slightly higher lactic acid content while the cheeses PA to pH 6.00 with citric or acetic acid seemed to have a slightly lower lactic acid content (Figure 4.3) although this was not significantly different (Table 4.2). The lactose content in the cheeses significantly decreased and the lactic acid and the galactose content slowly increased until the 2-wk timepoint as a result of some fermentation of residual lactose (Table 4.2). Moynihan et al. (2016) observed similar changes in lactose and lactic acid contents in their LMPS Mozzarella cheeses during storage.

4.3.2 Total and Insoluble Calcium Changes During Cheese Manufacture and Storage

The total and insoluble calcium contents in the milk sample were 32.1 ± 0.2 and 23.8 ± 1.4 mg/g protein, respectively. In unconcentrated milk, approximately, 68% of total calcium is in the insoluble calcium form (Lucey and Fox, 1993). The percentage of insoluble calcium content in our milk sample (concentrated to 4% casein) was 74%. This suggests that some of the soluble calcium in the aqueous phase of milk was removed via the permeate during the membrane concentration of the milk sample, thus altering the calcium balance (ratio of soluble to insoluble calcium) before the start of the cheese-making process.

The total calcium content decreased significantly in all the samples as cheese manufacturing progressed (Figure 4.4). The most significant decrease in total calcium was observed at the whey drainage step where most of the dissolved calcium in the sample was lost to whey; the moisture in the samples decreased approximately from 85% (milk sample) to 56% at the end of the whey drain step. The total calcium decreased only slightly, approx. 3-4 mg/g protein, after the whey drain step to cheese at 2 d timepoint as the moisture decreased further, by approx. 7-8% due to ongoing syneresis. There were no differences between the control and all of the

cheeses that were PA except for the cheese PA to pH 6.00 with citric acid. The cheese PA to pH 6.40 with citric acid had a slightly lower total calcium than the other samples PA to the same pH (Figure 4.4a). The cheeses that were PA to pH 6.00 with citric acid had a significantly lower total calcium as compared to all other samples (Figure 4.4b).

The insoluble calcium content decreased in all the cheese samples as the cheese-making progressed (Figure 4.5). The milks PA to pH 6.40 and 6.00 had lower amounts of insoluble calcium as compared to control milk as a result of lower pH dissolving more insoluble calcium. There were no differences in the amount of insoluble calcium in the milk samples PA to pH 6.40 (Figure 4.5a) whereas the milk samples PA to pH 6.00 (Figure 4.5b) using different acids had varying amounts of insoluble calcium content, where the milk PA using citric acid had the most dissolved insoluble calcium at each pH value. After the whey drain step, all of the curds had similar amounts of insoluble calcium except the cheese PA to pH 6.00 using citric acid (Figure 4.5b). The pH of the curd in all the samples after the whey drainage step was approx. 5.40 except for the cheese PA to pH 6.00 with citric acid, which was approx. 5.26. Since citric acid is a calcium chelator, it removes more calcium from the caseins as compared to other acids at any pH value. As more calcium and phosphate was removed by the citric acid as a result of CCP solubilization, the buffering capacity in the milk and curd samples of cheese made from milk that was PA to pH 6.00 with citric acid, was greatly reduced resulting in curds with a lower pH value after the whey drain step. The insoluble calcium content decreased slightly between the curds after whey drain and milling as the pH decreased by 0.2 units (Figure 4.5). The stretching and the brining process after milling also further decreased the insoluble calcium content in the curds as shown in Figure 4.5.

There was a significant difference in insoluble calcium amounts between control and PA cheeses during storage (Figure 4.6; Table 4.2). The control had the highest amount of insoluble

calcium compared to all other cheeses; within the PA cheeses, the cheeses that were PA with citric acid to pH 6.40 (Figure 4.6a) and pH 6.00 (Figure 4.6b) had the lowest amount of insoluble calcium. The cheeses PA to pH 6.00 had slightly lower insoluble calcium values as compared to cheeses PA to pH 6.40 at all storage timepoints, approx. 0.5 mg Ca/g protein for cheeses PA with acetic and carbonic acid and approx. 3 mg Ca/g protein for cheeses PA with citric acid. Similar effects with the different acids used for acidification during Mozzarella cheese manufacture have been observed by others (Keller et al., 1974; Metzger et al., 2001a; To et al., 2022) in cheese made from unconcentrated milk. During storage, the insoluble calcium content in the cheeses changed slightly but significantly (Table 4.2) due to calcium buffering (Figure 4.6).

4.3.3 pH Changes in Cheeses During Storage

The type of acid used for PA of cheese significantly ($P < 0.05$) affected the pH as shown in Table 4.2. The control and the cheese PA with carbonic acid had a slightly higher pH values during storage as compared to cheeses PA with acetic or citric acid (Figure 4.7). Differences in the pH of PA did not significantly affect the pH of the cheese made with acetic or carbonic acid but affected the cheeses PA with citric acid. The cheese PA with citric acid to pH 6.00 had a significantly lower pH values during storage compared to the cheese PA to pH 6.40 (Figure 4.7). The trend for the pH values were strongly correlated with the trends for the insoluble calcium content in the cheeses during storage (Figure 4.6), where a cheese with a higher pH value had a higher amount of insoluble calcium. There was a slight increase in pH values at the 2 d time point as compared to the milled curds on the day of manufacture. Similar observations have been made by Guinee et al. (2002). This increase in pH can be attributed to some additional lactic acid produced shortly after brining (Figure 4.3), which favors the solubilization of insoluble calcium phosphate (as seen in Figure 4.5 between the milled and cheese at 2 d). As the concentration of

phosphate ions increases in the aqueous phase of cheese due to the solubilization of CCP , the free H^+ ions in the cheese serum associate with the phosphate ions thereby increasing the pH of the cheese. There were very minor but not significant (Table 4.2) changes in pH values of cheeses during storage.

4.3.4 Proteolysis

Proteolysis in Mozzarella can be mostly attributed to the residual rennet activity (the rennet that was retained in the cheese during manufacture) and partly because of plasmin, an indigenous protease enzyme present in milk (Kindstedt and Fox, 1993). α_s- Casein is mostly hydrolyzed by chymosin while plasmin is more specific to β-casein. The starter cultures used during cheese manufacture do not contribute much to the proteolytic breakdown in LMPS Mozzarella as they are mostly inactivated by the high temperature used during the stretching process. Neither the difference in pH values used for PA or the types of acid used for cheese manufacture caused a significant difference in the pH 4.6 soluble N levels (proteolysis) (Table 4.2) in our samples. A slow (significant) increase in protein breakdown was observed with storage time as shown in Table 4.2 and Figure 4.8. The extent of proteolytic breakdown observed in our study was similar to observations made by Feeney et al. (2002).

4.3.5 Microstructure of Cheeses

The microstructural changes in LMPS Mozzarella cheeses over refrigerated storage period, as determined by CLSM, are shown in Figures 4.9 and 4.10. All young cheeses had visible protein fibers (green in color) distributed in parallel with entrapped fat (red) and serum channels (black), which is a characteristic of pasta-filata-style cheeses. This type of orientation is due to the high-temperature stretching process that the cheeses undergo during manufacture that makes them

pliable (some more than others depending on the insoluble calcium content in the cheese), allowing orientation of protein during extrusion. At 2 d of storage, the control (Figure 4.9a) and the acetic acid cheeses (Figure 4.9f and Figure 4.10a) had a very similar para-casein matrix with fat distribution typical of LMPS Mozzarella cheeses; similar to observations made by Guinee et al. (1999). The para-casein matrix in the cheeses PA with citric acid to pH 6.40 (Figure 4.9k) and pH 6.00 (Figure 4.10f) did not have very obvious fibers present and they had a more open structure with larger fat globules. This could be attributed to the lower insoluble calcium content in these cheeses (Figure 4.6) which made the protein matrix very soft and pliable (as observed when the cheese came out of the cooker/stretcher), and not elastic enough to keep the fat channels separate; hence the fat globules seemed to have coalesced during stretching in the cooker/stretcher leading to the formation of larger fat globules. A similar large fat droplet size was observed by Joshi et al. (2004) in their studies on lower calcium LMPS Mozzarella. Other studies (McMahon et al., 2005; Guo and Kindstedt, 1995) also suggested that the increased hydration and swelling of the protein matrix caused by lower calcium cheeses could weaken the curd matrix and thereby affect the microstructure of cheese. In contrast to the cheeses PA with citric acid, the cheese PA with carbonic acid in our study at 2 d time point (Figure 4.9p and Figure 4.10k) had a highly intertwined (but less obvious fibers) para-casein matrix with many smaller fat globules distributed within the protein channels (more evident in the cheese that was pre-acidified to a lower pH; Figure 4.10k). The smaller size of fat globules in the cheeses made with carbonic acid could be due to how the cheese milk was pre-acidified. The injection of CO_2 into the milk could have disrupted the milk fat globule membrane and allowed the fat to be broken down into smaller sizes. The relatively higher insoluble calcium content (Figure 4.6) and a relatively lower fat content (Table 4.1) in the cheeses made by PA with carbonic acid could have also contributed partly to this type of

microstructure. The cheeses made by PA with carbonic acid had a good stretch and feathering in the curds coming out of the cooker/ stretcher owing to this cheeses structure.

As the cheese age increased, the water channels and visible fibers in all of the cheeses disappeared, which suggested that the free moisture in the cheeses was absorbed back into the protein matrix. By the 1 mo time point, the fat in all the cheeses that were PA to pH 6.00 was completely encased within the protein matrix. By the 3 mo time point, all cheeses had lost all visible channels and pores in the protein network due to ongoing proteolysis (Figure 4.8). Similar changes in the microstructure of LMPS Mozzarella during refrigerated storage have been observed by Kiely et al. (1993), Kuo and Gunasekaran. (2009), and McMahon et al. (1998).

4.3.5 Textural Properties

The hardness of the cheese as measured by TPA was significantly affected by treatment (Table 4.3). There were only small differences between the control and the cheeses that were PA to pH 6.40 (Figure 4.11a). The cheese that was PA to pH 6.00 with citric acid (Figure 4.11b) had a significantly higher hardness value as compared to all the cheeses PA to pH 6.40 (Figure 4.11a) and the cheese PA to pH 6.00 with carbonic acid (Figure 4.11b). The high hardness of the cheese PA to pH 6.00 with citric was likely due to its much lower pH value (Table 4.1). Low pH values promote increased hardness. To et al. (2022) observed a lower hardness in their cheese PA with lactic acid as compared to the cheese PA with carbonic acid due to slightly higher moisture content (approx. 2% higher). We did not observe any significant difference in the hardness values in our cheeses with an increase in cheese age during the 90 d of refrigerated storage (Table 4.3). Natural cheeses can soften over time due to proteolytic breakdown and calcium solubilization (Lucey et al., 2003) but since the rate of proteolysis was very slow in our cheeses over the storage period

(Figure 4.8) and insoluble calcium hardly changed (Figure 4.6), we did not observe a significant change in cheese hardness over time (Figure 4.9). It is possible that we would have observed more textural change if we did a large compression (more than 30% compression).

4.3.6 Rheological Properties

The LT_{max}, crossover temperature, and G′ value at 70ºC were all significantly impacted by treatment (Table 4.3). The cheeses made from milk that was pre-acidified to a lower pH initially had a slightly lower LT_{max} (Figure 4.12b) compared to cheeses pre-acidified to a higher pH (Figure 4.12a). The cheese made from milk PA to pH 6.00 with citric acid had a significantly lower LT_{max} compared to all other cheeses. Typically, a cheese with lower insoluble calcium content has a higher LT_{max} compared to a cheese with higher insoluble calcium content (Choi et al., 2008). The pH value of cheese plays an important role in cheese meltability and the molecular interactions (mainly, electrostatic repulsion between similar charges on proteins, electrostatic attraction between opposite charges, salt bridges, CCP bridges, and hydrophobic interactions) involved in meltability can be modified with the pH of the cheese (Lucey et al., 2003). Since the pH in the cheese that was PA to pH 6.00 with citric acid was significantly lower and closer to the isoelectric point of casein (pH 4.6), compared to other cheeses (Figure 4.7), there was likely an increase in attraction between opposite charges (plus/minus) between caseins. This increased attraction would have reduced melt. LT_{max} significantly increased with an increase in storage time but there was no interaction between the treatment and age of the cheese (Table 4.3). As the storage time increased,

the LT_{max} of the cheese increased (Figure 4.12) likely due to slow increase in proteolytic breakdown.

The G′ value at 70ºC was significantly affected by the treatment and storage time (Table 4.3). The interaction between treatment and age was also significant (Table 4.3). At 2 wk timepoint, all cheeses had similar G′ values at 70ºC except for the cheese PA to pH 6.00 with citric acid (Figure 4.13), which had a significantly higher G′ value. The enhanced electrostatic attraction due to the lower pH value in this cheese could likely have caused it to have a stiffer gel at 70ºC, as compared to other cheeses. The G′ values at 70ºC slowly decreased over the storage period, a more significant decrease was observed in cheese PA to pH 6.00 with citric acid as compared to the other cheeses.

The crossover temperature (the temperature at which the cheese transitions from a solid-like to a liquid-like system) or melting point was significantly impacted by the treatment and age (Table 4.3). The interaction between the treatment and age of cheese was also significant. The cheeses that were PA with citric acid tended to have higher crossover temperatures for both PA pH values (Figure 4.14). The pH of the cheeses also could have impacted the crossover temperature in the cheeses. Cheeses that had a higher pH had a lower crossover temperature (cheese melted sooner) and vice versa. A slightly higher temperature was required to melt the cheese (LT=1) in lower pH cheeses due to their increased G′ values at high temperatures for the cheese PA with citric acid to pH 6.00 (Figure 4.13). The crossover temperature in all the cheeses decreased with an increase in storage time (some more so than others) except for the control cheese. The reduced casein crosslinking as a result of increased proteolytic breakdown during storage (Lucey et al., 2003), likely caused these cheeses to melt more readily.

4.3.7 Sensory Analysis

4.3.7.1 Unmelted Cheese

The extent of pre-acidification and the type of acid employed during cheese manufacture did not significantly impact any of the texture attributes when evaluated in cube form including firmness, cohesiveness, and adhesiveness (results not shown). These parameters were significantly impacted by storage time. The cheeses became less firm, more cohesive, and more adhesive with age. The changes in these parameters during aging can be attributed to the slow increase in the proteolytic breakdown of cheese. Similar trends in sensorial textural change in LMPS Mozzarella cheese during refrigerated storage have been observed by Moynihan et al. (2014) and Ozturk et al. (2018). Our sensory firmness results did not correlate with TPA hardness. It should be noted that although the sensory firmness of the cheeses was significantly lower between 4 and 12 wk time points, it was only a one-point change in the score as measured using the 15-point QDA scale (results not shown). The flavor attributes of the cubes including acid and sourness scores seemed to be slightly higher for cheeses PA with citric and acetic acid to pH 6.00 as compared to other cheeses although they were not significantly different. These acid and sourness values for all the cheeses were lower than slight (score of 3 or below) on the QDA scale.

The shred parameters including straightness, shred length, matting, and adhesiveness were significantly impacted by storage time but not by treatment (Table 4.4). Since the composition, pH, and the proteolytic breakdown rates were similar in all cheeses, there were no major differences in the shredding of cheese. All of the mentioned shred parameters significantly changed with age (Table 4.4) at 2 wk and 1 mo time points, most parameters were similar but changed significantly at 3 mo time point; straightness and shred length decreased whereas matting and

adhesiveness increased. These results correlated with changes in adhesiveness of the cheese (measured using sensory); where a significant increase in adhesiveness of the cheese with age (Table 4.4) caused the cheese to stick more to the blade during shredding, which resulted in the cheese bending and deforming around the blade reducing the straightness and strand length of the cheese (Chen et al., 2009). Surface oil or free moisture in the shreds was not significantly affected by either treatment or age (results not shown).

4.3.7.2 Melted Cheese

The melted cheese surface characteristics including blister quantity, blister color, free oil, and skin formation were not impacted by treatment, but they were significantly affected by storage time (results not shown). There was a complete melting of shreds in all of the cheeses (QDA score > 14.5). The free oil was relatively lower in the cheese that was PA to pH 6.00 with carbonic acid with slightly higher blister quantity as compared to other cheeses, although they were not significantly different (results not shown). Rudan and Barbano (1998) reported that an increase in free oil on the cheese surface during melting helps protect the top layer of cheese from excessive moisture loss (dehydration) resulting in less burning and blistering of the protein layer. This phenomenon is probably what we observed in our cheese that was PA to pH 6.00 with carbonic acid. Since this cheese had a lower FDM (Table 4.1), it probably had relatively lower free oil release resulting in greater moisture loss from the cheese surface (drying) and as a result increased blistering. There was only a relatively small decrease in these mentioned melted cheese surface parameters with storage time except for blister quantity, which dramatically increased with storage

time. The mean blister quantity scores of all cheeses at 2, 4, and 12 wk time points were 6.9, 8.3, and 11.0, respectively. During the storage period, as proteolysis progressed and the free moisture was absorbed by the proteins, there was an increased tendency for cheeses to dry out on the surface as it is heated in the oven. If the extent of proteolysis is not too high and cheese is still pliable, the top layer of the dried cheese protein burns before releasing steam, which causes more blisters to form as the cheese is aged (Dairy Pipeline, 2022).

The strand length of the cheeses PA to pH 6.40 and pH 6.00 with citric acid were slightly lower compared to other cheeses but not significant (results not shown). Strand thickness was significantly impacted by both treatment and storage time (Table 4.5). At 2 wk of storage, the cheese PA to pH 6.00 with citric acid exhibited slightly lower strand thickness compared to all other cheeses (Table 4.6). The insoluble calcium content in cheese has been shown to influence the melting and stretching characteristics of cheese when heated (Choi et al., 2008; Metzger et al., 2001b; Guinee et al., 2002). A lower insoluble calcium content in cheese helps increase melt but decreases the stretch quality of cheese due to weakened CN-CN interaction, i.e., reduction in CCP cross-linking between casein molecules (Lucey et al., 2003). Since our pH 6.00 pre-acidified citric acid cheese had approx. 5 mg lower insoluble Ca/g protein as compared to other cheeses (Figure 4.6), it had a relatively shorter strand length (results not shown) and a significantly smaller strand thickness as compared to other cheeses (Table 4.6). Proteolytic breakdown of cheese can further weaken the cheese matrix and could contribute to lower strand thickness as shown in Table 4.6. Strand thickness significantly decreased with storage time in most cheeses, with the cheese PA to pH 6.00 with citric acid having lower strand thickness values compared to most other cheeses.

The first chew hardness (force required to bite through a sample) and chewiness were significantly affected by both treatment and age (Table 4.5). There were some differences in first

chew hardness or chewiness between cheeses at 2 wk of storage time. At the 4 and 12 wk of storage, the cheese PA to pH 6.00 with citric acid exhibited slightly lower first chew hardness and chewiness as compared to most other cheeses probably due to lower insoluble calcium content along with proteolytic breakdown at these timepoints. Similar trends were observed by Metzger et al. (2001b) in their cheese PA with citric acid. The first chew hardness and chewiness of the cheeses decreased significantly in most of the cheeses with increase in storage time. Among the flavor attributes measured, sourness was the only parameter that was significantly impacted by treatment and storage time (Table 4.5). At 2 wk of storage, the cheese that was PA to pH 6.00 with citric and acetic acid was significantly sourer but at 12 wk storage only the cheese PA to pH 6.00 with citric acid had significantly higher sourness values as compared to cheese PA to pH 6.00 with carbonic acid (Table 4.6). Although there were significant differences between cheeses in terms of sourness values, the values were around threshold limits (< 1.5 on a QDA scale).

4.4 Conclusions

The extent of PA and type of acid used during LMPS Mozzarella cheese manufacture impacted the amount of total and insoluble calcium lost during cheese manufacture. Pre-acidifying cheese milk to pH 6.00 using acetic and carbonic acids resulted in a cheese with approx. 1 mg lower total Ca/g protein and approx. 0.5 mg lower insoluble Ca/g protein as compared to milk that was PA to pH 6.40. PA of milk to pH 6.00 with citric acid resulted in cheese that had approx. 3 mg lower total and insoluble Ca/g protein as compared to sample where the milk was PA to pH 6.40. The rate of proteolytic breakdown was slow in all cheeses with no differences between the cheeses as a result of a difference in cheese making procedure. There were no significant differences in textural, rheological, or sensory properties between the cheeses except for the cheese PA to pH 6.00 with citric acid, which had lower first chew hardness, lower chewiness, lower strand

thickness, and was sourer compared to other cheeses as analyzed using sensory panelists. Unless a calcium-chelating acid like citric acid is used for pre-acidification during cheese manufacture, the extent of pre-acidification did not seem to impact the calcium balances enough to cause a difference in cheese functional properties at the casein content studied.

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4.6 Tables and Figures

Table 4.1. Composition of cheeses for low-moisture part-skim Mozzarella cheeses manufactured from high casein milks using different pH values for pre-acidification and acid types, measured at 2 wk of storage (n=4)

Components	Control	Pre-acidification pH 6.40			Pre-acidification pH 6.00			SEM
		Acetic	Citric	Carbonic	Acetic	Citric	Carbonic	
Moisture, %	48.4^{a}	48.2^{a}	48.5^{a}	48.0^{a}	49.2^{a}	49.1^{a}	48.7^{a}	0.46
Fat, %	21.8^{a}	21.8^{a}	21.8^{a}	22.0^{a}	21.5^{ab}	21.4^{ab}	20.9^{b}	0.29
Fat in dry matter, %	42.2^{a}	42.2^{a}	42.4^{a}	42.2^{a}	42.2^{a}	42.0^{a}	40.6^{b}	0.27
Protein[1], %	25.0^{b}	25.1^{ab}	25.0^{b}	25.3^{ab}	24.7^{b}	25.0^{b}	25.9^{a}	0.29
Salt[2], %	1.5^{a}	1.5^{a}	1.5^{a}	1.4^{a}	1.7^{a}	1.6^{a}	1.5^{a}	0.08
Salt in moisture[2], %	3.1^{a}	3.2^{a}	3.2^{a}	3.0^{a}	3.4^{a}	3.3^{a}	3.0^{a}	0.15
pH @ 2d	5.19^{ab}	5.14^{dc}	5.12^{d}	5.17^{abc}	5.15^{bcd}	5.04^{e}	5.21^{a}	0.02
Total Ca[3]	25.0^{a}	24.2^{abc}	23.3^{c}	24.5^{ab}	23.3^{c}	19.9^{d}	23.6^{bc}	0.35

$^{a-e}$ Means within a row not sharing common superscript differ ($P < 0.05$)

[1]% N × 6.38

[2]Determined using chloride analyzer

[3]Measured by ICP-OES and expressed as mg/g protein

Table 4.2. Probabilities and R^2 values for pH, lactic acid, galactose, insoluble calcium content, and pH 4.6 soluble nitrogen for low-moisture part-skim Mozzarella cheeses manufactured from high casein milks using different pre-acidification pH values and acid type during 90 days of refrigerated storage at 4ºC (n=4)

Factor[1]	df	Lactose	Galactose	Lactic acid	pH	Insoluble calcium[2]	pH 4.6 Soluble N
Whole-Plot							
Treatment (T)	6	0.01	0.0037	0.07	0.0002	<0.0001	0.70
Day of Cheesemaking (D)	3	0.0058	<0.0001	<0.0001	0.08	0.0002	0.0009
Error (T × D)	18						
Split-Plot							
Storage time (S)	4	<0.0001	0.0033	<0.0001	0.07	0.03	<0.0001
S × T	24	0.10	0.85	0.96	0.18	0.21	0.43
R^2		0.87	0.73	0.83	0.82	0.98	0.97

[1] Split-plot design with 7 treatments were analyzed as a discontinuous variable and the cheesemaking day was blocked (7 × 4 blocked design). Subplot included the effect of storage time of cheese (S) and storage time × treatment as variables.

[2]The values indicate statistical differences observed between cheeses throughout 12 wk. of storage.

Table 4.3. Probabilities and R^2 values for TPA hardness, max loss tangent, crossover temperature (ºC), and G' value at 70ºC for low-moisture part-skim Mozzarella cheeses manufactured from high casein milks using different pre-acidification pH values and acid type during 90 days of refrigerated storage at 4ºC (n=4)

Factor[1]	df	TPA hardness[2]	LTmax[3]	Crossover temperature[4]	G' values at 70ºC
Whole-Plot					
Treatment (T)	6	0.02	0.0030	0.0022	0.0058
Day of Cheesemaking (D)	3	0.0043	0.18	0.21	0.66
Error (T×D)	18				
Split-Plot					
Age (A)	2	0.63	<0.0001	<0.0001	<0.0001
A×T	12	0.95	0.05	0.06	0.0018
R^2		0.62	0.88	0.89	0.83

[1] Split-plot design with 7 treatments were analyzed as a discontinuous variable and the cheesemaking day was blocked (7 × 4 blocked design). Subplot included the effect of storage time of cheese (S) and storage time × treatment as variables.

[2]Measured by texture analyzer

[3]Maximum loss tangent values

[4]Temperature at which loss tangent value = 1

Table 4.4. Probabilities and R^2 values for sensory properties of shreds for low-moisture part-skim Mozzarella cheeses manufactured from high casein milks using different pre-acidification pH values and acid types during 90 days of refrigerated storage at 4ºC (n=4)

		Shreds			
Factor[1]	**df**	**Straightness**	**Matting**	**Shred length**	**Adhesiveness**
Whole-Plot					
Treatment (T)	6	0.80	0.60	0.69	0.38
Day of Cheesemaking (D)	3	0.38	0.01	0.43	<0.0001
Error (T×D)					
Split-Plot					
Age (A)	2	<0.0001	0.02	<0.0001	0.0026
A×T	12	0.83	0.99	0.81	0.71
R^2		0.75	0.43	0.67	0.66

[1] Split-plot design with 7 treatments were analyzed as a discontinuous variable and the cheesemaking day was blocked (7 × 4 blocked design). Subplot included the effect of storage time of cheese (S) and storage time × treatment as variables.

Table 4.5. Probabilities and R^2 values for sensory properties of melted cheese for low-moisture part-skim Mozzarella cheeses manufactured from high casein milks using different pre-acidification pH values and acid types during 90 days of refrigerated storage at 4ºC (n=4), tested as melted on pizza in a forced air impinger oven.

Factor	df	Strand thickness	First chew	Chewiness	Sour
Whole-Plot					
Treatment (T)	6	0.0046	0.0001	<0.0001	<0.0001
Day of Cheesemaking (D)	3	0.02	0.01	0.0024	<0.0001
Error (T×D)					
Split-Plot					
Age (A)	2	<0.0001	<0.0001	<0.0001	0.03
A×T	12	0.74	0.06	0.0004	0.97
R^2		0.73	0.70	0.89	0.61

[1] Split-plot design with 7 treatments were analyzed as a discontinuous variable and the cheesemaking day was blocked (7 × 4 blocked design). Subplot included the effect of storage time of cheese (S) and storage time × treatment as variables.

Table 4.6. Sensory analysis results for low-moisture part-skim Mozzarella cheeses manufactured from high casein milks using different pre-acidification pH values and acid types during 90 days of refrigerated storage at 4ºC, tested as melted on pizza in a forced air impinger oven.

Attribute	Storage time (wk)	Control	Pre-acidification pH 6.40			Pre-acidification pH 6.00		
			Acetic	Citric	Carbonic	Acetic	Citric	Carbonic
Strand thickness	2	$7.49^{a,A}$	$6.06^{b,A}$	$5.87^{bc,A}$	$5.63^{bc,AB}$	$6.84^{ab,A}$	$4.80^{c,A}$	$6.63^{ab,A}$
	4	$5.89^{a,AB}$	$5.66^{a,A}$	$6.00^{a,A}$	$6.06^{a,A}$	$5.98^{a,A}$	$4.93^{a,A}$	$5.33^{a,AB}$
	12	$4.75^{a,B}$	$4.21^{ab,B}$	$3.94^{ab,B}$	$4.71^{a,B}$	$4.83^{a,A}$	$2.81^{b,B}$	$4.28^{a,B}$
First chew hardness	2	$5.45^{a,A}$	$4.88^{bc,A}$	$4.57^{c,B}$	$4.95^{abc,AB}$	$5.54^{a,A}$	$5.44^{a,A}$	$5.35^{ab,A}$
	4	$5.19^{a,A}$	$5.43^{a,A}$	$5.29^{a,A}$	$5.19^{a,A}$	$4.52^{ab,AB}$	$3.66^{b,B}$	$4.80^{ab,A}$
	12	$4.22^{ab,B}$	$4.52^{a,A}$	$3.91^{ab,C}$	$3.98^{ab,B}$	$3.51^{ab,B}$	$2.94^{b,B}$	$4.28^{ab,A}$
Chewiness	2	$6.65^{ab,A}$	$6.78^{a,A}$	$6.19^{b,AB}$	$6.52^{ab,A}$	$6.62^{ab,A}$	$6.23^{ab,A}$	$6.66^{ab,A}$
	4	$6.45^{ab,A}$	$6.73^{a,A}$	$6.54^{ab,A}$	$6.69^{a,A}$	$5.88^{bc,B}$	$5.23^{c,B}$	$6.32^{ab,A}$
	12	$5.76^{a,B}$	$5.80^{a,B}$	$5.80^{a,B}$	$6.03^{a,A}$	$4.54^{bc,C}$	$3.73^{c,C}$	$5.15^{ab,B}$
Sour	2	$1.22^{ab,A}$	$1.35^{ab,A}$	$1.17^{ab,A}$	$1.24^{ab,A}$	$1.73^{a,A}$	$1.75^{a,A}$	$1.10^{b,A}$
	4	$1.06^{a,A}$	$1.08^{a,A}$	$1.09^{a,A}$	$1.09^{a,A}$	$1.22^{a,A}$	$1.74^{a,A}$	$1.09^{a,A}$
	12	$1.29^{ab,A}$	$1.57^{ab,A}$	$1.66^{ab,A}$	$1.39^{ab,A}$	$1.60^{ab,A}$	$1.93^{a,A}$	$1.17^{b,A}$

$^{a-d}$Means within a row with different lowercase superscripts differ ($P < 0.05$), comparing the effect of treatment at a single storage time).

$^{A-B}$Means within a column (for a particular attribute) with different uppercase superscripts differ ($P < 0.05$, comparing the effect of storage time at a single treatment)

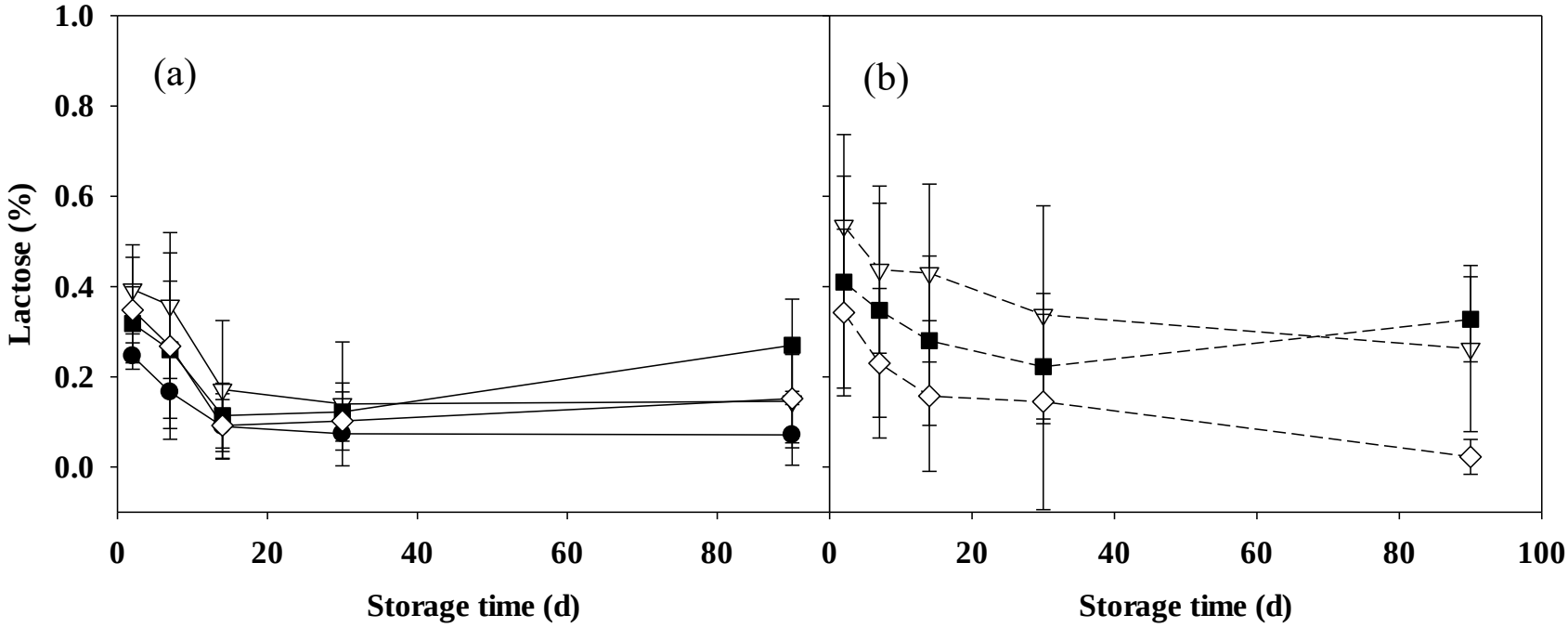

Figure 4.1 Lactose values for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC for control cheese (no pre-acidification) (●) or cheeses that were pre-acidified to a pH of 6.40 (a) or 6.00 (b) using acetic (∇), citric (■) or carbonic (◇) acid. Vertical bars represent standard deviations (n=4).

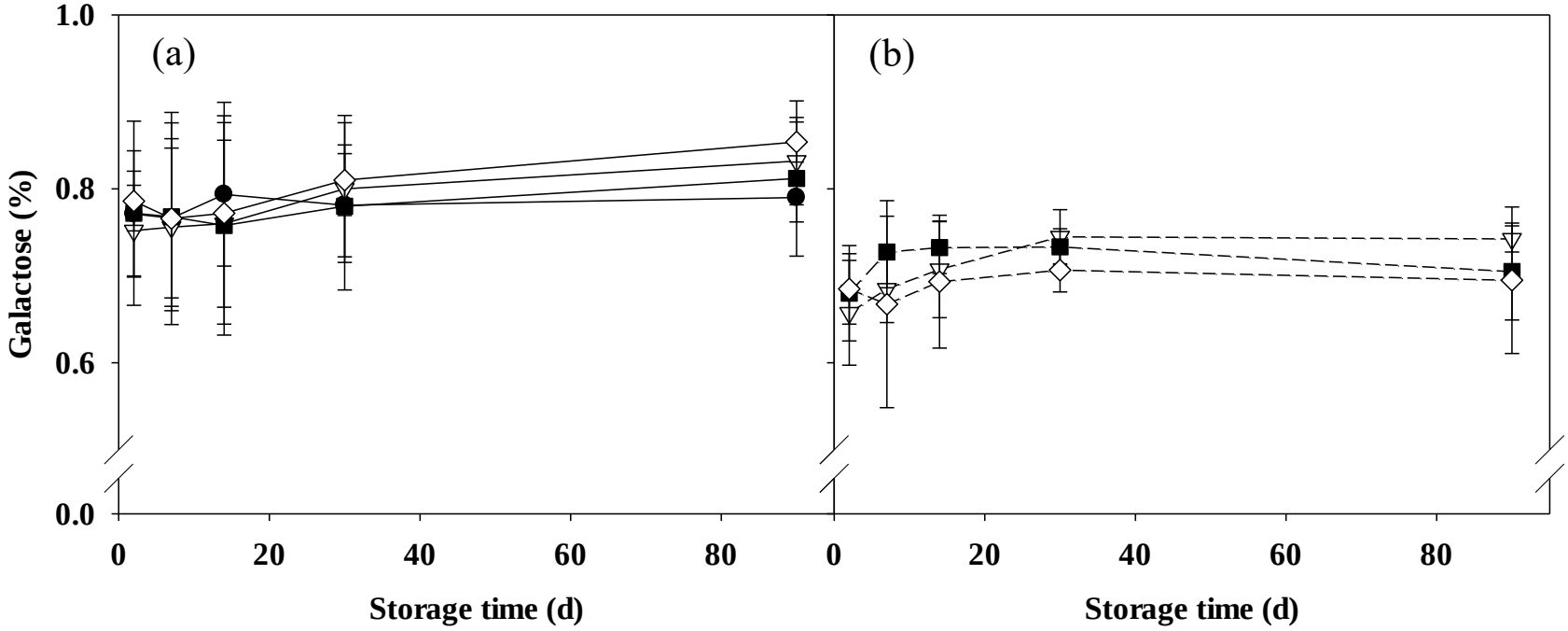

Figure 4.2 Galactose values for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC for control cheese (no pre-acidification) (●) or cheeses that were pre-acidified to a pH of 6.40 (a) or 6.00 (b) using acetic (▽), citric (■) or carbonic (◇) acid. Vertical bars represent standard deviations (n=4).

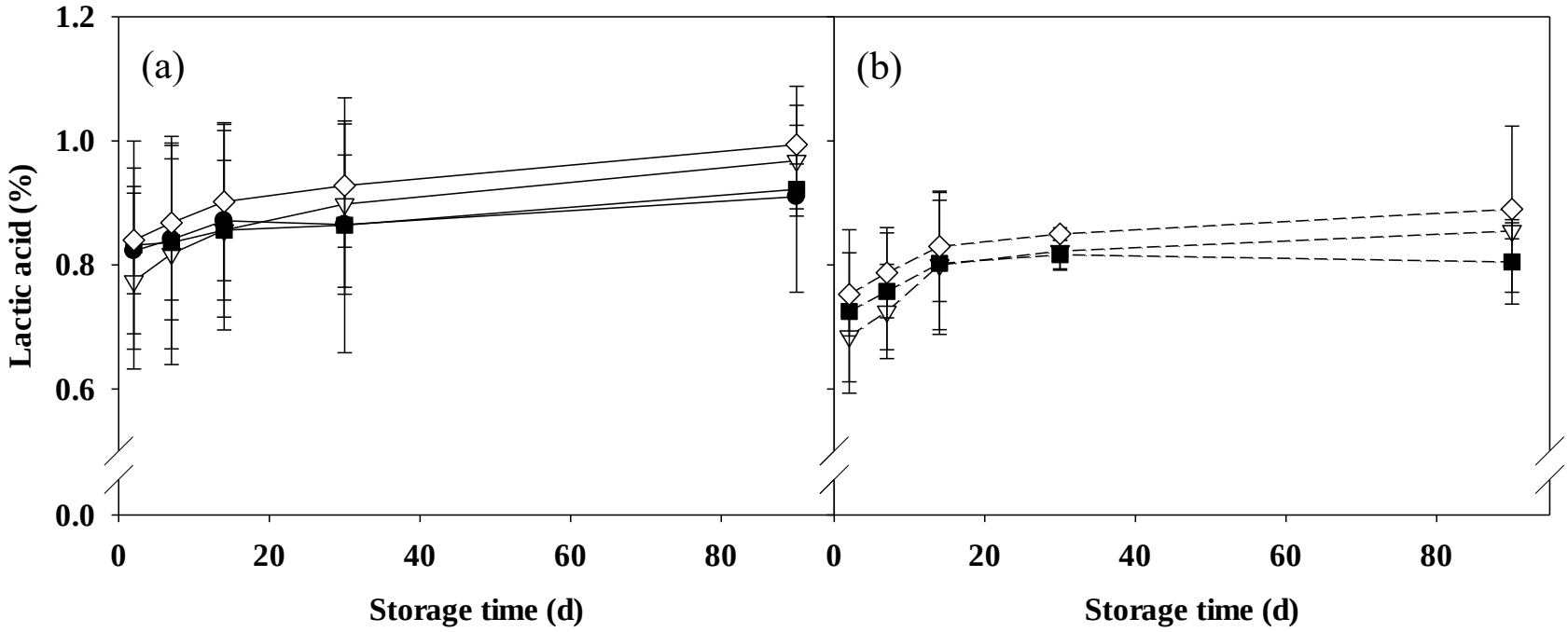

Figure 4.3 Lactic acid values for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC for control cheese (no pre-acidification) (●) or cheeses that were pre-acidified to a pH of 6.40 (a) or 6.00 (b) using acetic (▽), citric (■) or carbonic (◇) acid. Vertical bars represent standard deviations (n=4).

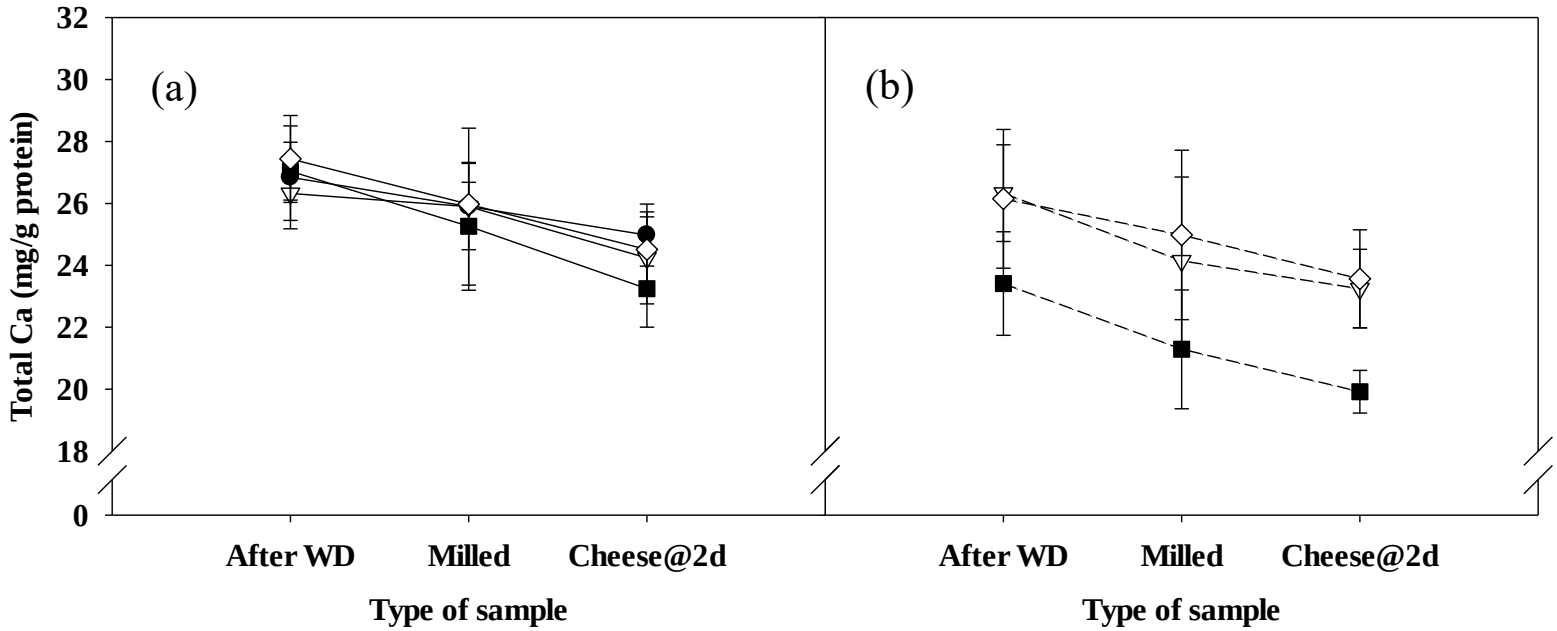

Figure 4.4 Total Ca content changes, expressed as mg/g protein, during the manufacture of low-moisture part skim Mozzarella for control cheese (no pre-acidification) (●) or cheeses that were pre-acidified to a pH of 6.40 (a) or 6.00 (b) using acetic (∇), citric (■) or carbonic (◇) acid. Vertical bars represent standard deviations (n=4). The total calcium in the control milk was approx. 38 mg/g casein. WD is curds after whey drain.

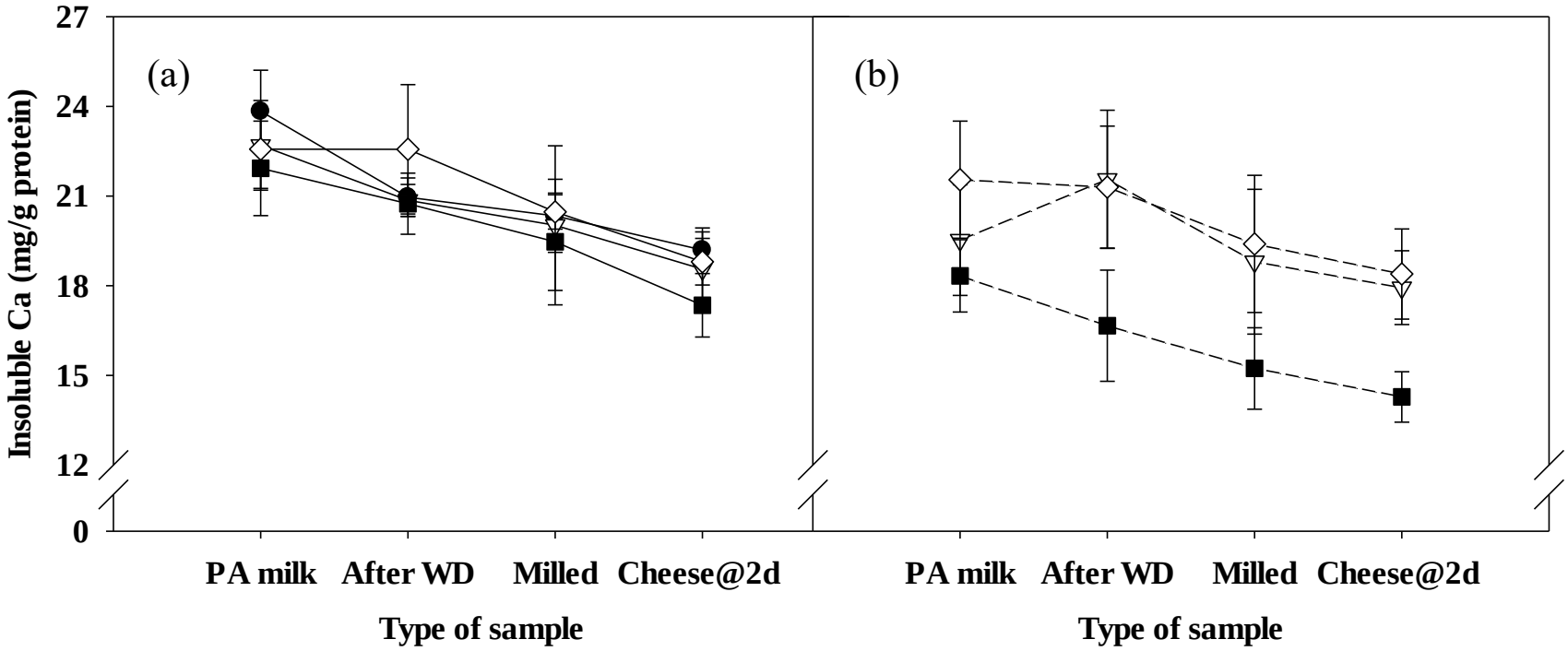

Figure 4.5 Insoluble Ca content changes, expressed as mg/g protein, during the manufacture of low-moisture part skim Mozzarella for control cheese (no pre-acidification) (●) or cheeses that were pre-acidified to a pH of 6.40 (a) or 6.00 (b) using acetic (∇), citric (■) or carbonic (◇) acid. Vertical bars represent standard deviations (n=4). The insoluble calcium in the control milk was approx. 29 mg/g casein. PA is pre-acidified milk and WD is curds after whey drain.

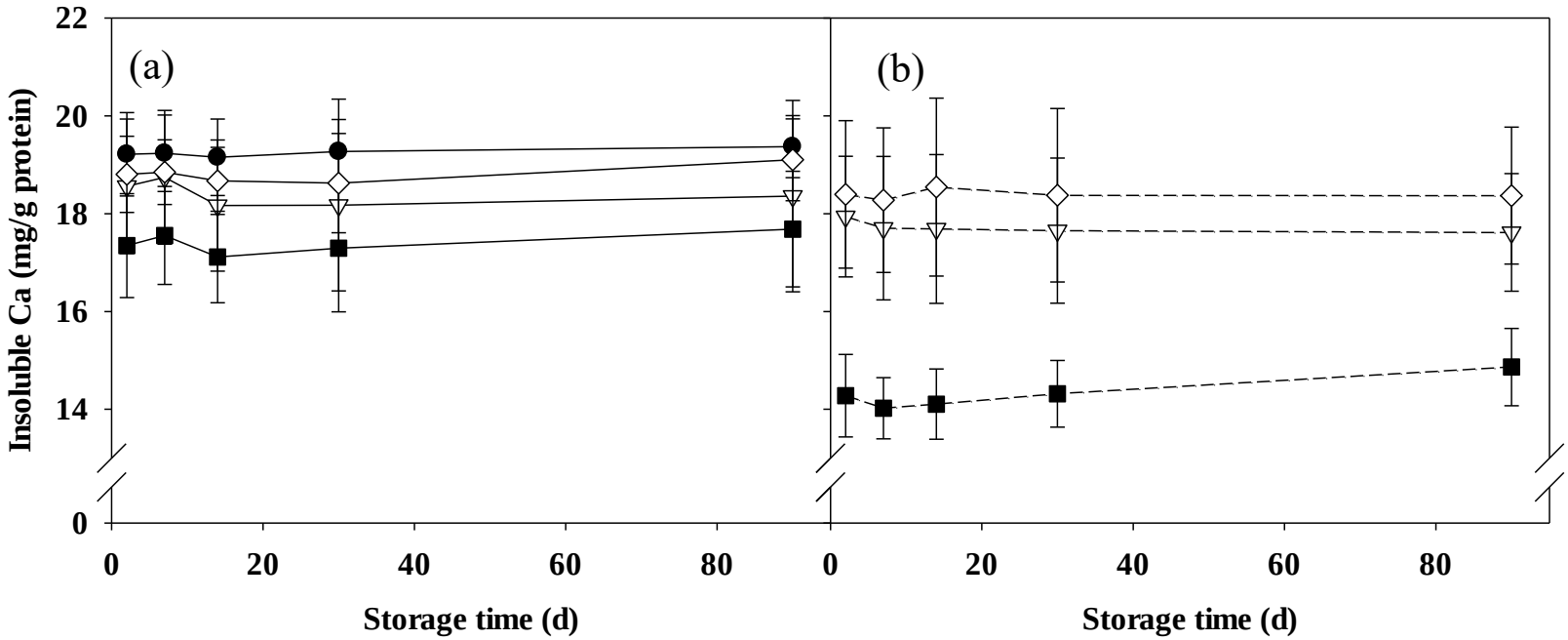

Figure 4.6 Insoluble Ca, expressed as mg/g protein, for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC for control cheese (no pre-acidification) (●) or cheeses that were pre-acidified to a pH of 6.40 (a) or 6.00 (b) using acetic (▽), citric (■) or carbonic (◇) acid. Vertical bars represent standard deviations (n=4).

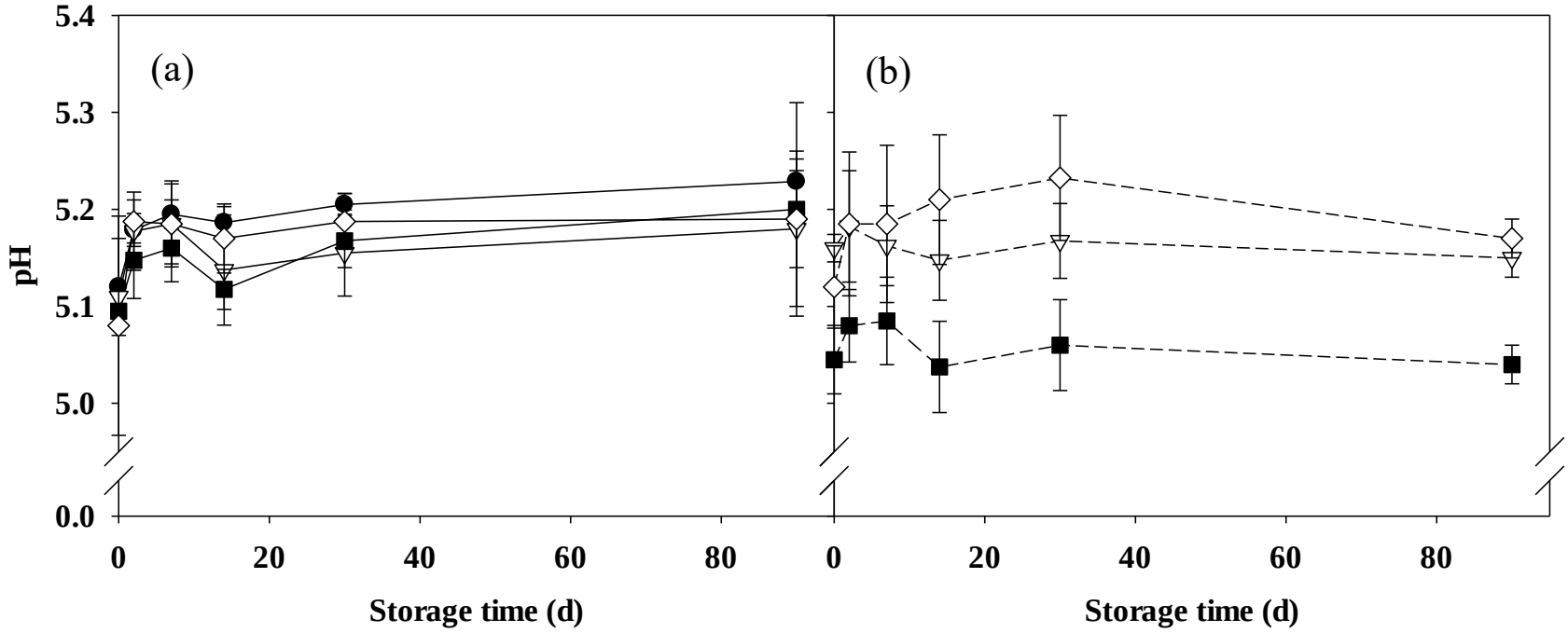

Figure 4.7 pH values for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC for control cheese (no pre-acidification) (●) or cheeses that were pre-acidified to a pH of 6.40 (a) or 6.00 (b) using acetic (▽), citric (■) or carbonic (◇) acid. Vertical bars represent standard deviations (n=4).

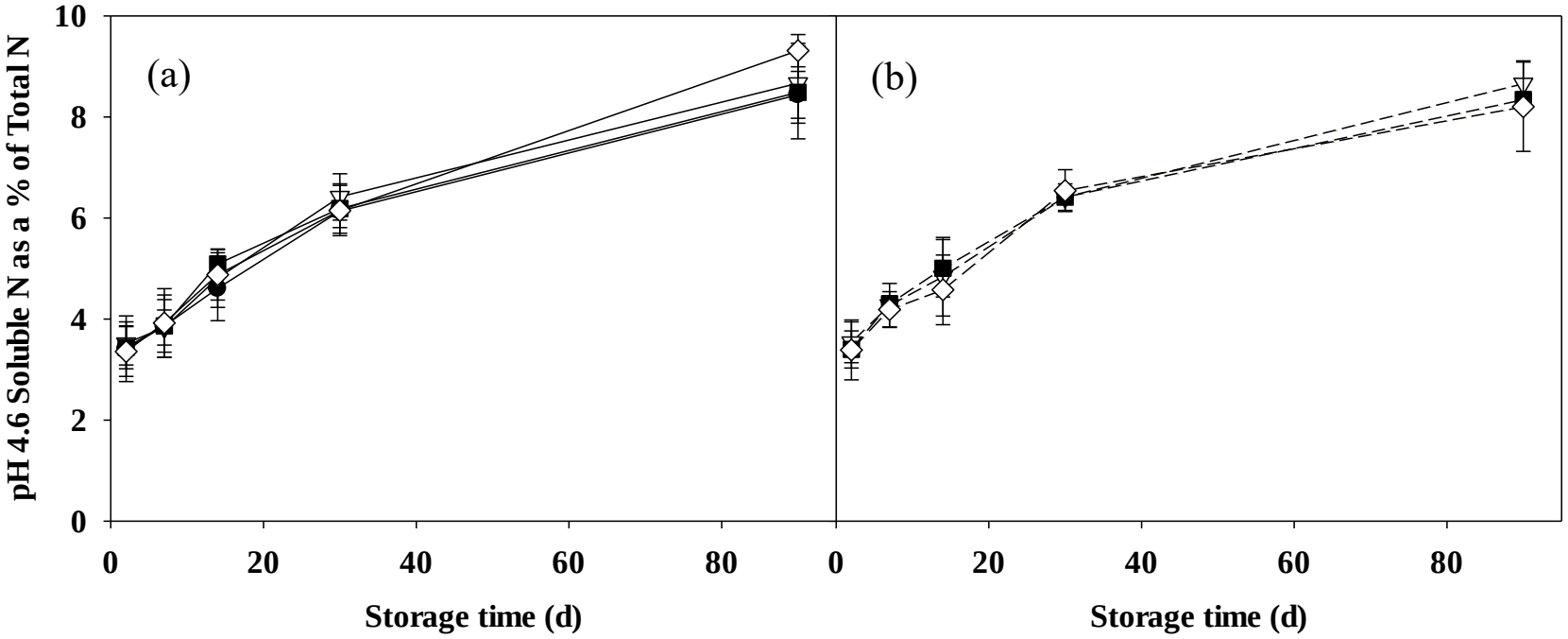

Figure 4.8 pH 4.6 soluble nitrogen as a percent of total nitrogen content for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC for control cheese (no pre-acidification) (●) or cheeses that were pre-acidified to a pH of 6.40 (a) or 6.00 (b) using acetic (▽), citric (■) or carbonic (◇) acid. Vertical bars represent standard deviations (n=4).

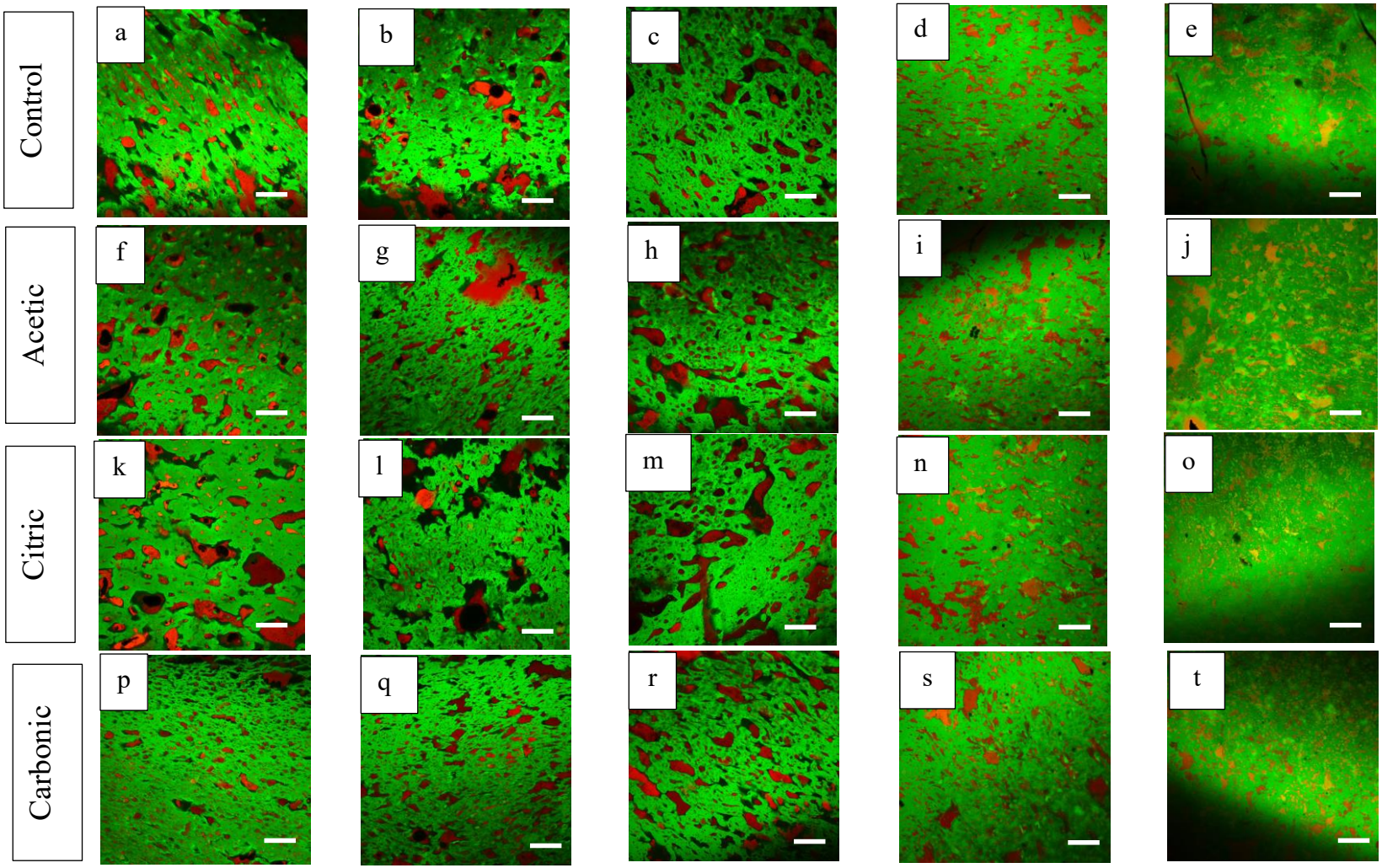

Figure 4.9 Confocal laser scanning microscopy images of LMPS Mozzarella cheeses at 2 d first column (a, f, k, p); 7 d second column (b, g, l, q); 2 wk third column (c, h, m, r); 1 mo fourth column (d, i, n, s); 3 mo of storage fifth column (e, j, o, t), stored at 4ºC for control cheese (no pre-acidification) (a, b, c, d, e) and cheeses that were pre-acidified to a pH of 6.40 using acetic (f, g, h, i, j), citric (k, l, m, n, o) or carbonic (p, q, r, s, t) acid; Nile Red and Fast Green FCF was used to dual stain the fats and proteins, respectively. Scale bar represents 100 µm.

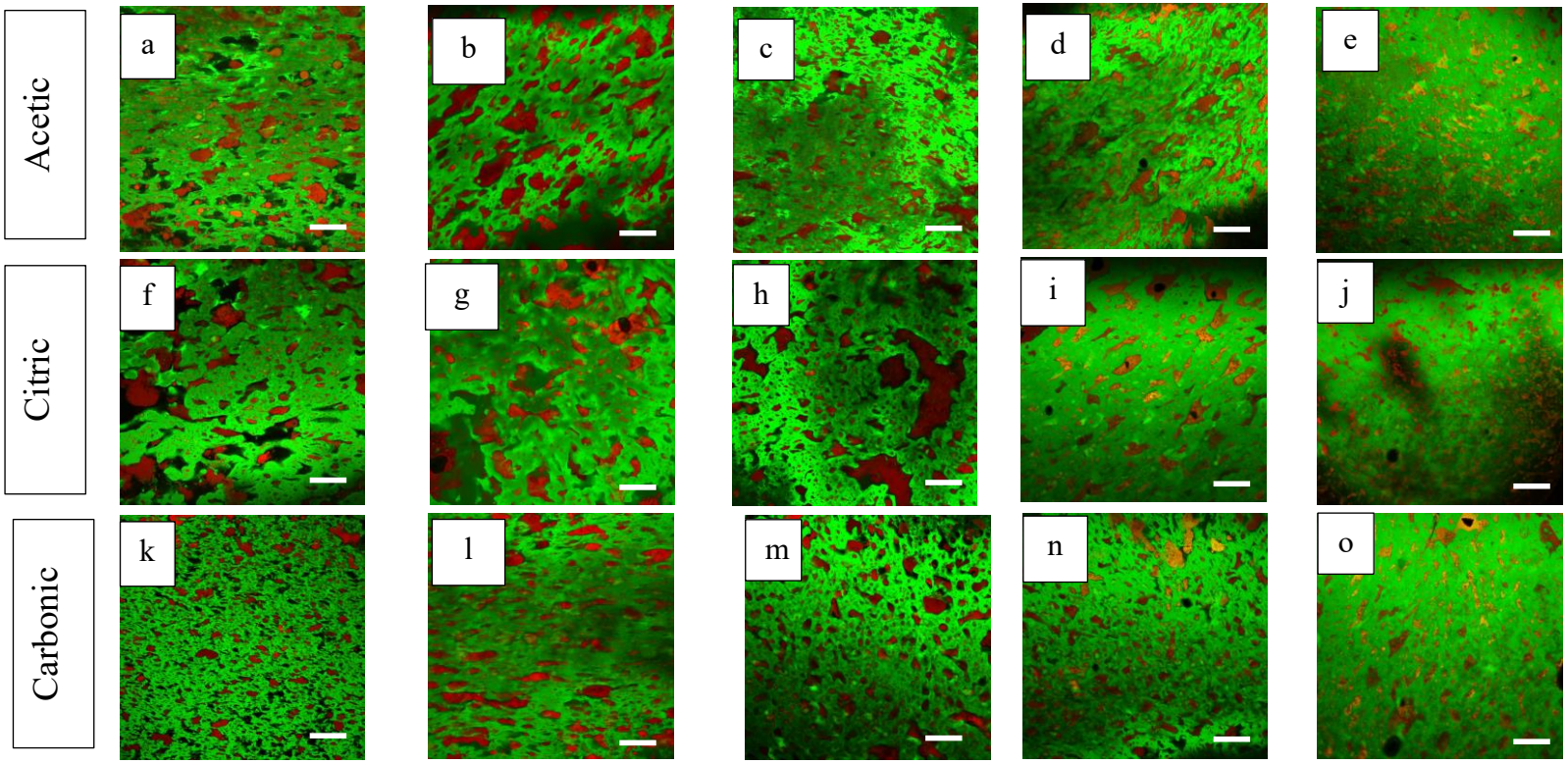

Figure 4.10 Confocal laser scanning microscope images of LMPS Mozzarella cheeses at 2 d first column (a, f, k); 7 d second column (b, g, l); 2 wk third column (c, h, m); 1 mo fourth column (d, i, n); 3 mo of storage fifth column (e, j, o), stored at 4ºC for cheeses that were pre-acidified to a pH of 6.00 using acetic (a, b, c, d, e), citric (f, g, h, i, j), or carbonic (k, l, m, n, o) acid; Nile Red and Fast Green FCF was used to dual stain the fats and proteins, respectively. Scale bar represents 100 µm.

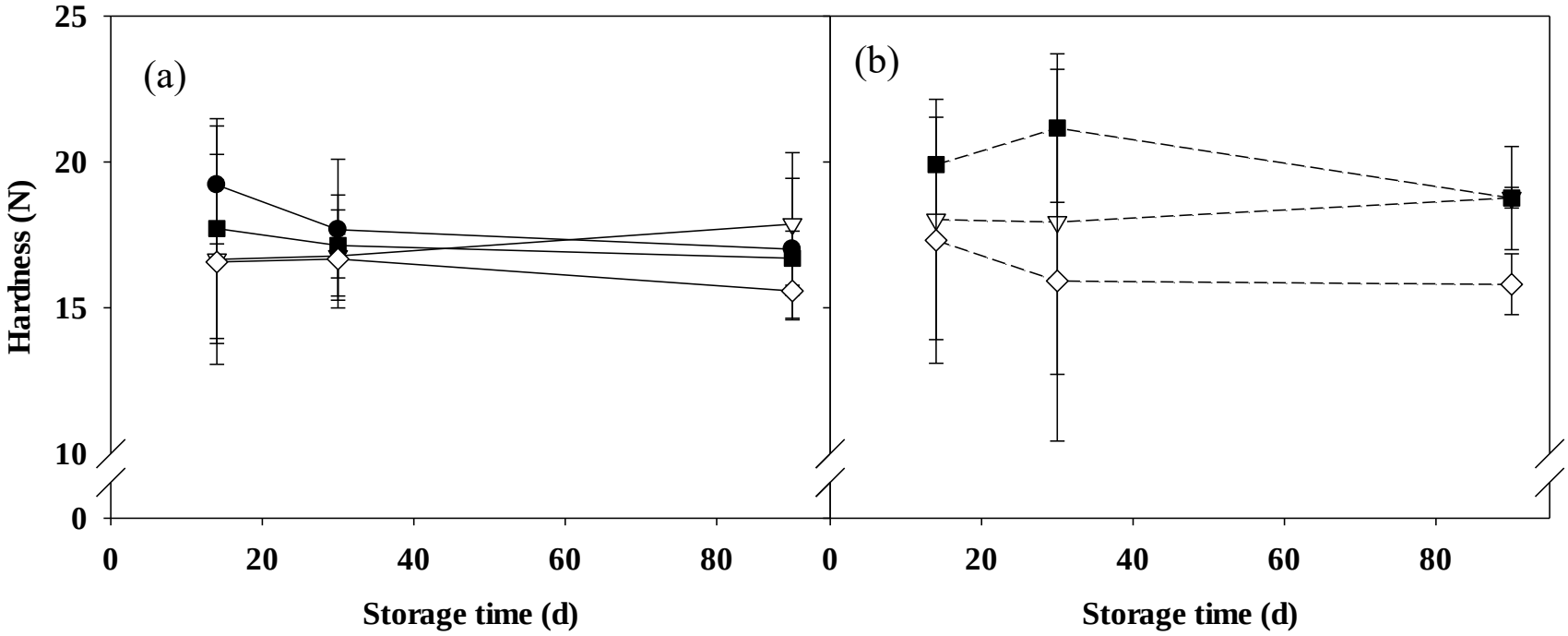

Figure 4.11 Hardness (N) from texture profile analysis for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC for control cheese (no pre-acidification) (●) or cheeses that were pre-acidified to a pH of 6.40 (a) or 6.00 (b) using acetic (▽), citric (■) or carbonic (◇) acid. Vertical bars represent standard deviations (n=4).

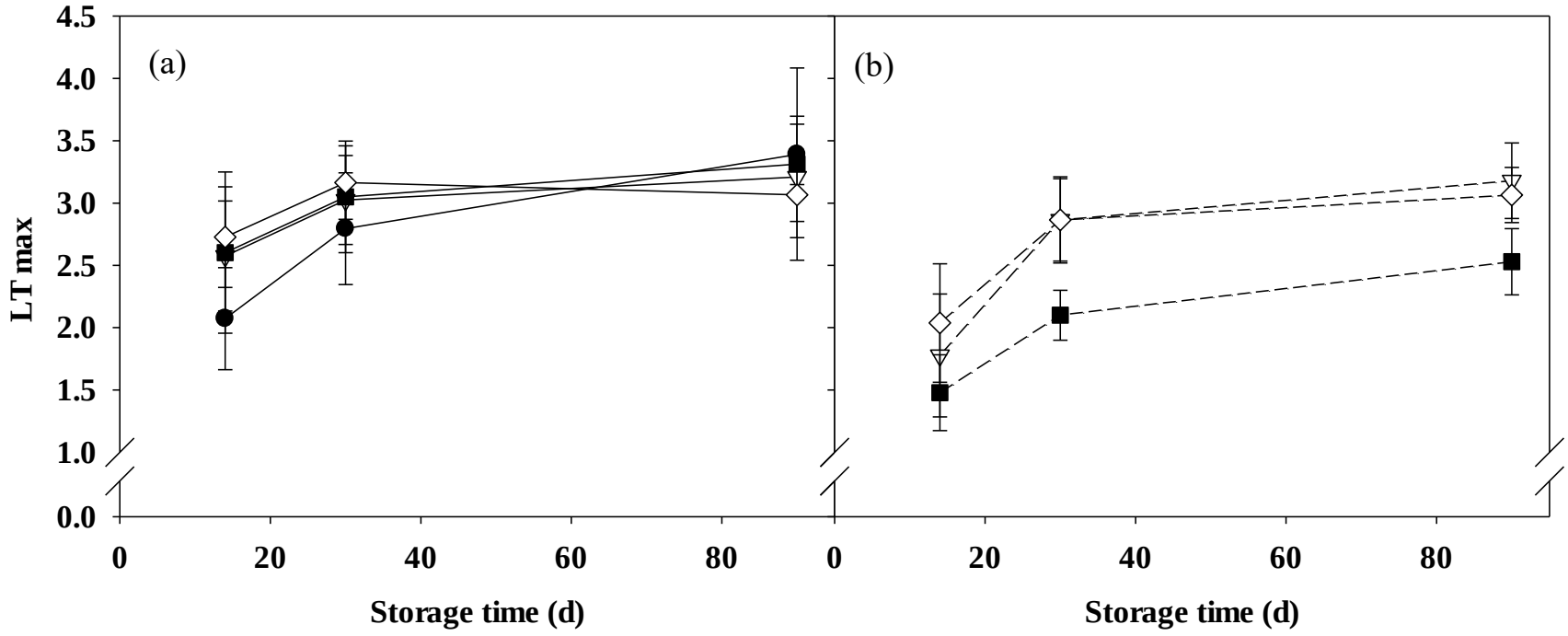

Figure 4.12 Maximum loss tangent values during heating using a dynamic low amplitude oscillatory strain test for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC for control cheese (no pre-acidification) (●) or cheeses that were pre-acidified to a pH of 6.40 (a) or 6.00 (b) using acetic (∇), citric (■) or carbonic (◇) acid. Vertical bars represent standard deviations (n=4).

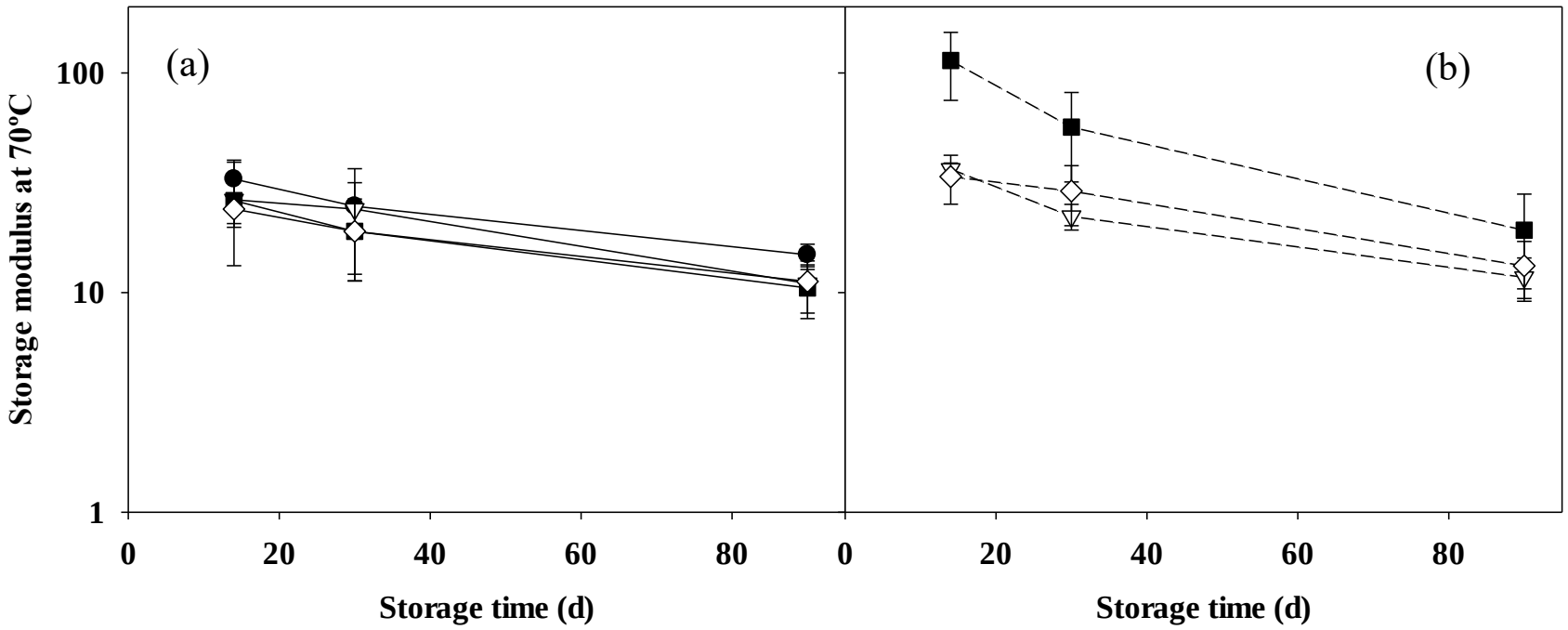

Figure 4.13 Storage modulus (G′) at 70ºC during heating using a dynamic low amplitude oscillatory strain test for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC for control cheese (no pre-acidification) (●) or cheeses that were pre-acidified to a pH of 6.40 (a) or 6.00 (b) using acetic (∇), citric (■) or carbonic (◇) acid. Vertical bars represent standard deviations (n=4).

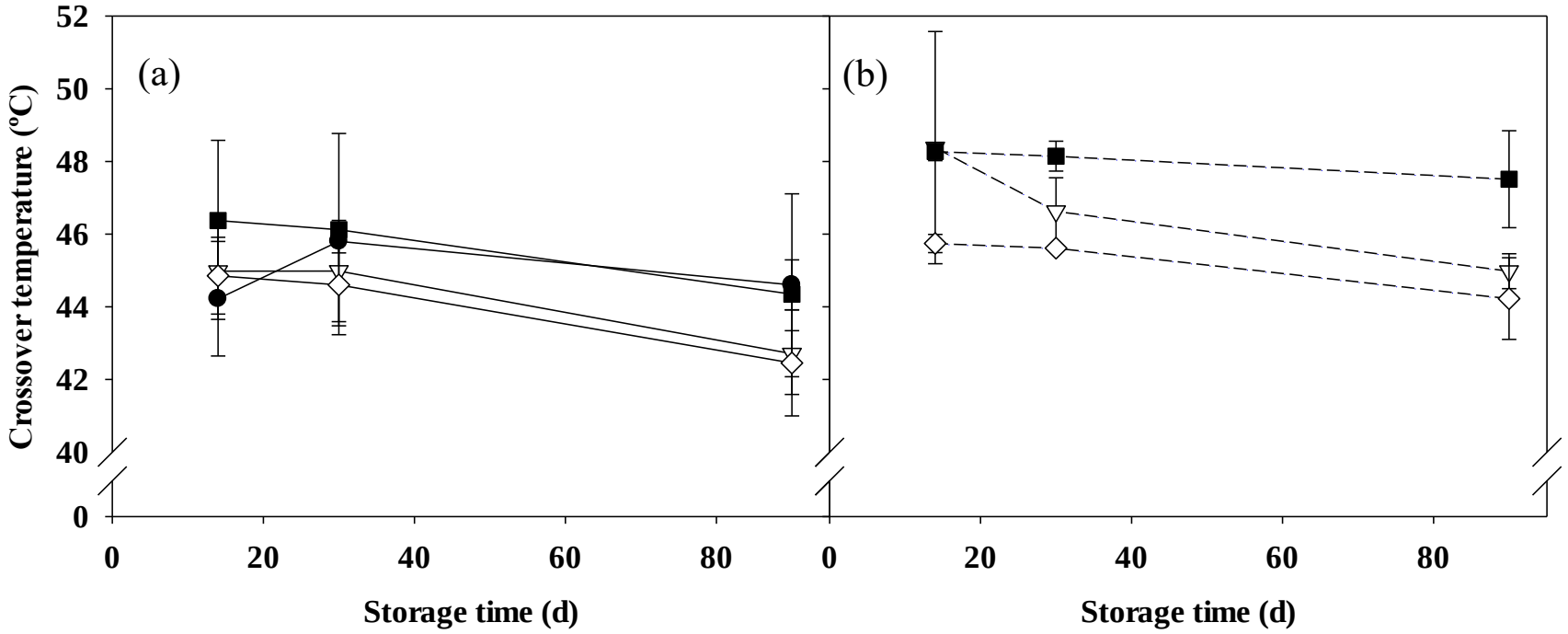

Figure 4.14 Crossover temperature (where loss tangent=1) during heating using a dynamic low amplitude oscillatory strain test for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC for control cheese (no pre-acidification) (●) or cheeses that were pre-acidified to a pH of 6.40 (a) or 6.00 (b) using acetic (∇), citric (■) or carbonic (◇) acid. Vertical bars represent standard deviations (n=4).

Chapter 5. Impact on the Calcium Balances and Functionality of Low-Moisture Part-Skim Mozzarella made from Milk with varying Casein Content and Milling pH Values

Abstract

Low-moisture part-skim (LMPS) Mozzarella made with membrane concentrated milk is of interest as more cheese is produced per vat. The amount of insoluble calcium lost from curds during the manufacturing process of LMPS Mozzarella made from milk with different casein (CN) contents is unknown. The final milling pH values of curds could potentially impact the overall amount of insoluble calcium lost before stretching in the cooker/stretcher, which could thereby impact the functionality of LMPS Mozzarella. Six vats of LMPS Mozzarella were made from milk with three different CN content (2.5, 4.0, 5.5%) and two milling pH values (pH 5.40 and pH 5.10) (n=4); all milk samples were pre-acidified (PA) to pH 6.00 using lactic acid and all curd samples had similar pH values at whey drainage. Cheese milks standardized to different CN contents had significantly different amounts of total calcium, but a similar amount of insoluble calcium content when expressed as mg per g of protein. The percentage of insoluble calcium dissolved in PA milks was significantly lower in the milk with higher CN content. Cheeses manufactured from milk with 2.5% CN had significantly higher moisture contents compared to cheeses made from milk with 5.5% CN milk. Cheeses made from concentrated milk had slightly, but significantly higher total calcium contents compared to cheeses made from unconcentrated milk. Difference in milling pH values did not cause a significant difference in the total calcium content of cheese but slightly reduced the insoluble calcium content (by 1 mg Ca per g protein in the final cheese) when the curds were milled at pH 5.10 compared to curds milled at pH 5.40. These differences in moisture and insoluble calcium contents in the cheeses affected the TPA hardness and rheological properties, i.e., the cheeses that had higher moisture had lower TPA hardness and the cheeses that had lower

insoluble calcium had higher meltability values (LT_{max}) values at 2 wk of storage. The microstructure of the cheeses was also slightly different where the curds milled at pH 5.10 (which had a lower amount of insoluble calcium), had larger fat droplets entrapped between the protein fibers as compared to curds milled at pH 5.40, which had smaller fat droplets. The functional performance of cheese was assessed in both unmelted (cubes and shreds) and melted form (on pizza). Cheeses that were made from milk with 2.5% CN had lower hand firmness and were less chewy with higher cohesiveness values due to their higher moisture content. The shreds from cheeses made from milk with 2.5% CN also had lower shred length and straightness values with increased adhesiveness and matting scores. In the melted form, the cheese made from milk with 2.5% CN had lower blister quantity at the 2 wk timepoint, with lower first chew hardness and chewiness likely due to higher moisture and lower insoluble calcium contents, respectively. The strand thickness was slightly lower in cheeses milled at lower pH values likely due to lower insoluble calcium content. Flavor attributes were similar between the cheeses except for salt, which was higher in the cheeses with higher moisture content. Manufacturing cheeses from milk standardized to different CN content and milling pH values slightly, but significantly, affected the insoluble calcium lost during cheese manufacture; there was also a variation in the moisture content between the cheeses, which had an impact on the functional performance of cheese.

5.1 Introduction

Cheese manufactured from membrane concentrated milk is of interest due to increased plant throughput, i.e., more cheese is produced per vat as a result of higher casein (CN) and fat content in the initial cheese milk. Membrane filtration is commonly used in the dairy industry for many applications, including removal of bacteria and spores, defatting of whey concentrates, whey demineralization, fractionation of milk proteins, milk protein standardization and concentration of

milk for cheese manufacture (Soodam and Guinee, 2018). The ultrafiltration (UF) concentrates fat, CNs (along with CCP or insoluble calcium attached to the CNs), and whey proteins in the retentate, while allowing some water, lactose, salts (soluble calcium), and non-protein nitrogen (NPN) to be removed via the permeate. The separation of the components into the retentate or permeate is not a precise cut-off but is dependent on many factors employed during the filtration run, including, the composition of feed, membrane material (polymeric vs ceramic), single state vs multi-stage, extent of concentration/diafiltration, temperature and back pressure.

There is a pseudo-equilibrium of calcium in the milk between the soluble and insoluble forms (Fox and McSweeney, 1998). When milk is concentrated to different extents, the amount of CN and insoluble calcium proportionally increases and the water/serum content in milk decreases, which results in an increase in the buffering capacity of the milk. The insoluble calcium probably does not dissolve as easily in the concentrated milk as compared to a unconcentrated milk because there is less serum for the calcium to dissolve into in the former. An increase in the CN and insoluble calcium content in milk has been shown to affect critical cheese-making properties including rennet coagulation properties (Lauzin et al., 2019), curd formation and syneresis, curd buffering capacity, rate of pH development, as well as the composition and ripening in cheese (Soodam and Guinee, 2018). Therefore, critical parameters, such as, pre-acidification, pH at rennet addition, rate and extent of acidification by lactic acid bacteria, the rennet coagulation time, etc., have to be adjusted to successfully make Mozzarella cheese with optimum functional performance, i.e., desirable stretch and melt characteristics (McSweeney, 2007). We are not aware of previous research that reported cheese made from milk with higher CN content and the impact on the solubilization of CCP during cheesemaking, such as milling stage. Therefore, in our study, we wanted to track the changes in calcium balances in cheese made from milk standardized to different

CN content and also to study the changes in compositional, textural, rheological, and sensory properties of these cheeses during storage.

LMPS Mozzarella undergoes a stretching process towards the end of manufacture since it is a pasta-filata style cheese. The stretching process allows the parallel distribution of protein fibers in the curds, with fat and serum entrapped between these fibers. This type of orientation impacts the stretching and melting properties of cheese when applied to pizza (Kindstedt and Fox, 1993). The final pH when curds are milled to facilitate stretching can influence how the cheese stretches in the cooker/stretcher. For LMPS Mozzarella made with starter cultures (from unconcentrated milk), the optimum milling pH is typically between 5.10-5.30 because this represents the ideal amount of insoluble calcium content in the curds for optimum stretching. The cheese texture observed during stretching is impacted by its milling pH value, i.e., if the milling pH is too high, where insufficient insoluble calcium has been dissolved, the cheese can have a mealy/rubbery texture, whereas, if the milling pH is too low and excessive amounts of insoluble calcium is dissolved, the cheese can become runny and soupy in the cooker/stretcher (McMahon and Oberg, 2017). Yun et al. (1993a, 1993b) studied the impact of milling pH values on the composition and functional properties of LMPS Mozzarella cheese made from unconcentrated milk. They found no significant differences in the total calcium content or the functional properties in their final cheeses as a result of differences in milling pH values (pH 5.10, 5.25, and 5.40). Cheeses can have similar total calcium contents but different insoluble calcium contents with a change in the milling pH values. The study by Yun et al. (1993a) did not measure the insoluble calcium content in their cheeses. The CCP solubilization in curds can be impacted not only by the milling pH values but also by the amount of moisture present in the curds. It is commonly observed that cheese made from concentrated milk can have lower moisture due to increased syneresis of gels, leading to

more moisture being expelled out of the curds during cheese manufacturing (Lu et al., 2017). This reduction in moisture (serum) content of curds we believe can help saturate the calcium content in the serum sooner which would restrict further CCP solubilization. It is therefore possible that during the manufacture of cheese from concentrated milk we might have to go lower than pH 5.20 at milling in order to reach the ideal amount of insoluble calcium content for optimum cheese stretching. Therefore, we wanted to study CCP solubilization during the manufacture of cheese made from milk with higher CN content (4% and above) as compared to control milk (approx. 2.5% CN), and where curds were milled at different pH values (pH 5.40 and pH 5.10).

Research Hypotheses

- The percent insoluble calcium lost during cheese manufacture will be lower in the cheese made from high CN milk as compared to cheese made from milk with lower CN content. This could be due to lower solubilization of insoluble calcium in a system with a lower moisture serum content. Calcium saturation in the reduced serum phase likely occurs more readily in curds made from high CN milk (more total and insoluble calcium).
- For the cheeses made from the milk with same CN content, the cheeses milled at a higher pH value will have higher amounts of insoluble calcium as compared to cheese milled at a lower pH, causing a difference in the functional performance of cheese.

5.2 Materials and Methods

5.2.1 Milk Processing and Standardization

Raw whole milk was skimmed using a cream separator. Raw part-skim milk with approx. 2.35% fat was ultrafiltered (10 kDa) using a 3.8” spiral wound polymeric membrane (8 elements in 4 vessels connected in series) at 4ºC with a boost pressure of 207 kPa. The concentration factor

achieved in the final retentate was 2.2 x (based on casein content), which had a protein content of approx. 5.8% (true protein). The target milk CN contents of 2.5, 4.0, and 5.5% was achieved by mixing the retentate from the filtration run with the liquid milk permeate and cream (CN to fat ratio of 1.05). The standardized cheese milk was then high-temperature, short-time (HTST) pasteurized at 74°C for 19 seconds.

5.2.2 Cheese Manufacturing

Six vats of LMPS Mozzarella cheeses were made by licensed Wisconsin cheesemakers at the Center for Dairy Research dairy plant on 4 separate days. Cheeses were manufactured from milk with three different CN content (2.5, 4.0, 5.5%) and two milling pH values (pH 5.40 and pH 5.10). Standardized pasteurized milk was transferred into each vat and pre-acidified (PA) to pH 6.0 (at 7ºC) with lactic acid (88% diluted 4:1 in water) keeping the rate of PA (45 min) constant in all vats. The amount of starter culture and chymosin were added to the milk samples in proportion to their CN content. The milk was warmed to 33°C before frozen direct vat-set *Streptococcus thermophilus* (Delvo CP® Cheese-201; DSM, Waukesha, WI) was added (66 g per 454 kg for 2.5% CN milk). A ripening time of 60 min was given for milk with 2.5% and 4.0% CN whereas a ripening time of 75 min was given for milk with 5.5% CN, before the addition of fermentation-produced calf chymosin (20 g/454 kg milk for 2.5% CN milk) with an activity of ≥620 IMCU per g (Maxiren® XDS, DSM, Waukesha, WI). These differences in ripening time were performed to achieve similar rates of acidification in all the vats and to compensate for the shorter gelation time in the 5.5% CN milk. The cultures and rennet in the 4.0 and 5.5% CN milk were added in proportion to casein content. Once the cheese milk was clotted and the desirable firmness was attained, as evaluated by a licensed cheese maker, the coagulum was cut using 2.54 cm knives, and the gel was allowed to heal for 15 minutes. The curds were cooked for 20 min at approx. 38°C.

The whey drainage was initiated when the curds reached a pH value of approx. 5.70 and was allowed to drain slowly (over 30 min). Once the whey was completely drained, the curds were cut into slabs and split into two portions. One portion of it was milled at pH 5.40 and the other portion was milled at pH 5.10. Milled curds were stretched (traditional Supreme 640 MM, Stainless Steel Fabricating Inc., Columbus, WI) at a water temperature of 75°C with the curd exit temperature approx. 60°C. All cheeses were extruded into molds, dipped in cold water baths for 60 min (to bring the curd temperature below 38°C), and later brined for 4 h in a 20% (w/w) salt solution held at 4ºC. The cheeses were vacuum sealed the next day and refrigerated until the analysis time points of 2, 7, 14, 30, and 90 d of storage.

5.2.3 Compositional Analysis

The milk and cheese samples were analyzed for total solids (Marshall 1992), fat by Mojonnier (AOAC International 2000), protein (total percentage N × 6.38) by Kjeldahl (AOAC International 2000), and CN (AOAC International 2000), and non-protein nitrogen (AOAC International 2000). The total calcium in milk, curds, and cheeses was analyzed using inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Agilent 5100, Agilent Technologies, Santa Clara, CA) (Park, 2000). The pH was measured using a spear tip pH probe (accuCap Capillary Junction pH combination electrode; Fisher Scientific, Itasca, IL). The salt content in all the cheese samples was measured using a Chloride Analyzer Model 926 (Corning Glass Works, Medfield, MA). The lactose, galactose, and lactic acid in cheeses during storage were measured by high-performance liquid chromatography as described by Zeppa et al. (2001).

5.2.4 Water-Soluble Calcium (WSC) Method

During cheese manufacture, curd samples obtained for WSC measurement were sheared for 30 sec using a food processor (Hamilton Beach®, Model 70730, Glen Allen, VA) before subjecting the mixture to WSC extraction. The WSC from the curd/cheese samples was extracted by blending 4 g of ground curds/cheese in 40 g Milli-Q water at 55ºC using an Ultra-Turrax T-25 Basic Homogenizer (IKA Works Inc., Willmington, NC) at a speed of 7,000 rpm. The heterogeneous mixture was centrifuged at 10,000 × g for 10 min at 22ºC (Selected from model rennet gel preliminary work; Swaminathan et al., 2023). The supernatant was filtered through a Whatman #1 filter paper (GE Healthcare Life Sciences, USA), and the filtrate was stored for subsequent analysis of soluble calcium. Curd samples drawn for WSC measurement during cheese manufacture were also measured for moisture and protein content as this is required for calculating the insoluble calcium content and for standardizing results in terms of mg Ca per gram protein. If the insoluble calcium content was not represented as per g protein, a comparison of results between curd samples drawn at various points in the process (for example, curds after whey drain and milled curds) would be difficult due to large differences in moisture contents between these samples. The WSC slurry needed to be immediately centrifuged after homogenization to avoid any potential shifts in calcium balances because moisture and pH differences in the curd samples could also affect the amount of calcium extracted.

The WSC in curds/cheese samples was calculated as shown in the equations below; the insoluble calcium content (mg/g protein) was calculated by subtracting the soluble calcium from the total calcium.

$$Soluble\ Ca\ (mg/100g) = \frac{Ca\ in\ WSC\ extract\ (mg)}{Weight\ of\ sample\ (g)} \times Moisture\ in\ cheese\ (\%)\ \times D \quad \text{(Eq.1)}$$

(Eq. 2)

$$Insoluble\ Ca\ (mg/g\ of\ protein) = \frac{Total\ calcium - Soluble\ calcium\ (mg/100g)}{\%\ Protein}$$

Where, D is the dilution factor, which was 11.

5.2.5 Rheological Analysis

The rheological properties of LMPS Mozzarella cheeses were measured using a dynamic low-amplitude oscillatory rheology with an Anton Paar Rheometer (MCR 302; Physica Messtechnik, Stuttgart, Germany) as described by Moynihan et al. (2014). The parameters measured during this test were storage modulus (G′), loss modulus (G″), and loss tangent (LT, which is G″/G′). The temperature at the crossover point, where G′ = G″, was calculated to determine where the cheese transitions from a solid to a liquid-like system. The maximum LT (LT_{max}) was used as a measure of cheese meltability, with a higher value indicating a more meltable (fluid) cheese (Lucey et al., 2003). The G′ value at 70ºC was used as a measure to evaluate the amount of bonds (mostly, electrostatic interactions as expressed by calcium crosslinking) remaining in the melted/hot cheese system. The analysis was performed in duplicate or until the rheograms were closely replicated.

5.2.6 Texture Profile Analysis

The hardness of the cheese was evaluated using a TA-XT2 Texture Analyzer (Texture Technologies Corp., Scarsdale, NY). The samples were cut to a diameter of 16 mm and a height of 17.5 mm and refrigerated overnight before analysis. A double compression test with the sample compressed to 70% of the original height was done at the speed of 0.8 mm/s at room temperature. Hardness is defined as the peak force achieved during the first compression. The test was replicated 10 times for each sample.

5.2.7 Proteolysis

Proteolysis was evaluated by measuring the pH 4.6 soluble nitrogen (Kuchroo and Fox, 1982).

5.2.8 Descriptive Sensory Analysis

Sensory texture and flavor properties of unmelted cheese cubes and shreds, in both unmelted and melted form (on pizza), were evaluated as described by Moynihan et al. (2014). The evaluation was done by trained sensory panelists (trained for 40 hours) ($n \geq 8$) using a mixture of sensory Spectrum® and quantitative descriptive analysis (QDA). The references used, corresponding to the range on the QDA scale for various attributes measured, are shown in Chapter 1 (Table 1.4 and 1.5).

The size dimensions of the cubes prepared for evaluation was 2 × 2 × 2 cm. The cheeses were shredded using a pilot scale shredder (Urschel CC-D, Urschel Laboratories Inc., Chesterton, IN). 100 g of shreds were used for evaluating the unmelted visual (straightness, matting, fusion, opacity) and surface (length, surface oil, surface moisture, adhesiveness) properties. Pizza was prepared by spreading 2 tablespoons of tomato sauce on 30 cm parbaked pizza base and 300 g of shredded Mozzarella cheese. The pizzas were baked in a forced-air commercial oven (2500 series Lincoln Impinger Oven, Fort Wayne, IN) at 260ºC for 5 minutes. The various parameters of melted cheese on the pizza were evaluated at different temperatures as the cheese cooled down as described by Moynihan et al. (2014). All sensory panels were conducted in duplicate.

5.2.9 Confocal Laser Scanning Microscopy

Cheese samples were prepared for confocal laser scanning microscopy by taking samples from the center of the cheese block. A very thin slice of cheese was cut perpendicular to the

direction of the parallel fibers using a Cool-cut Microtome (ThermoFisher Scientific, Waltham, MA) to visualize the para CN fibers in the cheese. The sliced cheese was then placed on a microscope slide and dual stained with Nile Red (0.1 mg/mL of acetone) and Fast Green FCF (1 mg/mL of water) (Sigma-Aldrich, St. Louis, MO) to stain fat and proteins, respectively. Nile Red was applied first to the cheese and allowed to stain for 1 min. The excess stain on the cheese was dabbed using Kimwipes (Kimberly-Clark professional, Roswell, GA) before Fast Green FCF was applied. A cover slide was placed on top of the cheese. The prepared slides (2 slides for each sample) were immediately evaluated for cheese microstructure using a Nikon A1R+ confocal microscope (Nikon Instruments Inc., Tokyo, Japan). Images were obtained using a 20× objective as described by Swaminathan et al. (2021).

5.2.10 Experimental Design and Statistical Analysis

Statistical analysis was performed using SAS (Version 9.4, SAS Institute Inc., Cary, NC). A randomized complete block design with 6 treatments and four replicates (6 × 4) was used to evaluate the differences in the composition of the cheeses. A split-plot design was used to evaluate the effect of treatment and storage time as well as their interactions on pH, insoluble calcium, proteolysis, textural, rheological, and functional properties. In the whole plot factor, the cheese-making days were blocked. For the subplot factor, storage time and the interaction of storage time and treatment were treated as variables. The interaction of treatment and cheese-making days was treated as an error term for the treatment effect. An ANOVA was carried out using the PROC GLM procedure of SAS and Tukey's multiple comparison test was used to evaluate the differences in the treatments at a significance level of $P < 0.05$.

5.3 Results and Discussion

5.3.1 Observations during Cheese Manufacturing

The overall manufacturing time for cheese made from 2.5% and 4.0% CN milk were similar but the cheese made from 5.5% CN milk took a significantly longer manufacturing time, approx. 25 min longer, due to the lower rate of acidification in the curds as a result of elevated buffering. The coagulation and cutting times were also significantly different between the milk samples. The first gelation observed, and the time that the gel was cut in the milk, with 2.5, 4.0, and 5.5% CN were 10, 8, 7 and 20, 12, 9 min, respectively. The milk with 5.5% CN content took only about 2 min from the first gelation to reach the desired gel firmness for cut. It is known that the milk with higher CN content needs lower degrees of κ-CN hydrolysis to aggregate because of the close proximity of CN micelles in concentrated milk as compared to unconcentrated milk (De Kruif, 1998). Lowering the pH of the milk increases rennet activity and reduces the negative charges on the CN micelles allowing for even faster aggregation in the milk with higher CN content (Lucey et al., 2003). We observed a significantly lower gelation time in our milk with 5.5% CN because it was also PA to pH 6.00. Our results are similar to trends observed by Waungana et al. (1998), Mishra et al. (2005), and Govindasamy-Lucey et al. (2011) in their studies of rennet coagulation with respect to pH, gelation time, and protein content in milk samples.

The time taken for the curds to reach the milling pH of 5.40 were similar in cheese made from milk with 2.5 and 4.0% CN (approx. 19 min after the whey was completely drained) whereas the curds in the cheese made from milk with 5.5% CN took 5 min longer to reach the target mill pH of 5.40. The curds in the cheese made from milk with 4.0 and 5.5% CN took significantly longer time to reach the target mill pH of 5.10 from pH 5.40 (46 and 49 min, respectively) compared to control cheese (31 min). The longer time taken by the curds in the cheese made from

high CN milk to reach the target mill pH could be due to higher buffering in the curds as a result of elevated insoluble calcium content and lower moisture content. It was observed that at various steps, the curds in the cheese made from milk with higher CN content (5.5% CN), including, the curds at whey drain, curds milled at pH 5.40 and 5.10 had 7-8%, 6-7%, 4-5% lower moisture contents as compared to the control cheese sample, respectively. The reason for the differences in moisture between the curds made from milk with different CN content is discussed in section 5.3.2.

The texture of the stretched cheese coming out of the cooker/stretcher was visually observed and evaluated by cheese maker. The curds milled at pH 5.40 had a tough, firm texture with nice String and squeaky chew as compared to curds milled at pH 5.10 that was clearly softer, flowed easily out of the cooker/stretcher, melted completely, and had a tear with no String. The curds in the cheese made from milk with 5.5% CN were the toughest and firmest of all cheeses with a mealy texture when milled at pH 5.40 whereas at pH 5.10, it had nice String along with tough, chewy, and squeaky texture. The observed differences in the texture between the curds milled at different pH values can be attributed to differences in insoluble calcium amounts between these samples, which is discussed in section 5.3.3.

5.3.2 Composition of Milk and Cheese Samples

The composition of milk samples is shown in Table 5.1. As the milk was concentrated, it had significantly higher total solids, fat, total and true protein, and CN. The target CN contents of 2.5, 4.0, and 5.5% were achieved in the respective milk samples. The CN to total protein and CN to true protein ratio slightly increased as the milk was concentrated due to some non-protein nitrogen lost in the permeate. A similar CN to fat ratio was achieved in all of the milk samples. The lactose content in the milk samples that was standardized to 4% CN and above had slightly but significantly lower lactose content probably due to some lactose removed during the membrane

filtration process. The total calcium content in milk sample increased with an increase in CN content. The total and the insoluble calcium content with respect to the protein content was higher in the milk standardized to 2.5% CN as compared to 4.0 and 5.5% CN milk. (Table 5.1). This is because when milk was standardized to a lower CN content, more liquid milk permeate (thereby more soluble calcium) was added to the UF retentate.

The composition of the experimental cheeses is shown in Table 5.2. The moisture, protein, fat, salt, and salt-in-moisture and total calcium were significantly different between the cheese samples made with milk of different CN levels. The cheeses made from milk with 5.5% CN had significantly lower moisture content resulting in the cheeses having a significantly higher protein and fat content with slightly lower salt-in-moisture as compared to cheeses made from milk with 2.5% CN. With an increase in total solids content in milk sample, Mishra et al. (2005) and Lu et al. (2017) observed an increase in rennet gel strength and Panthi et al. (2019) observed a coarser gel network. Similarly, an increase in CN content in our concentrated milk samples could have created a stronger and coarser gel network eventually leading to the formation of curds with higher firmness as the cheese making progressed. This increase in curd firmness during cheese manufacture could have allowed more moisture to be expelled resulting in a cheese with lower moisture content. There were no significant differences in composition between the cheeses made from milk with the same CN content but milled at different pH values, except for the fat content in the cheeses made from 4% CN milk. A slightly higher fat content in the cheese made from 4.0% CN milled at pH 5.10 was likely due to the slightly lower moisture content in this cheese as compared to the cheese milled at pH 5.40. The total calcium content in cheeses made from milk with 5.5% CN were significantly higher than the cheese made from milk with 2.5% CN at their respective milling pH values. The cheeses milled at pH 5.10 had a trend of slightly lower total

calcium values (mg Ca/g protein) as more insoluble calcium was dissolved due to lower pH compared to cheeses milled at pH 5.40. Similar observations were found by Yun et al. (1993a) in their study; they found no significant differences in total calcium content between their cheeses (made from unconcentrated milk) milled at different pH values.

The residual lactose content in the cheeses was not significantly different with treatment (Table 5.3). The cheeses that were milled at pH 5.40 had slightly higher lactose content (although not significant) as compared to cheeses milled at pH 5.10 because the fermentation by starter cultures was stopped at a higher pH value (when the cheese was stretched at higher temperature) resulting in less lactose being fermented (Figure 5.1). The cheese made from milk with 5.5% CN had fermented more lactose as compared to cheeses made from milk with lower CN content at the respective milling pH values. This was likely due to the increased buffering in the cheeses made from 5.5% CN milk, requiring the starter cultures to ferment more lactose to reduce pH to achieve the target milling pH values during cheese manufacture. Lactose content decreased significantly (Table 5.3) with age in all the cheeses (Figure 5.1). The galactose and lactic acid contents were significantly affected by both treatment and age (Table 5.3). The cheeses that were milled at pH 5.40 had significantly lower galactose (Figure 5.2) and lactic acid (Figure 5.3) content as compared to cheeses milled at pH 5.10 as less lactose was fermented at the higher milling pH (before curds were heated in the cooker/stretcher). It is well known that *S. thermophilus* can only utilize the glucose part of the lactose for metabolism resulting in an increase in the galactose content in cheese (Kindstedt and Fox, 1993). This explained why there was higher accumulation of galactose observed in the cheeses that were milled at pH 5.10 compared to cheeses milled pH 5.40 (Figure 5.2). The galactose and the lactic acid content slowly and significantly increased with age (Figures 5.2 and 5.3) as residual lactose was slowly fermented throughout storage (Figure 5.1).

5.3.3 Total and Insoluble Calcium Changes During Cheese Manufacture and Storage

The total calcium content were significantly different in all three CN level milk samples (Table 5.1). The calcium balance (i.e., the percentage insoluble to soluble calcium) in samples varied as the milk was standardized to different CN contents as shown in Figure 5.4. The insoluble calcium level in the control milk sample with 2.5% CN was around 68% of total calcium whereas in the milk standardized to 5.5% CN, approx. 78% of the calcium was in the insoluble calcium form (Figure 5.4). Although the amount of soluble to insoluble calcium changed in the milk samples standardized to different CN contents, the amount of insoluble calcium attached to the protein (i.e., mg insoluble calcium per g CN) was similar between the milk samples (Table 5.1). When the milk samples were PA to pH 6.00 with lactic acid, the proportion of insoluble calcium dissolved in the milk standardized to 2.5% CN was significantly higher than milk standardized to a higher CN content, i.e., approx. 21, 18, and 16% of insoluble calcium present in the initial milk dissolved in the PA milk standardized to 2.5, 4.0, and 5.5% CN, respectively (Figure 5.4). Even though less proportion of insoluble calcium was dissolved in the milk with higher casein content, when we represent the insoluble calcium as mg/100 g sample, the amount of insoluble calcium dissolved in the milk with higher casein content was higher as compared to milk that had 2.5% CN as it had a higher amount of insoluble calcium content in the initial milk sample (mg/100 g sample) to begin with. The serum calcium present in the initial and PA milk samples were 36, 37, 40 and 52, 56, 64 mg/100 g sample in the 2.5, 4.0, and 5.5% CN milk, respectively. The milk with high casein content dissolved more insoluble calcium content after PA probably due to its tendency to get back to its original equilibrium more quickly than the milk with 2.5% CN but as the calcium in the serum phase became too saturated, the solubilization of insoluble calcium was restricted.

The total calcium content was significantly reduced in the curds after the whey drainage step as compared to the initial milk sample, with approx. 34, 22, and 16% reduction in total calcium in the cheeses made from milk standardized to 2.5, 4.0, and 5.5% CN, respectively (calculated based on total calcium (mg/g protein) in the initial milk sample from Table 5.1). The largest decrease in total calcium was observed in the cheese made from milk standardized to 2.5% CN because it had the highest amount of soluble calcium, which was removed in the whey during the drainage step. The total calcium content in the curds after the whey drainage step was similar in all the samples (Figure 5.5), but the highest total calcium level was observed in the 5.5% CN sample when the curds were milled at pH 5.40 (Figure 5.5). Between the curds milled at different pH values, a slight further decrease in total calcium was observed in the curds of the cheeses made from milk standardized to 2.5 and 4.0% CN, but no obvious change was observed in the curds of cheese made from milk with 5.5% CN. This was likely because approx. 1.5% moisture was removed in the former whereas only about 0.6% moisture was removed in the latter; moisture removed from curd is the only mechanism to reduce the total calcium content. The stretching and brining steps also reduced the total calcium content further by 1-2 mg total Ca/g protein (comparing calcium values at milling and at 2 d timepoint, Figure 5.5 and Table 5.2, respectively).

There was a large significant decrease in the insoluble calcium content between the PA step and in the curds after whey drainage (when the insoluble calcium in the PA step was represented as mg/g casein), more so in the curds from the cheeses made from milk with higher CN content (results not shown). The decrease in pH (from pH 6.00 to approx. 5.50) and the ongoing removal of moisture from the curds as the cheese making progressed created a large change in the calcium equilibrium shifting to solubilization of insoluble calcium (as calcium from the saturated serum was removed along with moisture, more insoluble calcium could dissolve), more so in curds of the

cheese made from higher CN content. There was only a slight decrease in insoluble calcium content between the curds after whey drainage (pH 5.50) and curds milled at pH 5.40 as the pH decrease was approx. 0.1 pH units. When the curds were milled at pH 5.10, the insoluble calcium content in the curds decreased, more so in the curds of cheese made from 2.5 and 4.0% CN as compared to curds made from milk with 5.5% CN (Figure 5.6). The insoluble calcium content reduced further during the stretching and brining steps (comparing insoluble calcium values between milling and 2 d of storage, Figure 5.6 and 5.7, respectively). The curds milled at pH 5.40 and pH 5.10, which reduced by approx. 3 mg insoluble Ca/g protein, whereas the curds milled at pH 5.40 in the cheese made from 2.5% CN milk had a decrease of approx. 5 mg insoluble Ca/ g protein during these steps. The significantly larger reduction in insoluble calcium in the cheese made from milk with 2.5% CN could be due to the higher moisture content in the final cheese (Table 5.2), it was therefore able to dissolve more insoluble calcium in the moisture/serum phase of the cheese (before saturation was attained).

The insoluble calcium content of the cheeses during storage were significantly affected by treatment and storage time (Table 5.3). The cheeses made from milk with 2.5% CN had significantly lower insoluble calcium content as compared to cheeses made from milk with 4.0 or 5.5% CN (Figure 5.7). The cheeses where the curds were milled at pH 5.10 had a significantly lower insoluble calcium content as compared to cheeses where curds were milled at pH 5.40 (approx. 1-1.5 mg) although the total calcium content between these cheeses were similar (Table 5.2). The insoluble calcium content in all the cheeses reduced slightly (Figure 5.7) but significantly (Table 5.3) throughout storage due to ongoing buffering, as was also observed by Moynihan et al. (2016). Most changes occurred within the first few weeks of storage.

5.3.4 pH Changes in Cheeses During Storage

The pH values in the final cheese samples during storage were significantly affected by treatment but not storage time (Table 5.3). The cheeses where the curds were milled at pH 5.10 had a significantly lower pH as compared to cheeses where the curds were milled at pH 5.40 (Figure 5.8), which was similar to observations made by Yun et al. (1993a). The cheeses milled at pH 5.10 quickly increased in pH by 0.1 units by 2 d of storage as more calcium was solubilized increasing phosphate buffering (Hassan et al., 2004) except for the cheese made from milk with 2.5% CN which hardly changed. In contrast, the cheeses where the curds were milled at pH 5.40 slightly reduced in pH by 2 d timepoint. At each milling pH, the cheeses made from milk with 2.5% CN had a lower pH values during storage as compared to cheeses made milk with 4.0 or 5.5% CN. Although there were some minor changes in pH throughout storage due to some solubilization of insoluble calcium, these pH changes were not significant (Table 5.3). Yun et al. (1993a) also did not observe any significant changes in LMPS Mozzarella cheese pH during storage with a variation in milling pH values.

5.3.5 Proteolysis

The proteolytic breakdown of cheeses (as indicated by the pH 4.6 soluble N levels) were significantly impacted by treatment (Table 5.3), and slightly lower in the cheeses made from milk with 2.5% CN (Figure 5.9). The difference in milling pH values did not cause a significant difference in the proteolytic breakdown of cheese (Figure 5.9), which was similar to observations made by Yun et al. (1993a). The proteolytic breakdown of cheeses slowly increased (significantly) with age (Table 5.3) due to residual rennet and plasmin activity, which is typical for LMPS Mozzarella.

5.3.6 Textural Properties

The TPA hardness of the cheese was significantly affected by treatment and storage time (Table 5.4). The hardness of cheese is often correlated with the moisture and protein contents, where the lower protein level in the cheeses made from milk with 2.5% CN along with higher moisture content (Table 5.2) could have contributed to its lower TPA hardness values as compared to cheeses made from milk with 5.5% CN (Figure 5.10). The difference in milling pH values between the cheeses made from the milk with the same CN content did not result in any significant change in TPA hardness values (Figure 5.10). The slightly lower insoluble calcium content in the cheeses where curds were milled at pH 5.10 did not influence TPA hardness values. As the cheese age increased, the TPA hardness of the cheese decreased, especially at >30 d of storage. Ongoing proteolysis (Figure 5.9) likely caused the reduction in TPA hardness with prolonged storage.

5.3.7 Rheological Properties

The LT_{max} value of cheese was significantly affected by treatment and storage time (Table 5.4). At the 2 wk timepoint, the cheeses where the curds were milled at pH 5.40 had a lower LT_{max} value compared to the corresponding cheeses where the curds were milled at pH 5.10 (Figure 5.11), likely due to higher insoluble calcium content. As the cheese aged, the cheeses where the curds were milled at pH 5.40 had higher LT_{max} values at 1 and 3 mo of storage compared to cheeses where the curds were milled at pH 5.10. LT_{max} values typically increases as the texture of the cheese softens due to slow increase in proteolytic breakdown of cheese with storage time. We observed only a slight increase in the LT_{max} values in the cheeses milled at pH 5.10 probably due to the lower pH values in these cheeses. pH of the cheese can influence the LT_{max} values as it affects the electrostatic interactions between the CN molecules. Since the cheeses that were milled at pH 5.10 had a lower pH, the negative charges on the CN micelles could have been lower as the pH

was closer to the isoelectric point of CN micelles, reducing the electrostatic repulsion between the CN molecules enhancing electrostatic attraction and hydrophobic interactions (Lucey et al., 2003) thereby not melting as much as the cheeses that were milled pH 5.40.

The crossover temperature or melting point was not significantly affected by treatment but was significantly impacted by storage time (Table 5.4). The crossover temperature decreased significantly in all cheeses with an increase in storage time (Figure 5.12) as the cheese became softer due to increased proteolysis and melted more readily. The G′ value at 70ºC was significantly impacted by treatment and storage time (Table 5.4). The cheeses that were milled at pH 5.40 exhibited slightly higher G′ values at 70ºC as compared to cheeses that were milled at pH 5.10 (Figure 5.13), probably due to slightly higher insoluble calcium content in these cheeses leading to stronger CN-CN interactions at high temperatures (Lucey et al., 2003).

5.3.8 Microstructure of Cheeses

The microstructure of the cheeses made from milk with different CN contents and milled at pH 5.40 and 5.10 are shown in Figures 5.14 and 5.15, respectively. It was observed that when the cheese was milled at pH 5.10 (Figure 5.15), the cheeses had larger fat droplets trapped in the serum phase of the cheese as compared to the cheeses milled at pH 5.40 (Figure 5.14). This difference in fat distribution was observed more so in the cheeses made from milk with 2.5% CN (Figure 5.14a and Figure 5.15a) and 4.0% CN (Figure 5.14f and Figure 5.15f) at the 2 d timepoint. The slightly lower insoluble calcium content and final pH values in the cheeses milled at lower pH could have allowed the protein fibers in the cheese to be more pliable, allowing more fat to be coalesced during the stretching process resulting in larger fat globules being distributed in these cheeses. The fat distribution in the cheeses made from milk with 5.5% CN milled at two different milling pH values (Figure 5.14k and Figure 5.15k) were not that different because they had higher

insoluble calcium content; likely the protein fibers were more rigid during stretching allowing the smaller fat globules entrapped between the protein fibers to remain in place. Serum channels (black in color) were observed in the cheeses in the 2 d and 7 d timepoint cheeses but were less visible with further aging as the free moisture was absorbed back into the protein matrix (Kiely et al., 1993).

5.3.9 Sensory Properties

5.3.9.1 Unmelted Cheese

The sensory hand firmness, chewiness, and cohesiveness of unmelted cheese (in cube form) were significantly impacted by treatment and storage time (Table 5.5). At 2 and 4 wk of storage, the cheeses made from milk with 2.5% CN had significantly lower hand firmness as compared to cheeses made from milk with 5.5% CN (Table 5.6) due to the former cheeses having a significantly higher moisture content than the latter. This data was also consistent with the TPA hardness values (Figure 5.10). The cheeses made from milk with 2.5% CN also had significantly lower chewiness and higher cohesiveness as compared to cheeses made from milk 5.5% CN at 2 and 4 wk storage timepoint (Table 5.6). This was likely because the protein content in the cheeses made from milk with 2.5% CN decreased with an increase in moisture, they became softer and had to be chewed less than the cheese with higher protein content, like the cheeses made from milk with 5.5% CN (Table 5.2). A lower chewiness values in the unmelted cheeses was correlated with the cheeses having a higher cohesiveness values. Changing the milling pH values did not cause a significant difference in the unmelted cheese attributes of cubes (Table 5.6); protein and moisture content seemed to have more of an impact. As the cheese aged, the sensory hand firmness of the cheese decreased slightly throughout storage for cheeses made with 2.5% CN milk and milled at pH 5.40. This decrease was likely due to an increase in proteolytic breakdown. The chewiness

values significantly decreased with age for all samples, and the cohesiveness values increased in cheeses with an increase in storage time (Table 5.6). The flavor attributes including salt and acid were significantly higher in cheeses made from milk with 2.5% CN compared to cheeses made from milk with 5.5% CN (results not shown), likely due to higher moisture content with slightly higher salt and lactic acid content in the former.

Straightness, shred length, and adhesiveness values of shredded cheese were significantly impacted by treatment (Table 5.7). The straightness and shred length of cheeses made from milk with 2.5% CN were significantly lower whereas the adhesiveness values were significantly higher compared to cheeses made from milk with 5.5% CN, at most storage timepoints (Table 5.6). Higher moisture or lower protein contents in the cheese made from milk with 2.5% CN increased the adhesiveness of cheese. This likely caused the shreds to have lower straightness and shred length values; as the adhesiveness values were higher, these cheeses also exhibited increased matting (Table 5.6). The straightness and shred length decreased with increase in storage time, more dramatically in the cheeses made from milk with 2.5% CN as compared to cheeses made from milk with 5.5% CN. The cheeses made from milk with 2.5 and 4.0% CN did not exhibit any significant changes in shred adhesiveness values with age; the higher moisture with lower insoluble calcium content in these cheeses made from 2.5% and 4.0% CN caused it to become more adhesive even at 2-wk timepoint (Table 5.6). These cheeses stuck to the blade while shredding thereby significantly reducing the machinability of the cheeses as observed in our cheeses made from milk with 2.5% CN. Since the cheeses made from milk with 5.5% CN had slightly higher protein content along with higher insoluble calcium content as compared to other cheeses, the straightness of the shreds of these cheeses even at 3 mo timepoint were higher, i.e.,

the cheese shredded well without large amount of fines. The shred adhesiveness values slightly but significantly increased with age in cheeses made from milk with 5.5% CN.

5.3.9.2 Melted Cheese

Blister quantity was significantly impacted by treatment and age (Table 5.8). The blister quantity of the cheeses made from milk with 2.5% CN content were significantly lower at the 2 and 4 wk storage timepoint as compared to cheeses made from milk with 5.5% CN, likely due to higher moisture content in the cheese made with 2.5% CN (Table 5.9). As the cheese was heated in the impinger oven, the cheese that had higher moisture content probably lost more moisture as the matrix contracted thereby wetting the cheese surface reducing blistering on the pizza (Dairy Pipeline, 2022). As the cheese aged, the blister quantity increased significantly in all the cheeses (Table 5.8), more so in the cheese that was made from milk with 2.5% CN. Since the cheeses made from milk with 2.5% CN also had significantly lower insoluble calcium content during storage (Figure 5.7), the cheeses became more pliable as the cheese was aged resulting in higher blister quantity along with forming blisters that were larger in size (results not shown). The free oil release in the cheese slightly but significantly was impacted by treatment and age (Table 5.8). The cheeses made from milk with 5.5% CN and milled at pH 5.10 had significantly higher free oil release and larger fat droplets (Figure 5.15 m, n, o) as compared to cheeses made from milk with 2.5% CN milled at pH 5.40 (Figure 5.14 c, d, e).

Strand thickness was significantly impacted by treatment and age (Table 5.8). Strand thickness was significantly higher in the cheeses that had higher moisture content and lower protein content, i.e., cheeses made from milk with 2.5% CN (Table 5.9). The higher moisture to protein ratio and lower insoluble calcium content in these cheeses could have allowed the CNs to interact with more of the neighboring CN molecules resulting in a thicker strand when the cheese

was initially stretched (Lucey et al., 2003). When the cheeses were made from milk with similar CN content but different milling pH values, the cheeses milled at lower pH tended to exhibit lower strand thickness likely due to their lower insoluble calcium contents. Similar observations were found in our previous study (Chapter 4). As the cheese aged, the strand thickness hardly changed with an increase in proteolysis except for cheese made from milk with 5.5% CN and milled at pH 5.10, which decreased significantly.

Texture attributes of melted cheese including the first chew hardness and chewiness were impacted by treatment and age (Table 5.8) and were slightly lower in some cheeses milled at pH 5.10 as compared to cheeses milled at pH 5.40. The first chew hardness slightly decreased (but not significantly) and the chewiness of the cheeses significantly decreased with an increase in storage time, more so in the cheeses that were milled at pH 5.10 (Table 5.9). The flavor attributes of the cheeses including acid, milky, and buttery notes were around the threshold with sour values below the threshold (results not shown). The salt attribute was slightly higher in the cheeses made from milk with 2.5% CN as compared to other cheeses.

5.4 Conclusions

Cheeses that were made from milk with 5.5% CN retained more insoluble calcium during the cheese-making process, possibly due to altered calcium solubility as a result of higher serum calcium content in the milk and lower moisture in the curd samples. The significantly lower moisture content in the curds of the cheese made from milk with 5.5% CN was likely due to the stiffer gel network formed by the CNs after rennet addition, which altered syneresis and moisture losses. The difference in milling pH values did not significantly affect the total calcium content in the cheeses; the initial insoluble calcium content were approx. 1 mg (per g protein) lower in the curds were milled at pH 5.10 as compared to curds milled at pH 5.40, when the pH at whey

drainage was kept consistent between the cheeses during manufacture. Differences in the moisture, moisture-to-protein ratio, and insoluble calcium content in these cheeses impacted their microstructure, textural, rheological, and functional performance on pizza.

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Tables and Figures

Table 5.1. Composition of standardized cheese milk with various CN levels (n=4)

Composition	2.5% Casein	4.0 % Casein	5.5 % Casein	SEM	*P*-value
Solids, %	11.1[c]	14.0[b]	17.6[a]	0.11	<0.0001
Fat, %	2.3[c]	3.5[b]	5.0[a]	0.04	<0.0001
Total Protein[1], %	3.2[c]	4.9[b]	6.8[a]	0.06	<0.0001
True Protein[2], %	3.0[c]	4.7[b]	6.6[a]	0.06	<0.0001
Casein[3], %	2.5[c]	3.9[b]	5.5[a]	0.06	<0.0001
Casein to Total Protein Ratio	77.6[c]	79.8[b]	80.7[a]	0.26	<0.01
Casein to True Protein Ratio	82.7[a]	83.3[a]	83.6[a]	0.28	0.07
Casein to Fat Ratio	1.1[a]	1.1[a]	1.1[a]	0.01	0.48
Lactose, %	4.6[a]	4.5[b]	4.4[b]	0.03	<0.01
Total Ca[4] (mg/100 g sample)	109.6[c]	146.1[b]	190.1[a]	0.93	<0.0001
Total Ca[4] (mg/g protein)	36.2[a]	31.0[b]	28.6[c]	0.33	<0.0001
Insoluble Ca (mg/g protein)	24.7[a]	23.1[b]	22.4[b]	0.21	0.0008
Insoluble Ca (mg/g casein)	36.0[a]	31.1[a]	31.0[a]	0.74	0.84

[a,b,c] Means within a row not sharing common superscript differ ($P < 0.05$)

[1]Total nitrogen % × 6.38

[2](Total nitrogen % - non-protein nitrogen %)

[3](Total nitrogen % - non-casein nitrogen %)

[4]Measured by ICP-OES

Table 5.2. Composition of LMPS Mozzarella cheeses made from milk with different casein content and two different milling pH values (n=4)

Composition	**2.5 % Casein**		**4.0 % Casein**		**5.5 % Casein**		**SEM**	***P*-value**
	pH 5.40	**pH 5.10**	**pH 5.40**	**pH 5.10**	**pH 5.40**	**pH 5.10**		
Moisture, %	50.0^{a}	49.9^{a}	49.2^{ab}	48.2^{b}	46.6^{c}	46.5^{c}	0.39	<0.0001
Fat, %	21.3^{c}	21.8^{bc}	21.2^{c}	22.4^{ab}	22.5^{a}	22.8^{a}	0.23	<0.001
Fat in dry matter, %	42.6^{ab}	43.4^{a}	41.7^{b}	43.3^{ab}	42.2^{ab}	42.6^{ab}	0.53	0.12
Protein[1], %	24.1^{c}	24.3^{c}	24.5^{bc}	25.1^{b}	26.1^{a}	26.3^{a}	0.23	<0.0001
MNFS[2], %	63.6^{a}	63.8^{a}	62.4^{ab}	62.2^{ab}	60.2^{b}	60.2^{b}	0.43	<0.001
Salt[3],%	1.7^{ab}	1.8^{a}	1.7^{ab}	1.6^{bc}	1.4^{c}	1.4^{c}	0.08	0.01
Salt in moisture, %	3.4^{abc}	3.6^{a}	3.5^{ab}	3.2^{abc}	3.1^{bc}	3.0^{c}	0.14	0.04
Total Ca[4], mg/g protein	21.6^{bc}	21.4^{c}	22.6^{a}	21.8^{bc}	22.6^{a}	22.1^{ab}	0.21	<0.01
Total Ca[4], mg/100 g sample	521.2^{c}	520.0^{c}	553.3^{b}	546.5^{b}	590.0^{a}	581.6^{a}	6.50	<0.001

[a,b,c] Means within a row not sharing common superscript differ ($P < 0.05$)

[1]% N × 6.38

[2]Milk non-fat solids

[3]Determined using chloride analyzer

[4]Measured by ICP-OES

Table 5.3. Probabilities and R^2 values for pH, lactic acid, galactose, insoluble calcium content, and pH 4.6 soluble nitrogen for low-moisture part-skim Mozzarella cheeses manufactured from milk with different casein content and milling pH values during 90 days of refrigerated storage at 4ºC (n=4)

Factor[1]	**df**	**pH**	**Insoluble calcium**	**Lactose**	**Galactose**	**Lactic acid**	**pH 4.6 Soluble N**
Whole-Plot							
Treatment (T)	5	<0.0001	<0.0001	0.09	0.0002	0.0006	0.03
Day of Cheesemaking (D)	3	<0.0001	0.08	0.01	0.46	0.45	0.15
Error (T × D)							
Split-Plot							
Storage time (S)	4	0.30	<0.0001	<0.0001	0.0018	<0.0001	<0.0001
S × T	20	0.52	0.73	0.03	0.89	0.27	0.68
R^2		0.85	0.97	0.84	0.67	0.79	0.97

[1] Split-plot design with 6 treatments were analyzed as a discontinuous variable and the cheesemaking day was blocked (6 × 4 blocked design). Subplot included the effects of storage time of cheese (S) and storage time × treatment as variables.

Table 5.4. Probabilities and R^2 values for TPA hardness, max loss tangent, crossover temperature (ºC), and G' value at 70ºC for low-moisture part-skim Mozzarella cheeses manufactured from milk with different casein content and milling pH values during 90 days of refrigerated storage at 4ºC (n=4)

Factor[1]	**df**	**TPA hardness[2]**	**LTmax[3]**	**Crossover temperature [4]**	**G′ values at 70ºC**
Whole-Plot					
Treatment (T)	5	0.0002	<0.01	0.14	0.0003
Day of Cheesemaking (D)	3	0.91	0.0004	0.15	0.05
Error (T×D)					
Split-Plot					
Age (A)	2	<0.0001	<0.0001	<0.0001	<0.0001
A×T	10	0.90	0.0022	0.01	0.80
R^2		0.81	0.87	0.83	0.88

[1]Split-plot design with 6 treatments were analyzed as a discontinuous variable and the cheesemaking day was blocked (6 × 4 blocked design). Subplot included the effects of storage time of cheese (S) and storage time × treatment as variables.

[2]Measured by texture analyzer

[3]Maximum loss tangent values

[4]Temperature at which loss tangent value = 1

Table 5.5 Probabilities and R^2 values for sensory properties of cubes for low-moisture part-skim Mozzarella cheeses manufactured from milk with different casein content and milling pH values during 90 days of refrigerated storage at 4ºC (n=4)

		Unmelted cheese (Cubes)		
Factor[1]	**df**	**Firmness**	**Chewiness**	**Cohesiveness**
Whole-Plot				
Treatment (T)	5	<0.0001	<0.0001	0.0008
Day of Cheesemaking (D)	3	<0.0001	0.0039	0.53
Error (T×D)				
Split-Plot				
Age (A)	2	0.0025	<0.0001	<0.0001
A×T	10	0.93	0.98	0.59
R^2		0.72	0.75	0.79

[1] Split-plot design with 6 treatments were analyzed as a discontinuous variable and the cheesemaking day was blocked (6 × 4 blocked design). Subplot included the effects of storage time of cheese (S) and storage time × treatment as variables.

Table 5.6 Sensory analysis results for low-moisture part-skim Mozzarella cheeses manufactured from high casein milk using different pre-acidification pHs and acid types during 90 days of refrigerated storage at 4ºC, tested as unmelted cheeses in cube and shred form (n=4)

Attribute	Storage time (wk)	2.5% CN		4.0% CN		5.5% CN	
		pH 5.40	pH 5.10	pH 5.40	pH 5.10	pH 5.40	pH 5.10
Cubes							
Firmness	2	$4.75^{c,AB}$	$4.59^{c,A}$	$5.14^{bc,A}$	$5.77^{ab,A}$	$6.52^{a,A}$	$6.09^{a,A}$
	4	$5.08^{c,A}$	$5.07^{c,A}$	$5.36^{c,A}$	$4.90^{c,A}$	$7.28^{a,A}$	$6.39^{b,A}$
	12	$3.60^{c,B}$	$4.43^{a,A}$	$4.10^{bc,A}$	$4.34^{b,A}$	$5.92^{a,A}$	$5.39^{a,A}$
Chewiness	2	$5.00^{cd,A}$	$4.89^{d,A}$	$5.33^{bc,A}$	$5.53^{ab,A}$	$5.72^{a,A}$	$5.65^{ab,A}$
	4	$4.74^{c,AB}$	$4.69^{c,AB}$	$5.13^{bc,A}$	$4.84^{c,AB}$	$5.73^{a,A}$	$5.36^{ab,AB}$
	12	$4.03^{e,B}$	$4.09^{de,B}$	$4.37^{cd,B}$	$4.44^{bc,B}$	$4.96^{a,B}$	$4.70^{ab,B}$
Cohesiveness	2	$10.30^{a,A}$	$9.88^{ab,B}$	$9.31^{abc,B}$	$9.30^{bc,B}$	$8.79^{c,B}$	$9.26^{bc,B}$
	4	$11.01^{ab,A}$	$11.35^{a,A}$	$10.45^{bc,A}$	$11.53^{a,A}$	$9.39^{d,AB}$	$10.19^{cd,AB}$
	12	$11.56^{ab,A}$	$11.76^{a,A}$	$11.12^{b,A}$	$11.44^{ab,A}$	$10.49^{c,A}$	$11.28^{ab,A}$
Shreds							
Straightness	2	$9.19^{b,A}$	$9.21^{b,A}$	$9.45^{ab,A}$	$9.81^{ab,A}$	$10.24^{a,A}$	$9.89^{ab,A}$
	4	$8.06^{ab,B}$	$7.09^{b,A}$	$8.74^{ab,A}$	$9.16^{a,A}$	$9.86^{a,AB}$	$9.93^{a,A}$
	12	$1.79^{d,C}$	$2.41^{cd,B}$	$5.19^{b,B}$	$5.15^{bc,B}$	$9.03^{a,B}$	$8.82^{a,B}$
Matting	2	$7.43^{a,A}$	$6.60^{a,A}$	$7.36^{a,AB}$	$5.64^{ab,A}$	$4.27^{b,A}$	$4.41^{b,A}$
	4	$6.35^{a,A}$	$3.74^{b,A}$	$2.58^{b,B}$	$4.38^{ab,A}$	$2.18^{b,A}$	$2.41^{b,A}$
	12	$10.35^{a,A}$	$9.59^{ab,A}$	$10.11^{a,A}$	$9.78^{ab,A}$	$8.13^{bc,A}$	$6.86^{c,A}$
Shred length	2	$3.25^{bc,A}$	$3.10^{c,A}$	$3.60^{ab,A}$	$3.77^{a,A}$	$3.82^{a,A}$	$3.82^{a,A}$
	4	$3.10^{bc,A}$	$2.60^{c,AB}$	$3.65^{a,A}$	$3.42^{ab,A}$	$3.82^{a,A}$	$3.95^{a,A}$
	12	$0.96^{d,B}$	$1.83^{cd,B}$	$2.43^{bc,A}$	$2.03^{cd,B}$	$3.70^{a,A}$	$3.45^{ab,B}$
Adhesiveness	2	$9.70^{a,A}$	$9.41^{a,A}$	$9.01^{a,A}$	$8.78^{a,A}$	$7.30^{b,B}$	$7.27^{b,B}$
	4	$8.96^{a,AB}$	$8.74^{a,A}$	$8.36^{ab,A}$	$8.39^{ab,A}$	$7.23^{c,B}$	$7.62^{bc,B}$
	12	$7.96^{b,B}$	$8.72^{ab,A}$	$9.01^{a,A}$	$9.25^{a,A}$	$8.73^{ab,A}$	$9.54^{a,A}$

[a-d]Means within a row with different lowercase superscripts differ ($P < 0.05$, comparing the effect of treatment at a single storage time). [A-B]Means within a column (for a particular attribute) with different uppercase superscripts differ ($P < 0.05$, comparing the effect of storage time at a single treatment)

Table 5.7 Probabilities and R^2 values for sensory properties of shreds for low-moisture part-skim Mozzarella cheeses manufactured from milk with different casein content and milling pH values during 90 days of refrigerated storage at 4ºC (n=4)

		Unmelted cheese (Shreds)			
Factor[1]	**df**	**Straightness**	**Matting**	**Shred length**	**Adhesiveness**
Whole-Plot					
Treatment (T)	5	0.0003	0.0004	<0.0001	0.0005
Day of Cheesemaking (D)	3	0.37	<0.0001	0.0018	0.0011
Error (T×D)					
Split-Plot					
Age (A)	2	<0.0001	0.0002	<0.0001	0.0306
A×T	10	0.0008	0.99	0.02	0.0035
R^2		0.88	0.55	0.82	0.71

[1] Split-plot design with 6 treatments were analyzed as a discontinuous variable and the cheesemaking day was blocked (6 × 4 blocked design). Subplot included the effects of storage time of cheese (S) and storage time × treatment as variables.

Table 5.8 Probabilities and R^2 values for sensory properties of melted cheese on pizza for low-moisture part-skim Mozzarella cheeses manufactured from milk with different casein content and milling pH values during 90 days of refrigerated storage at 4ºC (n=4)

		Melted cheese					
Factor[1]	**df**	**Blister Quantity**	**Free oil**	**Strand thickness**	**First chew**	**Chewiness**	**Salt**
Whole-Plot							
Treatment (T)	5	0.0099	0.0079	0.02	0.02	<0.0001	<0.0001
Day of Cheesemaking (D)	3	0.16	0.02	0.31	0.0024	<0.0001	0.0012
Error (T×D)							
Split-Plot							
Age (A)	2	<0.0001	0.0006	0.0012	0.0002	<0.0001	0.0826
A×T	10	<0.0001	0.05	0.58	0.95	0.27	0.73
R^2		0.89	0.77	0.60	0.68	0.81	0.78

[1] Split-plot design with 6 treatments were analyzed as a discontinuous variable and the cheesemaking day was blocked (6 × 4 blocked design). Subplot included the effects of storage time of cheese (S) and storage time × treatment as variables.

Table 5.9 Sensory analysis results for low-moisture part-skim Mozzarella cheeses manufactured from high casein milk using different pre-acidification pHs and acid types during 90 days of refrigerated storage at 4ºC, tested as melted on pizza in a forced air impinger oven at 250ºC for 5 min (n=4)

Attribute	Storage time (wk)	2.5% CN		4.0% CN		5.5% CN	
		pH 5.40	pH 5.10	pH 5.40	pH 5.10	pH 5.40	pH 5.10
Blister quantity	2	$5.77^{b,B}$	$5.90^{b,B}$	$6.90^{b,B}$	$6.95^{b,B}$	$9.00^{a,B}$	$9.22^{a,B}$
	4	$6.52^{b,B}$	$6.82^{b,B}$	$8.07^{b,AB}$	$10.02^{a,A}$	$10.00^{a,AB}$	$10.40^{a,A}$
	12	$11.16^{a,A}$	$10.50^{a,A}$	$9.29^{a,A}$	$10.94^{a,A}$	$10.49^{a,A}$	$9.32^{a,B}$
Free oil	2	$5.37^{d,A}$	$5.62^{bcd,A}$	$5.42^{cd,B}$	$5.82^{b,A}$	$5.72^{bc,B}$	$6.27^{a,A}$
	4	$5.90^{b,A}$	$5.87^{b,A}$	$6.05^{ab,AB}$	$6.25^{ab,A}$	$6.32^{ab,A}$	$6.67^{a,A}$
	12	$4.57^{c,B}$	$5.12^{bc,A}$	$6.20^{a,A}$	$5.63^{ab,A}$	$6.32^{a,A}$	$5.93^{ab,A}$
Strand thickness	2	$7.05^{a,A}$	$6.10^{ab,A}$	$6.25^{ab,A}$	$5.67^{ab,A}$	$5.40^{b,AB}$	$4.87^{b,B}$
	4	$5.60^{ab,A}$	$5.72^{ab,A}$	$6.07^{ab,A}$	$5.50^{b,A}$	$6.20^{a,A}$	$5.47^{b,A}$
	12	$5.47^{ab,A}$	$4.48^{bc,A}$	$5.82^{a,A}$	$4.55^{bc,A}$	$4.67^{abc,B}$	$4.06^{c,C}$
First chew hardness	2	$5.90^{a,A}$	$5.30^{b,A}$	$5.80^{ab,A}$	$5.25^{b,A}$	$5.97^{a,A}$	$5.57^{ab,A}$
	4	$4.65^{bc,A}$	$4.25^{c,A}$	$5.22^{ab,A}$	$4.72^{bc,A}$	$5.67^{a,A}$	$5.30^{ab,A}$
	12	$4.83^{abc,A}$	$4.20^{c,A}$	$4.99^{ab,A}$	$4.50^{bc,A}$	$5.22^{a,A}$	$4.76^{abc,A}$
Chewiness	2	$6.72^{ab,A}$	$6.32^{c,A}$	$6.77^{a,A}$	$6.42^{bc,A}$	$6.87^{a,A}$	$6.87^{a,A}$
	4	$6.15^{c,AB}$	$6.30^{bc,A}$	$6.60^{ab,A}$	$6.10^{c,AB}$	$6.77^{a,A}$	$6.75^{a,A}$
	12	$5.45^{cd,B}$	$5.01^{d,B}$	$6.20^{ab,A}$	$5.70^{bc,B}$	$6.30^{a,A}$	$5.69^{c,B}$
Salt	2	$6.82^{a,A}$	$6.92^{a,A}$	$6.55^{a,A}$	$6.35^{ab,A}$	$5.95^{b,A}$	$5.80^{b,A}$
	4	$7.10^{a,A}$	$7.37^{a,A}$	$6.80^{ab,A}$	$6.32^{b,A}$	$6.15^{b,A}$	$6.10^{b,A}$
	12	$6.75^{a,A}$	$6.74^{a,A}$	$6.94^{a,A}$	$6.29^{b,A}$	$5.79^{c,A}$	$6.04^{b,A}$

$^{a-d}$Means within a row with different lowercase superscripts differ ($P < 0.05$, comparing the effect of treatment at a single storage time).

$^{A-B}$Means within a column (for a particular attribute) with different uppercase superscripts differ ($P < 0.05$, comparing the effect of storage time at a single treatment)

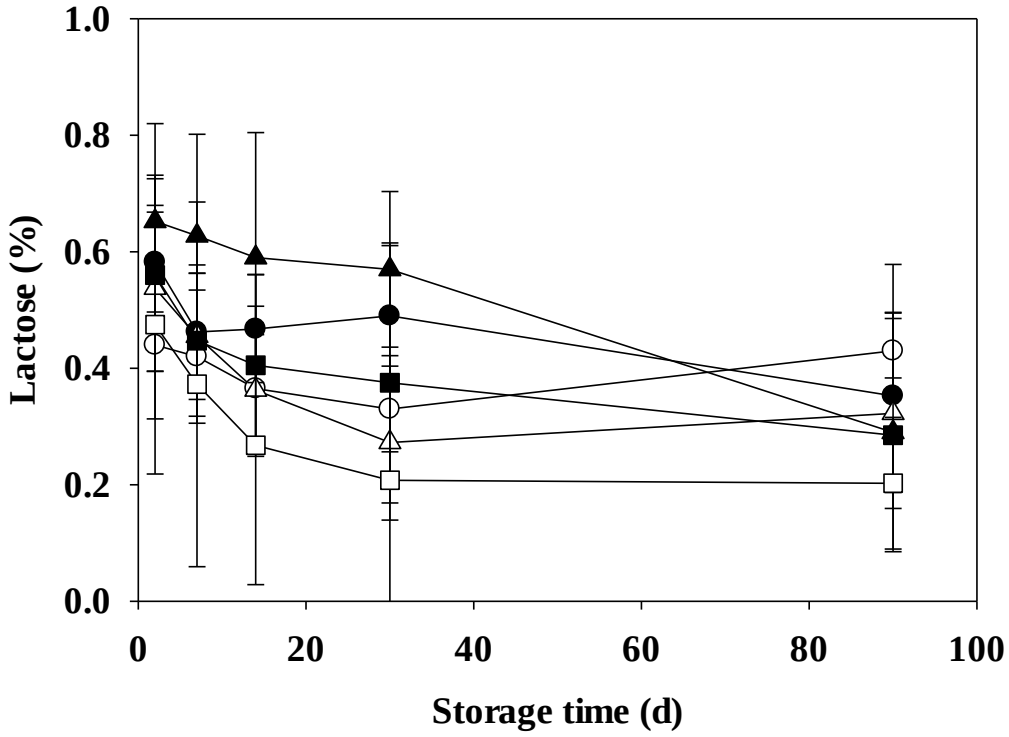


Figure 5.1 Lactose values for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC made from milk with 2.5% (circle), 4.0% (triangle), 5.5% casein (square) and milled at either pH 5.40 (filled) or 5.10 (open) (n=4).

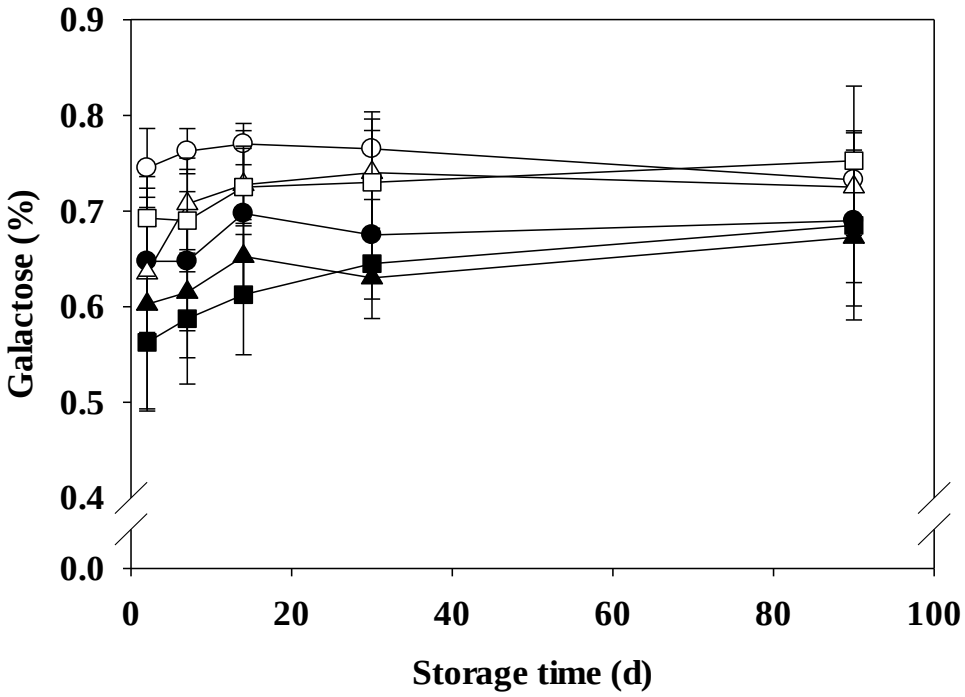


Figure 5.2 Galactose values for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC made from milk with 2.5% (circle), 4.0% (triangle), 5.5% casein (square) and milled at either pH 5.40 (filled) or 5.10 (open) (n=4).

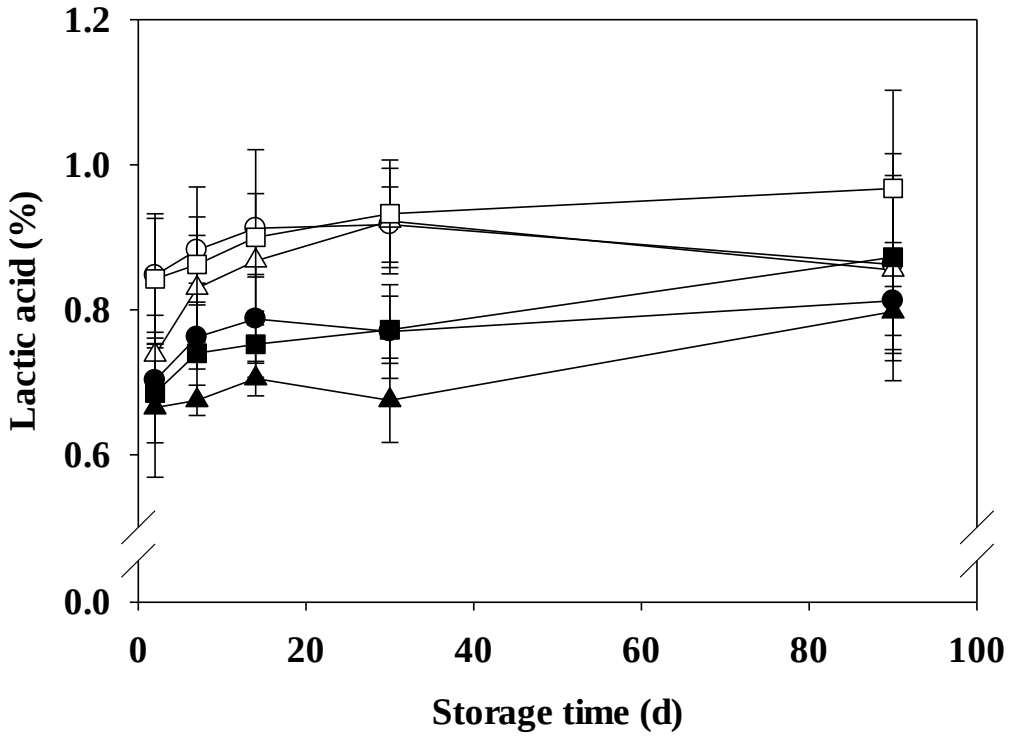


Figure 5.3 Lactic acid values for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC made from milk with 2.5% (circle), 4.0% (triangle), 5.5% casein (square) and milled at either pH 5.40 (filled) or 5.10 (open) (n=4).

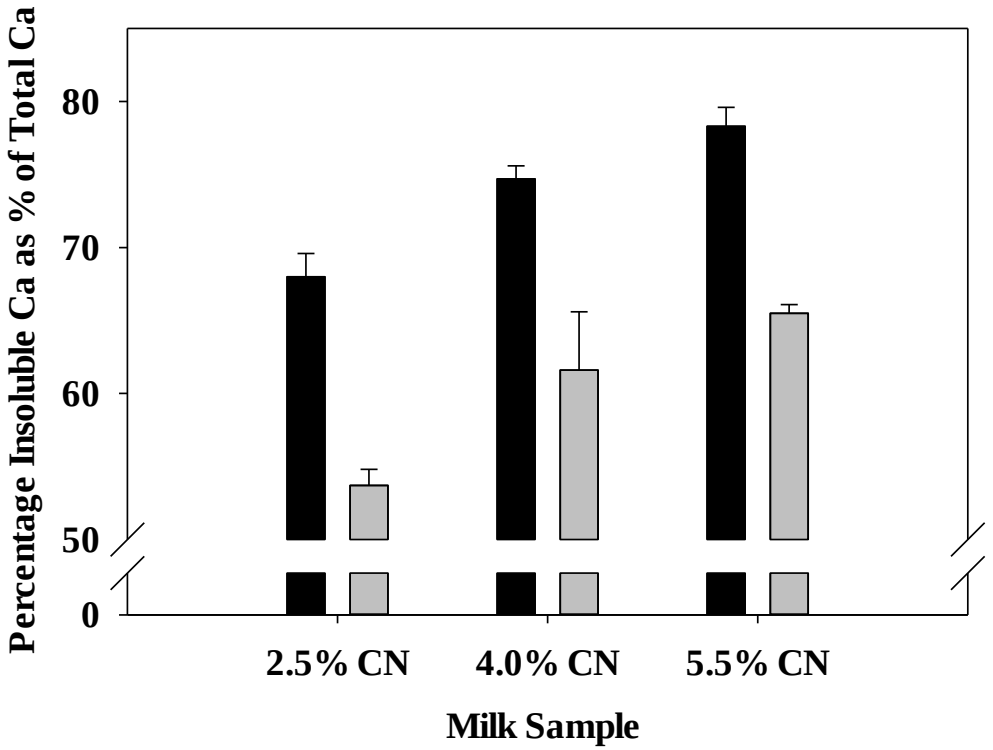


Figure 5.4 The percentage insoluble calcium as a function of total calcium present in standardized milk samples (black column) of different CN contents and these milks pre-acidified to pH 6.00 with lactic acid (grey column) (n=4).

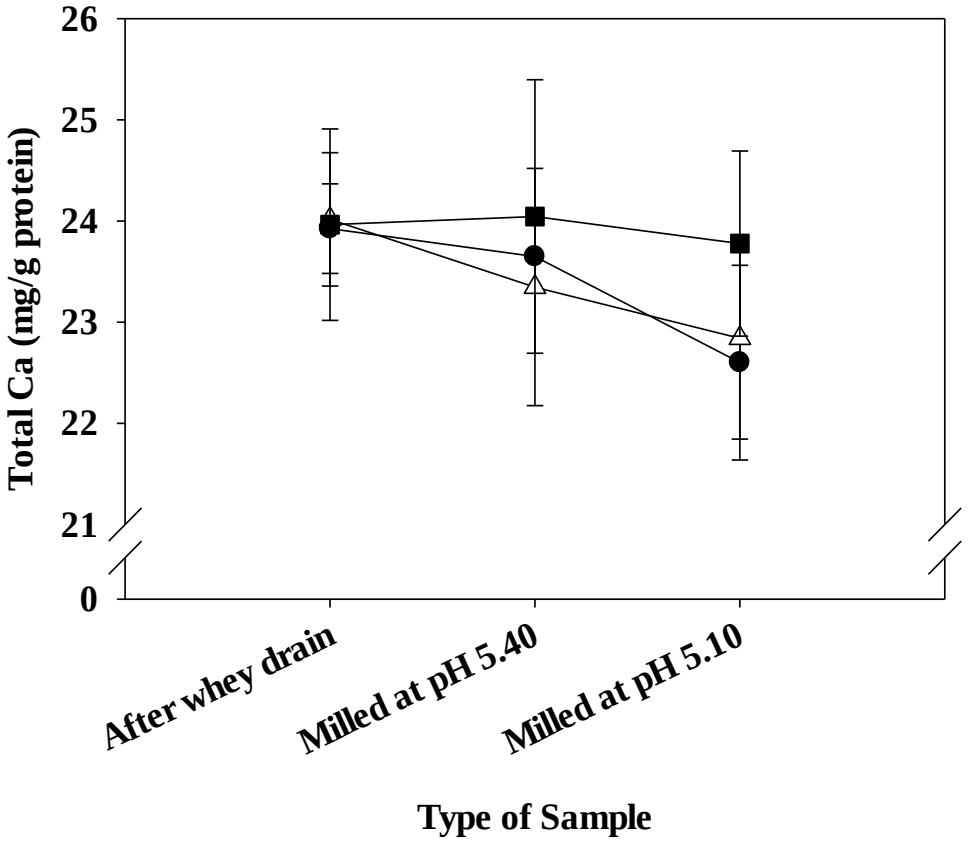


Figure 5.5 Total Ca changes (expressed as mg/g protein) during the manufacture of low-moisture part skim Mozzarella for cheese made from milk with 2.5% (circle), 4.0% (triangle), 5.5% casein (square) (n=4).

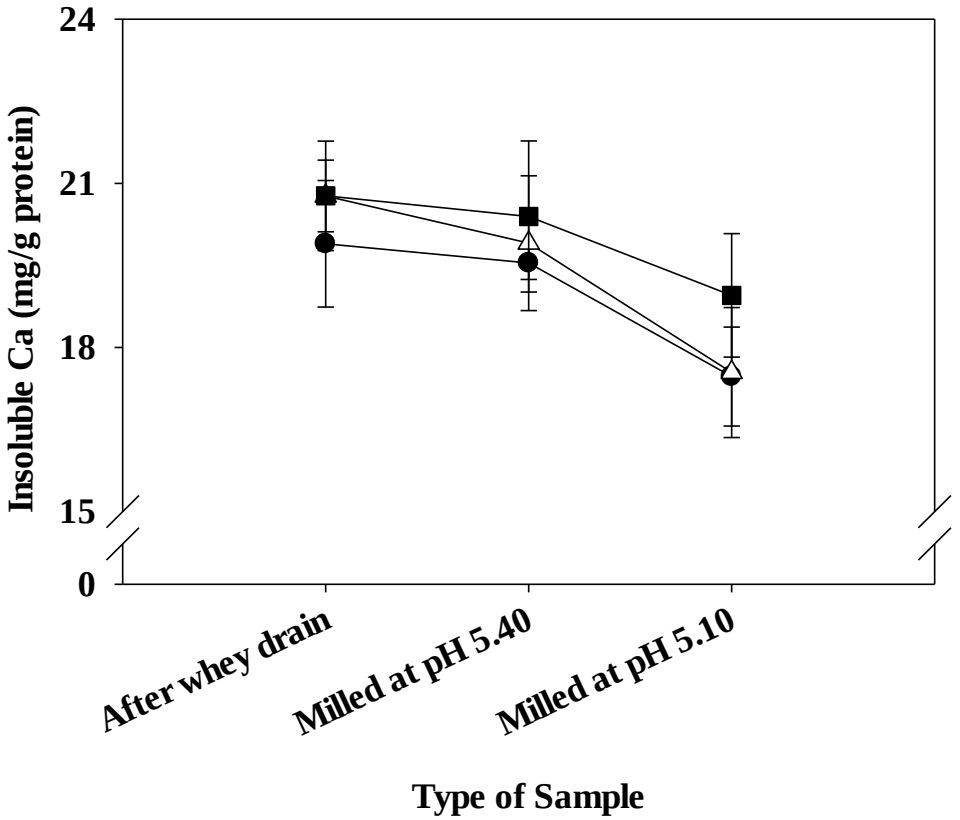


Figure 5.6 Insoluble Ca changes, expressed as mg/g protein, during the manufacture of low-moisture part skim Mozzarella for cheese made from milk with 2.5% (circle), 4.0% (triangle), 5.5% casein (square) (n=4).

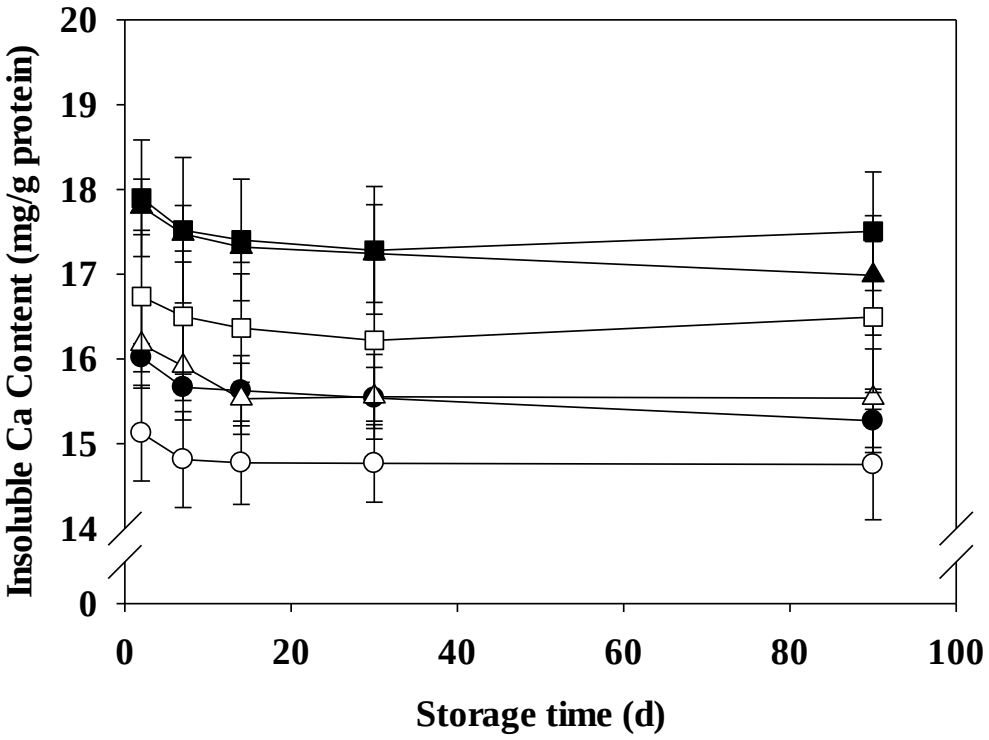


Figure 5.7 Insoluble calcium content (expressed as mg/g protein) for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC made from milk with 2.5% (circle), 4.0% (triangle), 5.5% casein (square) and milled at either pH 5.40 (filled) or 5.10 (open) (n=4).

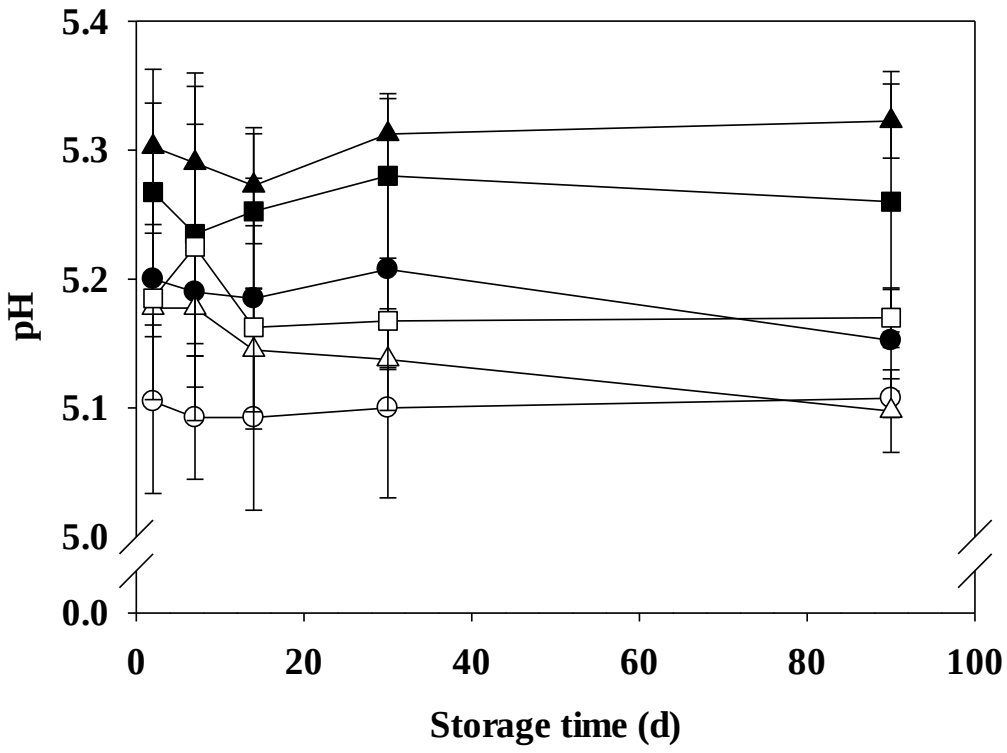


Figure 5.8 pH values for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC made from milk with 2.5% (circle), 4.0% (triangle), 5.5% casein (square) and milled at either pH 5.40 (filled) or 5.10 (open) (n=4).

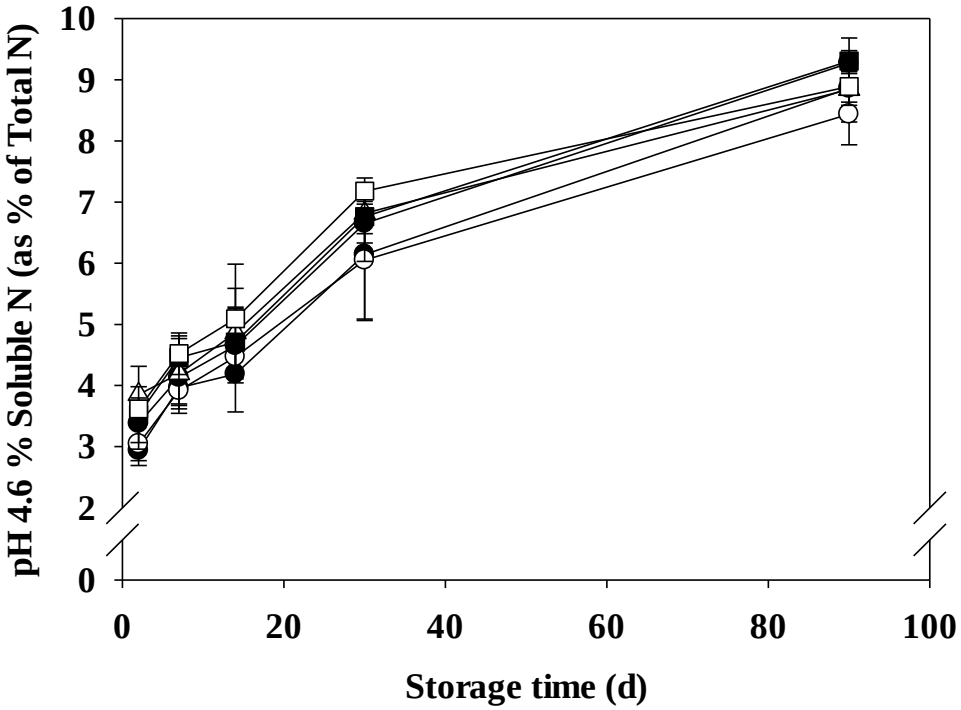


Figure 5.9 pH 4.6 soluble nitrogen as a percent of total nitrogen content for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC made from milk with 2.5% (circle), 4.0% (triangle), 5.5% casein (square) and milled at either pH 5.40 (filled) or 5.10 (open) (n=4).

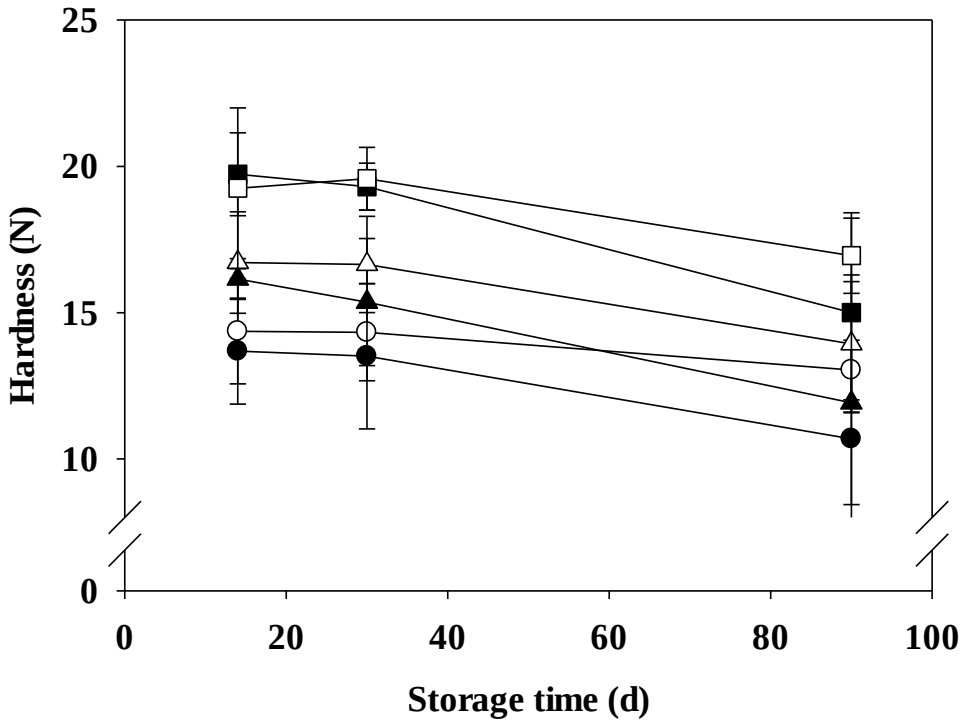


Figure 5.10 Hardness (N) from texture profile analysis for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC made from milk with 2.5% (circle), 4.0% (triangle), 5.5% casein (square) and milled at either pH 5.40 (filled) or 5.10 (open) (n=4).

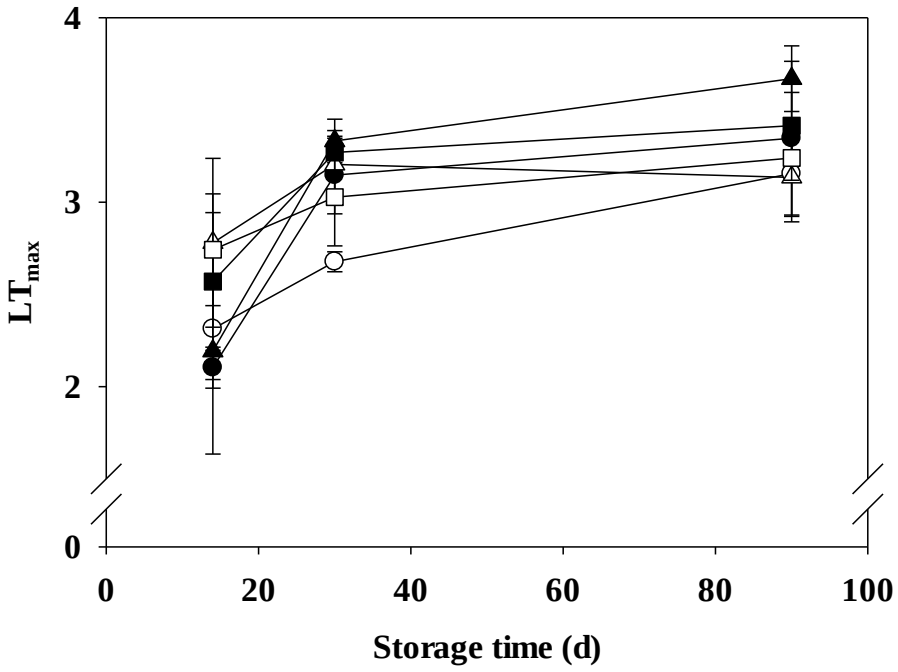


Figure 5.11 Maximum loss tangent (LT_{max}) values during heating using a dynamic low amplitude oscillatory strain test for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC made from milk with 2.5% (circle), 4.0% (triangle), 5.5% casein (square) and milled at either pH 5.40 (filled) or 5.10 (open) (n=4).

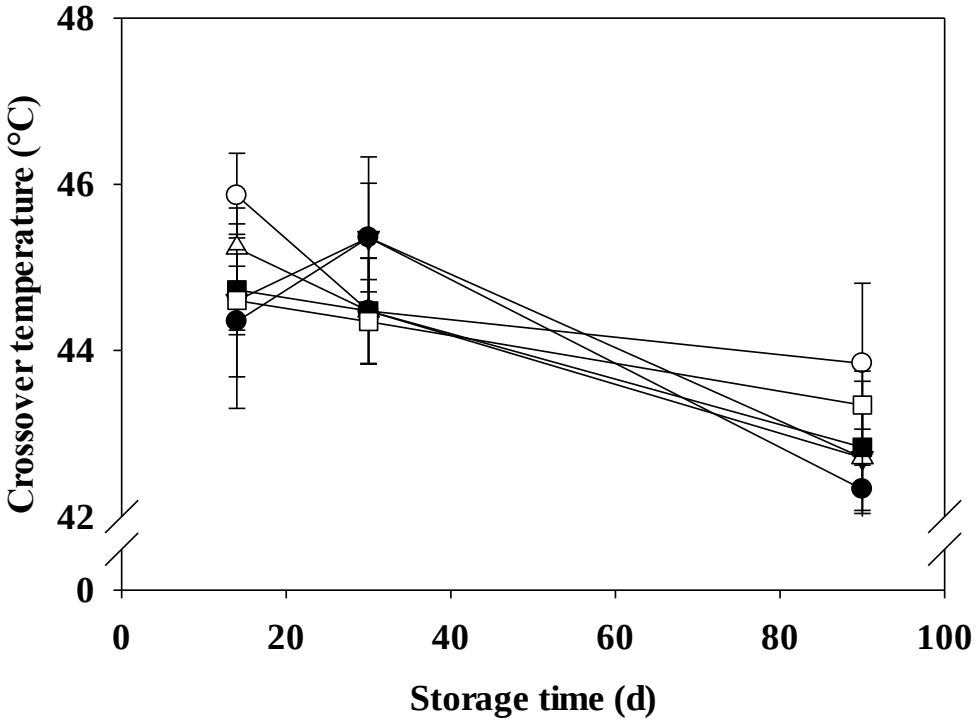


Figure 5.12 Crossover temperature (where loss tangent=1) during heating using a dynamic low amplitude oscillatory strain test for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC made from milk with 2.5% (circle), 4.0% (triangle), 5.5% casein (square) and milled at either pH 5.40 (filled) or 5.10 (open) (n=4).

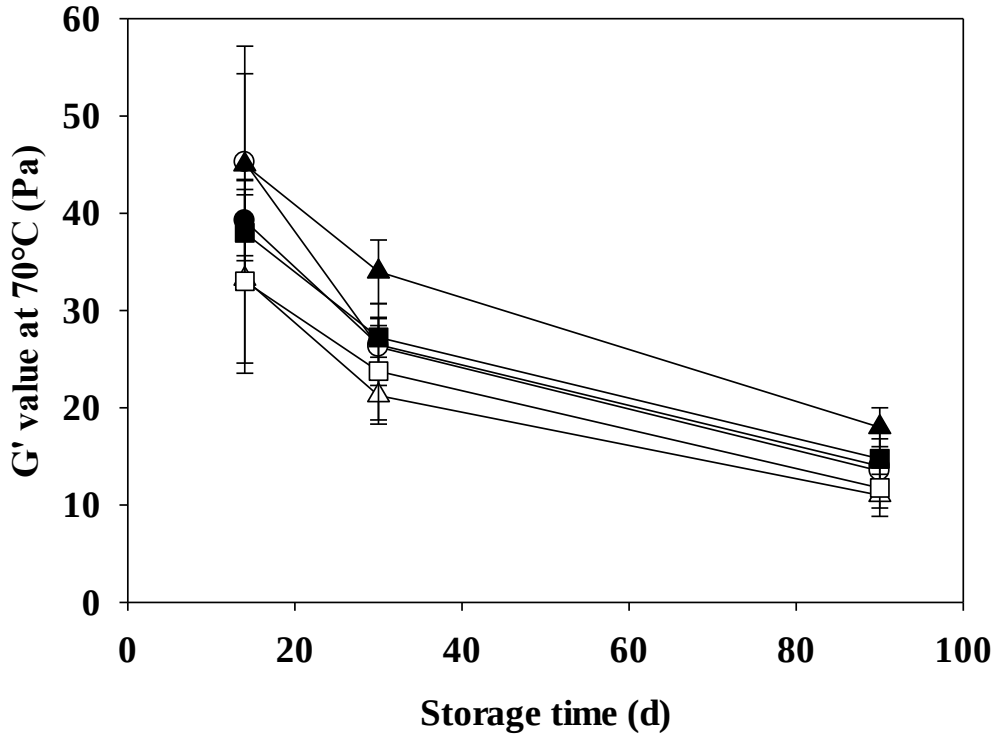


Figure 5.13 Storage modulus (G′) at 70ºC during heating using a dynamic low amplitude oscillatory strain test for low-moisture part skim Mozzarella cheeses during 90 days of refrigerated storage at 4ºC made from milk with 2.5% (circle), 4.0% (triangle), 5.5% casein (square) and milled at either pH 5.40 (filled) or 5.10 (open) (n=4).

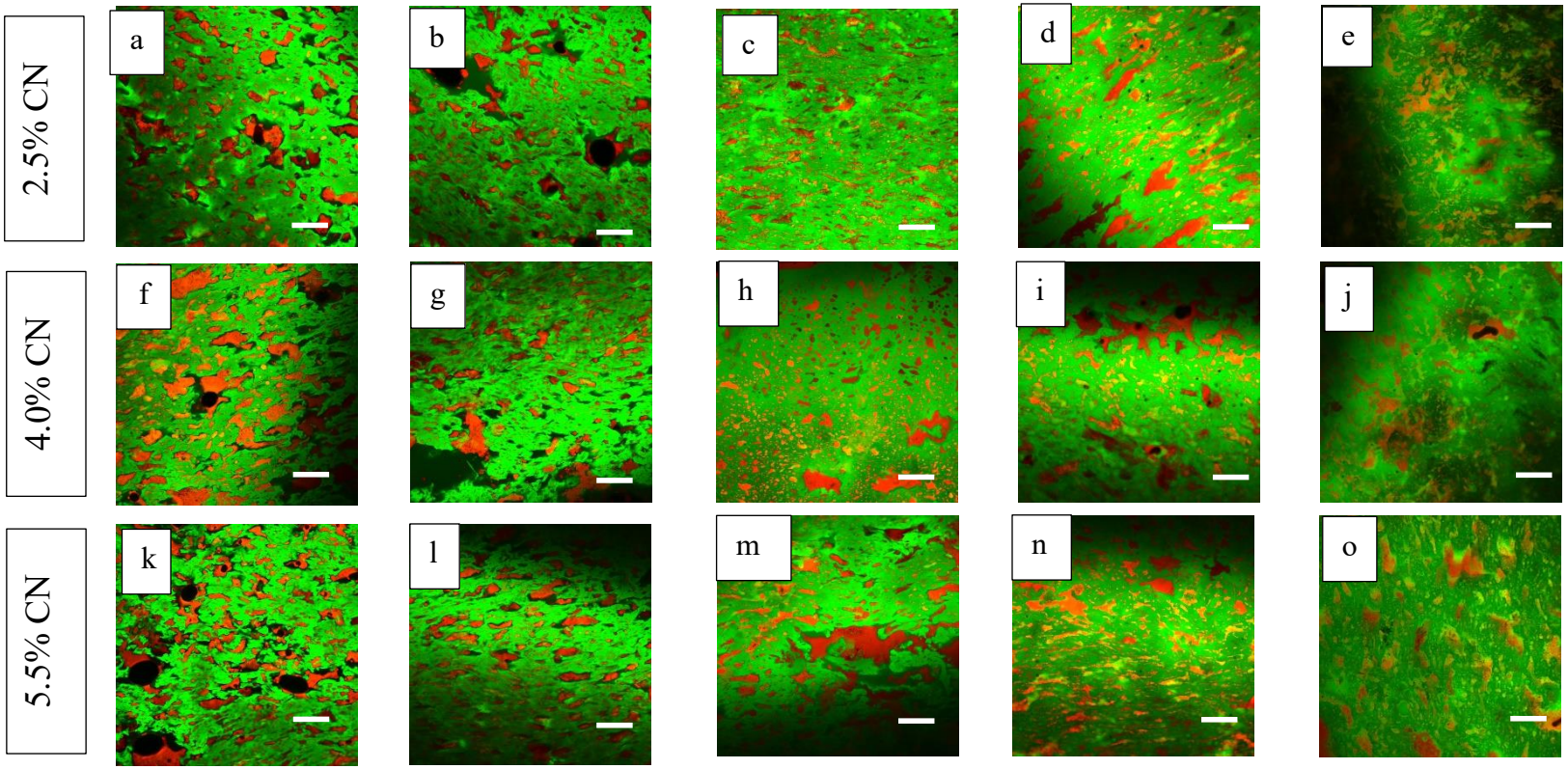


Figure 5.14 Confocal laser scanning microscopy images of LMPS Mozzarella cheeses milled at pH 5.40 observed at 2 d first column (a, f, k); 7 d second column (b, g, l); 2 wk third column (c, h, m); 1 mo fourth column (d, i, n); 3 mo of storage fifth column (e, j, o), stored at 4ºC for cheese made from milk with 2.5% casein (a, b, c, d, e), 4% casein (f, g, h, i, j) and 5.5% casein (k, l, m, n, o); Nile Red and Fast Green FCF was used to dual stain the fats and proteins, respectively. Scale bar represents 100 µm (n=4).

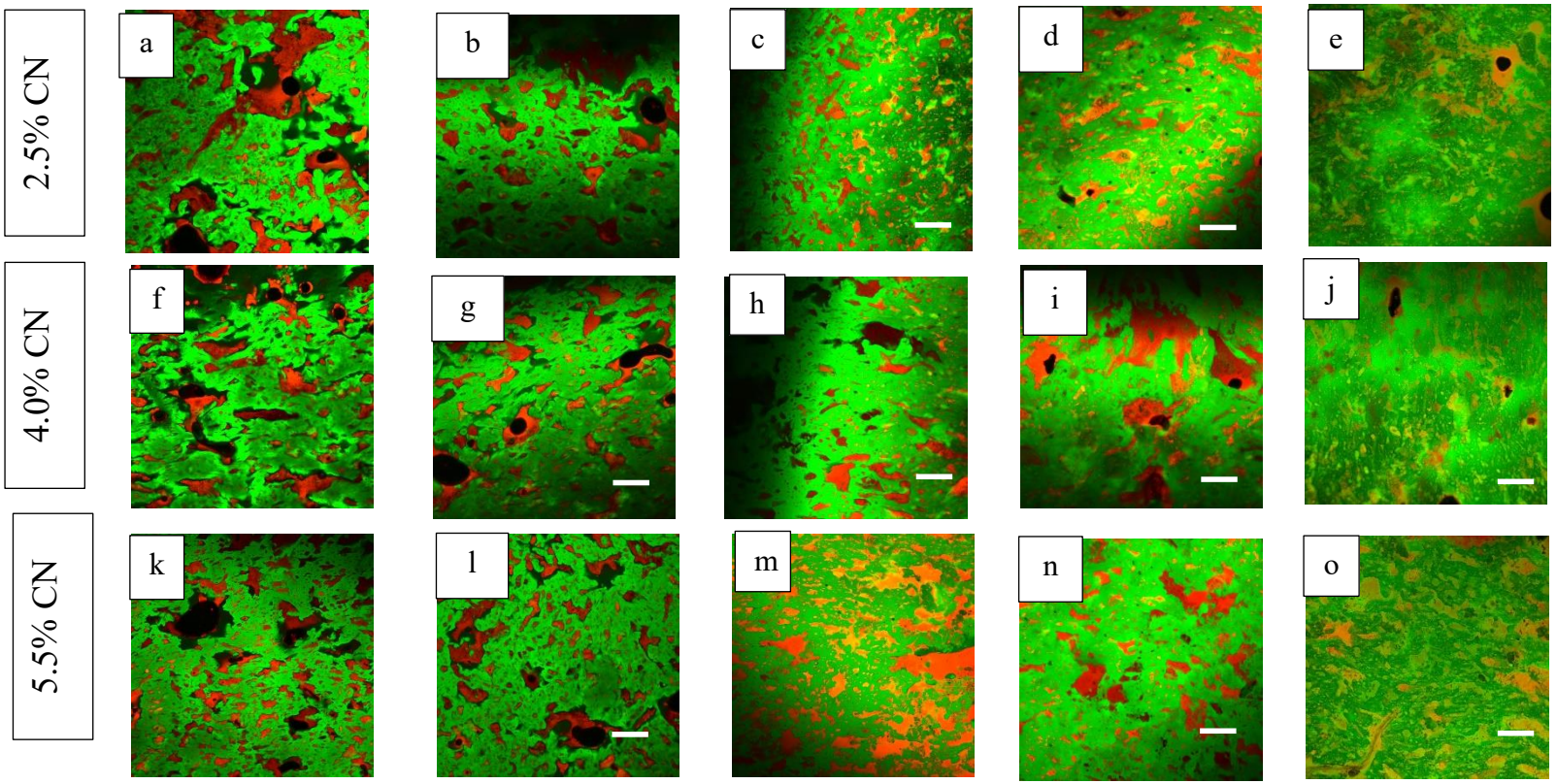


Figure 5.15 Confocal laser scanning microscopy images of LMPS Mozzarella cheeses milled at pH 5.10 observed at 2 d first column (a, f, k); 7 d second column (b, g, l); 2 wk third column (c, h, m); 1 mo fourth column (d, i, n); 3 mo of storage fifth column (e, j, o), stored at 4ºC for cheese made from milk with 2.5% casein (a, b, c, d, e), 4% casein (f, g, h, i, j) and 5.5% casein (k, l, m, n, o); Nile Red and Fast Green FCF was used to dual stain the fats and proteins, respectively. Scale bar represents 100 µm (n=4).

Chapter 6. Impact of Various Factors on the Soluble/Insoluble Calcium Content and Functional Performance of Manufactured Cheese

Abstract

The final insoluble calcium content in the cheese (at 2 d timepoint) is affected by various manufacturing factors including milk composition, pH at rennet addition, type of acid used for pre-acidification (PA), pH at whey drainage (WD), milling pH, and salting method. The data from our previous trials were used to try to better explore the effects of individual factors on the approx. amount of insoluble calcium in the final manufactured cheese. Out of the factors that were studied, concentrated milk, the pH value at WD (changing it by ≥ 0.2 pH units), citric acid used for PA to pH 6.00, and salting method (pre-salting vs brining) seemed to cause the largest difference in the final insoluble calcium in the cheeses. These changes caused an overall range of differences of approx. 2-4 mg insoluble Ca/g protein, which was sufficient to modify the functional performance of cheese. Changing the milling pH values, or PA to pH 6.00 using acids, such as, acetic and carbonic acid, lowered the insoluble calcium content to a lesser extent, approx. 1-2 mg/g protein. However, the changes in the functional performance of the cheese on pizza as a result of this smaller difference in the insoluble calcium content were still noticeable. There was only a slight, or not significant, difference in insoluble calcium content between the control sample and the cheese PA to pH 6.40 using carbonic and acetic acid. The amount of insoluble calcium content that is needed to be altered to cause a difference in functional performance of cheese is important information for cheese manufacturers; they can use this very data to help select cheese making procedures to produce cheese with desired functional performance.

6.1 Introduction

The impact of individual manufacturing factors on calcium losses during manufacture and the impact on the final total and insoluble calcium content in cheese, along with its associated functional performance, were discussed in previous chapters. The experiments were replicated four times, and the data was averaged from these trials to analyze the data. But it is well known that even when we try to keep the cheese-making parameters consistent there can be variability during the manufacturing process, which can affect the final cheese composition, specifically, in our research, the insoluble calcium content in the final cheese. Therefore, in this chapter, we wanted to individually look at the impacts of various factors, in addition to the variability observed between trials, to better understand the impact of these individual factors. We also tried to relate the cheese functional performance to its insoluble calcium content in these respective cheese samples.

6.2 Materials and Methods

The insoluble calcium content values as impacted by different factors including the pH value at WD in relation to PA pH, moisture content in relation to casein content of cheese milk, and milling pH values were graphed as a scatter plots using Sigma plot 13.0 (SYSTAT, San Jose, California) and PCA (principal component analysis) plots using JMP Pro 17 (JMP, Cary, NC) to explore possible correlations between individual factors. Four cheeses with similar composition, but different insoluble calcium content were chosen to describe its impact on the functionality of cheese at 2 wk and 1 mo of storage (typically before which the LMPS Mozzarella is commercially shredded for its application on pizza).

6.3 Results and Discussion

6.3.1 Impact of pH at rennet addition and whey drainage on calcium solubilization

pH at rennet addition plays an important role in calcium solubilization during cheese manufacturing. Calcium equilibrium during cheese making is impacted by two main factors: pH and moisture in milk or curds. As the pH of the milk, or curd, is reduced as cheese making progresses, the insoluble calcium phosphate is solubilized. The rate of calcium solubilization is highly pH-dependent, where a pH below 6.00 increases calcium solubility to a greater extent than pH > 6.0. In contrast, if the moisture content in these samples (milk/curd) is reduced (by synereses), the solubility of the calcium phosphate is restricted because there is less moisture for the insoluble calcium to dissolve into. Calcium phosphate in the serum phase becomes more easily saturated due to the lower moisture of the curd/milk, which should restrict or significantly slow down the calcium phosphate solubilization process.

An example of this situation is comparing a Mozzarella cheese made with starter culture (SC) and direct acid (DA). The rennet is usually added at around pH 6.60 and pH 5.60 for SC and DA cheese, respectively. The whey is also drained off at a much higher pH value in the SC cheese (pH 5.90-6.00) as compared to DA cheese (pH 5.60). Before milling and stretching the SC cheese is allowed to reach a pH of 5.20 but in the DA cheese, the curds are typically stretched at pH 5.60. Even though a lower pH is reached in the SC cheese as compared to DA cheese, the SC cheese has significantly higher insoluble calcium values, typically, approx. 19-20 mg/g protein, whereas the DA cheeses have insoluble calcium values around 11-12 mg/g protein (depending on the type of acid used). Because the pH at rennet addition and WD were much higher in the SC cheese, the milk was converted to a gel and then into curds at higher pH values or earlier on during the cheese-making process. Decreasing the pH in curd after WD as opposed to in milk or in curds before WD

has a large impact on calcium solubilization due to moisture differences between the respective samples; the soluble calcium dissolved from the colloidal calcium phosphate (CCP) saturates quickly in the serum phase of the curds restricting or slowing further calcium solubilization. This example, of course, is an extreme scenario but this concept similarly applies when we pre-acidify the milk sample. If we pre-acidify the milk sample to a lower pH before rennet addition, it will lose more calcium during manufacture, as compared to a control (no PA) or a milk sample PA to a higher pH because the milk is converted to curds at a lower pH in the former that allows more calcium to solubilize due to higher moisture content in the milk as compared to curds (which quickly synereses and loses a significant amount of moisture as whey). A caveat with prior research is we do not know how much total or insoluble calcium was lost due to the difference in pH at rennet addition and the differences in pH values at WD. Typically, when a cheese is PA to lower pH values, the pH at WD is also lower, because the cheese making is based on time and not necessarily on pH alone. So, it is hard to segregate the impact of these two individual factors.

In our research that dealt with the extent of PA and different types of acids for PA, we tried to drain the whey at similar pH values, but it is difficult to reach the same pH value every time we make cheese. This is because the rate of acidification by starter culture can be variable. Owing to this, we saw slight differences in the pH of curds at WD in our curd samples during manufacture (Figure 6.1). It is well known that a difference in the pH value of curd at WD causes a difference in insoluble calcium content because this is considered the most critical step where the whey is separated from the curds. There was a significant reduction in the insoluble calcium content when the pH of curds at whey drainage decreased to pH $\leq$ 5.6 (Figure 6.1).

The question that we tried to answer was does the pH at rennet addition/extent of PA impact the insoluble calcium content in the final cheese, if the curds at WD had similar pH values. Before

we discuss this, it is important to keep in mind that the rate of PA can also influence the amount of insoluble calcium dissolved as we observed in one of our experiments in Chapter 3. We kept the rate of PA constant (approx. 1 h) in all our cheeses.

The variability in pH at WD was mostly in the range of pH 5.80 to 6.00 for all control (SC) and samples that were PA to pH 6.40 (Figure 6.2). In the cheeses where the pH at WD was around 6.00 (Figure 6.2), there was no clear trend observed for differences in insoluble calcium content, but when the curds had a WD pH of around 5.8, the control cheeses exhibited slightly higher insoluble calcium content, approx. 0.5-1 mg, compared to cheeses PA to pH 6.40 using acetic and carbonic acid. The cheeses that were PA to pH 6.40 with citric acid tended to have lower insoluble calcium values, approx. 1-2 mg lower, because of the calcium chelating ability of citric acid. Between the control and cheeses PA to pH 6.40 with acetic and carbonic, no differences in functionality were observed because the difference in insoluble calcium between these samples was not large enough.

In the cheeses where the milk was PA to pH 6.00 before rennet addition, when the curds had pH of 5.80 and 5.60 at WD, the cheeses PA with carbonic acid was similar to control cheese, whereas the cheeses that were PA with acetic had a lower insoluble calcium value, i.e., approx. 2–4 mg difference (Figure 6.3). The cheeses that were PA with citric acid tended to reach a lower pH at WD due to less buffering, which allowed the cheeses to have a significantly lower insoluble calcium content. In some cases where the cheese that were PA with citric acid had similar pH values at WD, for example, when the pH at WD was 5.60 (Figure 6.3), we observed that the citric acid had significantly lower insoluble calcium content due to its calcium chelating ability. A significant difference in functionality was observed only for cheeses that were PA with citric acid to pH 6.00 (Figure 6.4). Cheese PA with citric acid to pH 6.00 was differentiated from the other

cheeses by principal component 1. Principal component 2 separated the cheeses PA with citric acid, the cheeses PA to pH 6.40 with carbonic and acetic as well as control cheese from the cheeses that were PA to pH 6.0 with acetic and carbonic acid. The distribution of different samples on the biplot shows that the cheese PA to pH 6.00 with citric had lower pH value at WD resulting in the final cheese having a lower pH at 2 d timepoint with reduced insoluble calcium content and slightly higher moisture as compared to other cheeses; functional attributes such as strand thickness and chewiness values (as measured at 1 mo timepoint) were lower for this cheese due to lower insoluble calcium content. The remaining cheeses PA to pH 6.00 with acetic and carbonic, some trends in difference in functionality like lower chewiness and lower first chew hardness values were observed but these were not significant.

It is important to note that the observations described above are for the cheeses made from concentrated milk, i.e., 4.0% CN. The total solids content in the milk can also have an impact on calcium solubilization and it is therefore possible that even though a similar trend may be observed, the extent to which the PA and the pH value at WD can impact the calcium losses during cheese manufacture can vary and this is discussed in the next section.

6.3.2 Impact of casein content in milk on calcium solubilization

Cheese made from milk with higher casein content (>4.0% CN) tends to have slightly higher insoluble calcium content, which can impact the functional performance of cheese. We observed in our previous chapter that the milk with higher casein content had significantly higher serum calcium content, which dramatically restricted CCP solubilization. The resulting lower moisture content in the curd samples throughout the cheese making process in the cheese made from milk with higher casein content could have also restricted calcium from solubilizing. We now wanted to see if the final moisture content has an influence on the soluble and insoluble calcium

content in these cheeses. Figure 6.5 and 6.6 shows the insoluble calcium content with respect to the final moisture content in our cheese samples made from milk with varying casein content milled at pH 5.40 and 5.10, respectively. The final lower moisture content in the cheese made from milk with 5.5% CN may have enabled or contributed to its higher insoluble calcium content when the cheeses were milled at pH 5.40 (Figure 6.5) and pH 5.10 (Figure 6.6). No clear relationship was observed when the soluble calcium content in the final cheese was plotted against moisture content in the cheeses milled at pH 5.40 (Figure 6.7) or when the cheeses were milled at pH 5.10 (Figure 6.8). The most significant difference between the cheeses made from milk with different CN content was the altered moisture content in the final cheese. The lower final pH attained in the cheeses when milled at a lower pH value could also influence the serum calcium content and this is discussed in the next section.

It is hard to attain typical moisture content requirements for LMPS Mozzarella (47-52%) in cheese made high casein milk (unless very specific adjustments are made) due to the stiffer rennet gel network formed during cheese making that expels more moisture (synereses). We were able to achieve similar moisture requirements in our cheese made from 5.5% CN milk milled at pH 5.40 and 5.10, i.e., approx. 46-47%. The following are the changes/adjustments that we made to our cheese make procedure to obtain a cheese with desired moisture requirements: (1) pre-acidifying the milk sample to pH 6.00 before rennet addition, (2) lowering the pH value at WD, i.e., the whey was separated from the curds when the curd pH reached 5.70-5.80 (typically with SC cheese, the whey is drained at a curd pH of 5.90), (3) brining the cheese for 4 hours instead of pre-salting and brining for 1 hour (reason for this is discussed later in the chapter). Other adjustments that could have been explored include (1) lowering the set/renneting temperature - lowering the temperature at rennet addition can allow a softer gel to be formed rather than a stiffer

gel that is mostly the case in milk with high casein content (and with less syneresis ability); the proximity of CN casein micelles allows for faster aggregation; (2) lower the cooking temperature - this allows for less moisture being expelled from the curds earlier on in the process so that more CCP could be solubilized into the serum. Most of these suggested or recommended changes to the cheese-making procedure (cheese made from concentrated milk) indicate that these steps if employed would retain more moisture in the curds earlier on during the cheese-making process, which could also help to allow additional insoluble calcium to be dissolved from the curds.

6.3.3 Impact of milling pH on calcium solubilization

Changing the milling pH of the curds during cheese manufacture impacted the insoluble calcium content significantly but not the total calcium content. Figure 6.9 shows the differences in insoluble calcium content obtained in cheeses made from milk with varying casein content and milled at different pH values. Changing the milling pH values by 0.3 pH units lowered the insoluble calcium content by 1-1.5 mg/g protein in cheeses made from milk with varying casein content. The cheese made from milk with 2.5% CN took approx. 20 mins to reduce the pH from 5.40 to 5.10 whereas the cheese made from milk 4.0 and 5.5% CN took about 45 min for the same pH reduction. Since the latter cheese had higher serum calcium with respect to moisture, the phosphate content would have also been higher (CCP is the colloidal calcium phosphate complex, so as calcium dissolves, so does phosphate), which would have increased the buffering capacity of this cheese (as H^+ ions become complexed with PO_4^{2-}), taking a much longer time to achieve the desired target milling pH value. If the goal was to dissolve additional insoluble calcium from the curds made from cheese manufactured from high casein milk, pre-acidifying the milk sample or reducing the pH at WD can allow for faster solubilization of insoluble calcium content, as compared to reducing the milling pH value.

Figure 6.10 and 6.11 shows the impact of final cheese pH on the soluble and insoluble calcium content in the cheese sample at 2 d of storage, respectively. A linear inverse relationship between pH and soluble calcium content was observed, where a cheese with lower pH had higher soluble calcium content and vice versa. When the pH was plotted against insoluble calcium content, similar positive relationship was observed, where the cheese with lower pH had lower insoluble calcium content; more variability was observed in this case as the calcium values were normalized to protein content. Similar variability was not observed in Figure 6.10 because the values are represented based on 100 g sample; there are significant differences in moisture content between the cheeses.

The overall impact of these factors on cheese functionality has been shown in Figure 6.12 and Table 6.1 using biplot and Pearson correlation, respectively. Principal component 1 separated the cheeses with low moisture from cheeses with high moisture content (Figure 6.12). As the cheese made from milk with 2.5% casein (LCHpH and LCLpH) had higher moisture content, this resulted in cheeses having lower insoluble and higher soluble calcium content; these cheeses when shredded had lower shred length and shred straightness with increased adhesiveness and matting at 1 mo of storage (Table 6.1). The cheeses made from milk with 5.5% casein (HCHpH and HCLpH) exhibited more blistering (Figure 6.12) with higher first chew hardness values as a result of higher insoluble calcium content (Table 6.1). Principal component 2 separated the cheeses milled at higher pH value from the cheese milled at lower pH value. In the cheeses made from milk with same casein content but milled at different pH values, the cheeses milled at pH 5.40 (HpH) had higher strand thickness values due to higher insoluble calcium content as compared to cheeses milled at pH 5.10 (LpH) (Figure 6.12).

6.3.4 Impact of salting method on calcium solubilization

The most commonly used salting methods for LMPS Mozzarella involve pre-salting (directly adding dry salt to the cheese immediately after milling) with brining or just directly brining the cheese (for approx. 4 hours or longer). Figure 6.2 shows the impact of salting methods on the final insoluble calcium content in cheese. The circled cheeses in Figure 6.2 made from milk with 4.0% CN that was pre-salted and brined for 1 hour had significantly higher amounts of insoluble calcium, i.e., 2-3 mg higher insoluble calcium per g protein as compared to cheeses where only brining was employed as salting method. In our preliminary experiment, pre-salting the cheeses decreased the moisture in the curds significantly during the stretching step and these cheeses had a moisture content of around 45-46%. But when we skipped pre-salting, the curds were able to retain more moisture during the stretching step which resulted in cheeses having slightly higher moisture content (approx. 49%) with lower insoluble calcium content. We understand that calcium solubilization is a slow process; therefore, longer brining time in our cheeses, i.e., 4 hours, could have aided in slightly increased solubilization of insoluble calcium. The dilution of serum phase in the cheese as the cheese was brined could have also reduced calcium saturated and allowed more CCP to solubilize.

6.3.5 Impact of insoluble calcium content on functional performance of cheese

In our research, we have made LMPS Mozzarella cheeses that varied in moisture content between 45-50% with insoluble calcium contents varying from 14-22 mg/g protein. Table 6.1 shows the impact of various manufacturing variables on the final amount of insoluble calcium content in the cheese. Some examples of how this change in insoluble calcium content can impact the cheese functional performance are shown in Table 6.1. All of these experimental cheeses have similar moisture and protein contents with varying insoluble calcium content. One of the cheeses,

i.e., cheese PA to pH 6.00 with citric acid has a slightly lower pH value compared to other cheeses. If the moisture content or the pH value in the cheese changes, the functionality of the cheese can also vary due to the difference in bond mobility and calcium interactions at the very high temperatures the cheese is exposed to during heating on a pizza. Some general trends observed with the difference in insoluble calcium content between the cheeses are as follows (Table 6.1):

Visual attributes of melted cheese on Pizza

- **Blister quantity, blister color, and melt** were similar in all of the cheeses at 2 wk and 1 mo of storage.
- **Blister size:** We did not directly measure this attribute in our cheeses but at 1 mo and 3 mo of storage, we have observed trends where the cheeses with lower insoluble calcium form large blisters. As the cheese is more pliable, a larger surface area of the cheese expands before it releases steam forming larger blisters. A significant difference was not observed in young cheese at 2 wk of storage.
- **Flow off crust:** There appeared to be a greater tendency for the cheese to flow off crust when the insoluble calcium content was lower. All of these cheeses at one point or another showed a tendency to flow off crust even when the insoluble calcium was higher in some cases because of its relatively higher moisture content.
- **Free oil release:** At 2 wk of storage, the free oil release seemed to be higher in the cheeses with lower insoluble calcium content. This was likely due to how the fat was distributed within the cheese microstructure. We have observed in our experiments that the cheeses with lower insoluble calcium content tended to have larger coalesced fat droplets trapped between the protein fibers, which when heated was released on the cheese surface. Typically, when a greater amount of free oil is released, the blister quantity is significantly

reduced. We did not observe these trends in the cheeses shown in Table 6.1, probably due to the high moisture content in these cheeses. The higher moisture content in itself would significantly reduce the blistering in young cheeses as a result of evaporative cooling during baking.

- **Strand thickness** is an attribute that seems to be significantly impacted by the insoluble calcium content, where the cheese with lower insoluble calcium seems to have a significantly lower strand thickness value in cheeses with similar moisture content. If the moisture content of the cheese was changed and the insoluble calcium content was lowered, the cheeses tend to exhibit higher strand thickness values due to increased bond mobility which increases the opportunity for the insoluble calcium to interact with more neighboring casein molecules (this was observed in cheese made from milk with 2.5% CN milled at pH 5.10, Chapter 5).
- **Strand length** can also be influenced by the insoluble calcium content. At 2 wk of storage, the cheeses with around 17 mg insoluble calcium/g protein seemed to have the highest strand length but at 1 mo of storage, the cheeses with the lowest amount of insoluble calcium (14 mg) seemed to stretch the most. Typically, if the insoluble calcium content is too high or too low, the strand length is lower because there is limited flexibility for the casein molecules to interact with each other in the former whereas there was not enough calcium for the casein interactions to occur in the latter. In our experiments, we were not able to observe a clear trend on the impact of insoluble calcium, as there was too much variability in results. The variability can be due to temperature differences when the cheese is stretched (the sensory panel tries to stretch the cheese at the same temperature but sometimes the cheese cools down too fast or too slow), the person stretching the cheese

(stretching too quickly or too slow), the location where the cheese is stretched (is the cheese layer too thick or thin), etc. All of the cheeses stretched well, above 6 inches, which is the basic minimum requirement for pizza cheese.

Texture attributes of melted cheese on Pizza

- **First chew hardness and chewiness** were typically lower when the insoluble calcium content was lower. This was because the cheese was softer with a lower insoluble calcium content. The trends for this were obvious in the cheeses at 1 mo of storage but not at 2 wk.
- **Cohesiveness** of cheese is inversely correlated with insoluble calcium content where a cheese with lower insoluble calcium content had a much higher cohesiveness value as can be observed in cheeses in Table 6.1. This trend was more evident at 1 mo of storage than 2 wk.

Flavor attributes of melted cheese on Pizza

- The flavor attributes including salt, acid, milky, and buttery notes were similar in all of the cheeses and were typical of an LMPS Mozzarella cheese. In cheeses where the PA with acetic or citric was employed, especially to a lower pH value, i.e., pH 6.00, there were sour and slight astringency notes detected by the panelists.

We have seen some trends indicating how the insoluble calcium content in the cheese can influence the functional performance of cheese on pizza. A more difficult question is what is the ideal range of insoluble calcium content in cheese for optimal functional performance, i.e., a cheese with complete melt, optimum blistering, strand thickness, strand length, chewiness, and cohesiveness. Based on our observations, the ideal range for the insoluble calcium content in cheese with moisture around 47-49% and pH around 5.10-5.20 would be 17-19 mg/g protein. The

consumer preference and acceptance can of course vary but this range suggests the visual and textural attributes are around just right and does not have too much or too little of an attribute. It may seem as though a 1-2 mg difference in insoluble calcium content (represented per g protein) was not a lot. But when we represent this value as mg/100 g of sample, it turns out to be 25-50 mg insoluble calcium/100 g of sample, considering the protein content in the cheese is approx. 25%. In our work, we standardized the insoluble content based on protein content in the sample to normalize the value so that we could compare differences between samples even when the moisture contents were variable.

6.4 Conclusions

A lot of different manufacturing factors were employed during cheese manufacturing to study its impact on the insoluble calcium content in cheese at 2 d of storage. We observed that pH value at WD has the most significant effect on calcium solubilization as compared to other factors including PA pH or milling pH values. PA of milk to pH 6.00 lowered the insoluble calcium content in the final cheese as compared to control cheese; the cheese where milk was PA with citric acid to pH 6.00 caused a dramatic reduction in the insoluble calcium content in the cheese, by approx. 3-4 mg/g protein. Changing the milling pH by 0.3 pH units reduced the insoluble calcium content by approx. 1 mg/ g protein. With all of these observations, we can conclude that if cheeses have a difference of only 0.5-1 mg/g protein in the insoluble calcium content, we do not see any obvious difference in cheese functional performance. Some differences or trends in functionality were observed if the difference in insoluble calcium content was between 1-2 mg/g protein, but the most obvious differences in cheese functional performance were observed when the differences in insoluble calcium contents were greater than 2 mg/g protein.

6.5 Tables and Figures

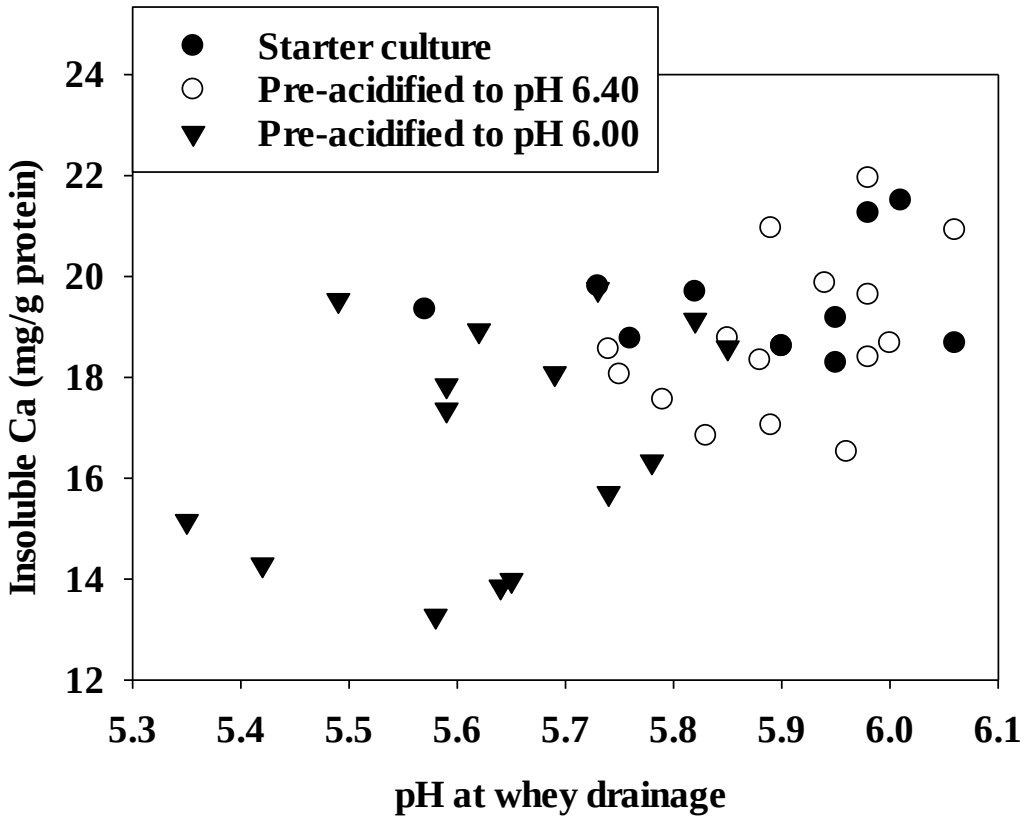


Figure 6.1. Impact of pH of curds at whey drainage on insoluble calcium content in the final LMPS Mozzarella cheese. All cheeses were made from milk with 4.0% CN and were milled at pH 5.20.

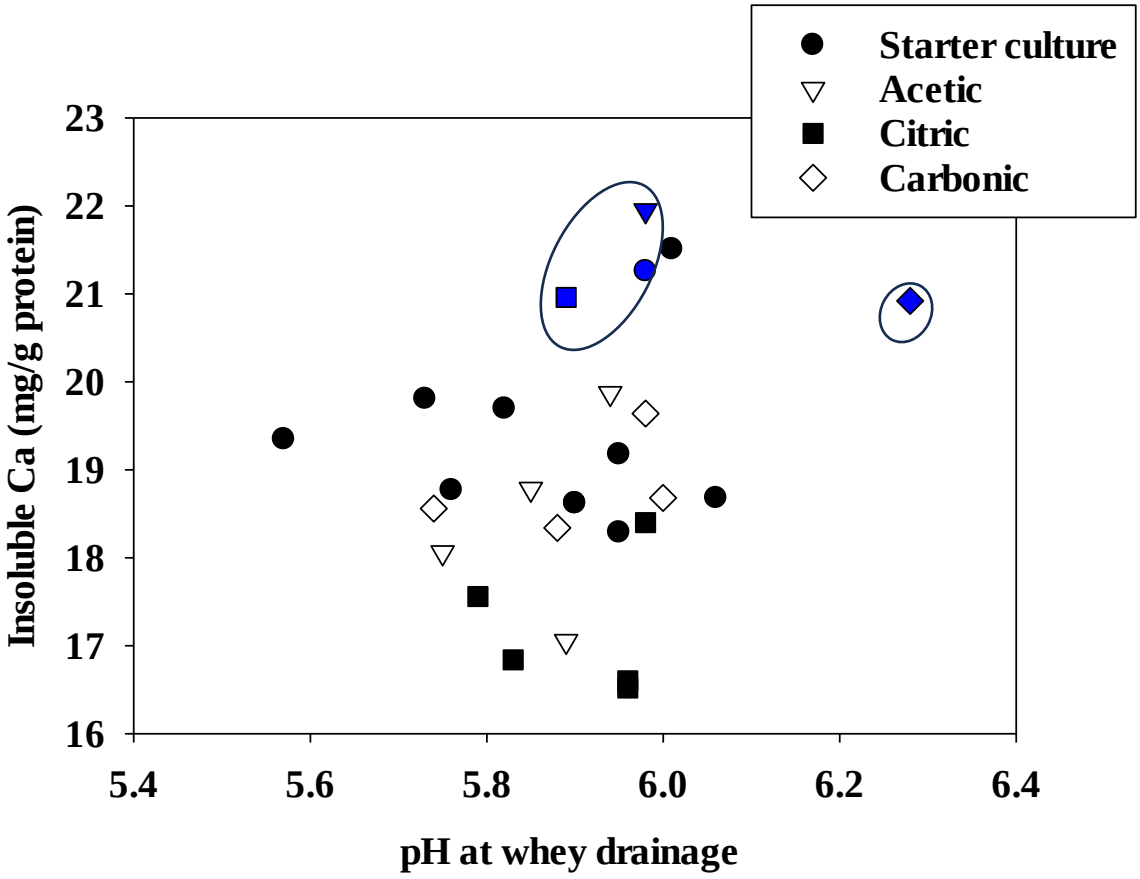


Figure 6.2. Impact of pH of curds at whey drainage on insoluble calcium content in the final LMPS Mozzarella cheese. All cheeses were made from milk with 4.0% CN and pre-acidified to pH 6.40 using various acids or not pre-acidified (starter culture cheese) and milled at around pH 5.20. The symbols in black and white are cheeses that have moisture of approx. 49%. The circled symbols are data from preliminary trial where the cheeses were pre-salted and brined- resulting in moisture around 45-46%.

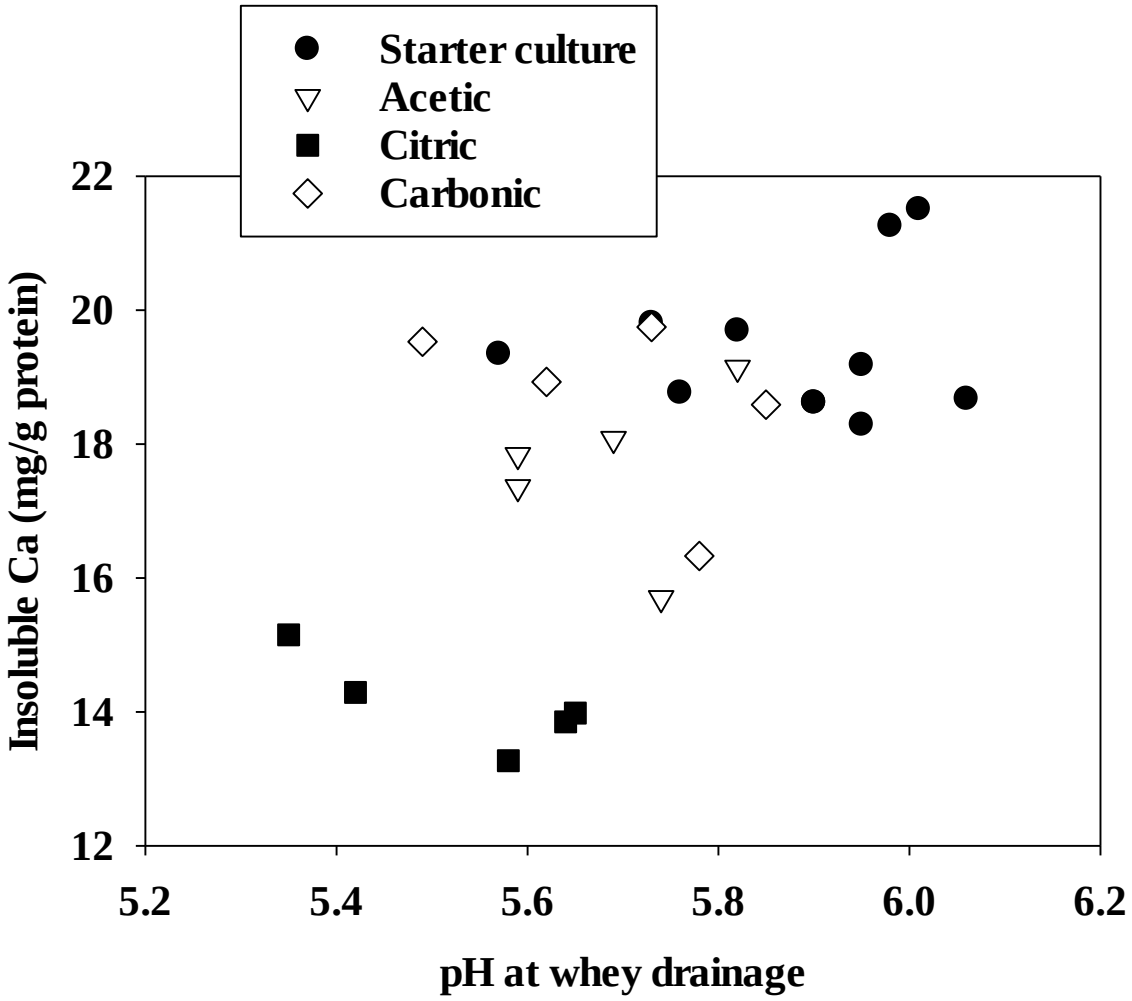


Figure 6.3. Impact of pH of curds at whey drainage on insoluble calcium content in the LMPS Mozzarella cheese. All cheeses were made from milk with 4.0% CN and pre-acidified to pH 6.00 using various acids or not pre-acidified (starter culture cheese) and milled at pH 5.20.

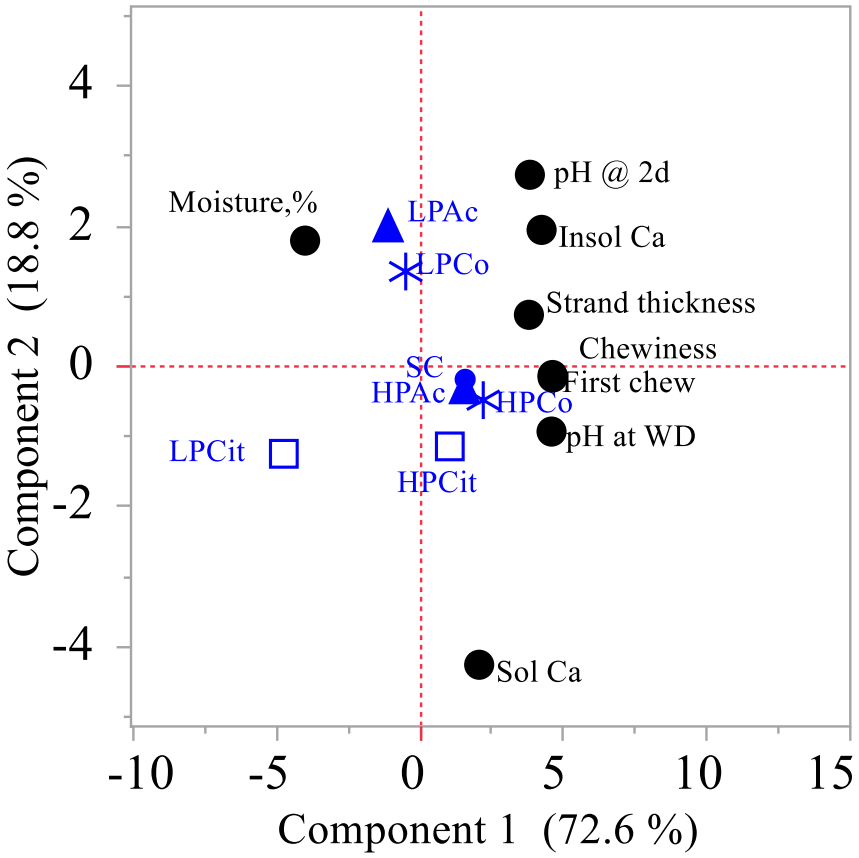


Figure 6.4. Biplot showing the impact of compositional parameters on cheese functionality between different LMPS Mozzarella cheese samples made from milk 4.0% casein and pre-acidified to different extents (pH 6.40 is HP- high pH, pH 6.00 is LP- low pH) using various acid including acetic (Ac), citric (Cit), and carbonic (Co). SC is starter culture cheese with no pre-acidification.

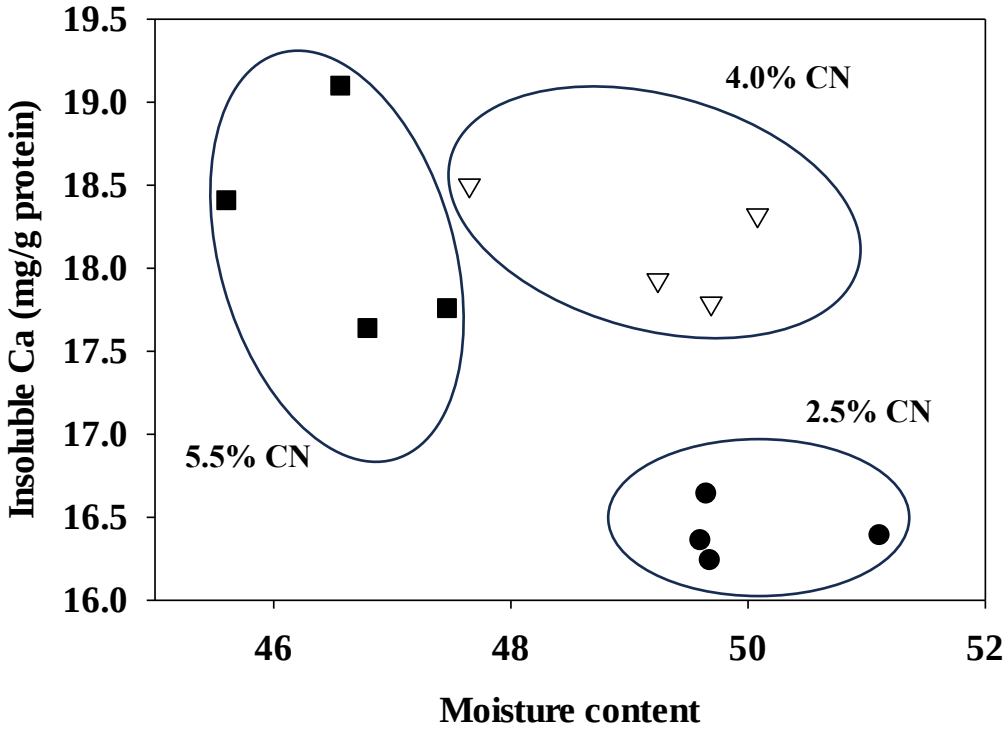


Figure 6.5. Impact of moisture content on the insoluble calcium content in LMPS Mozzarella cheeses at 2 d timepoint made from milk with varying casein content. All milk were pre-acidified to pH 6.00 using lactic acid and pH at whey drainage were similar varying between 5.70-5.87, with milling pH at 5.40.

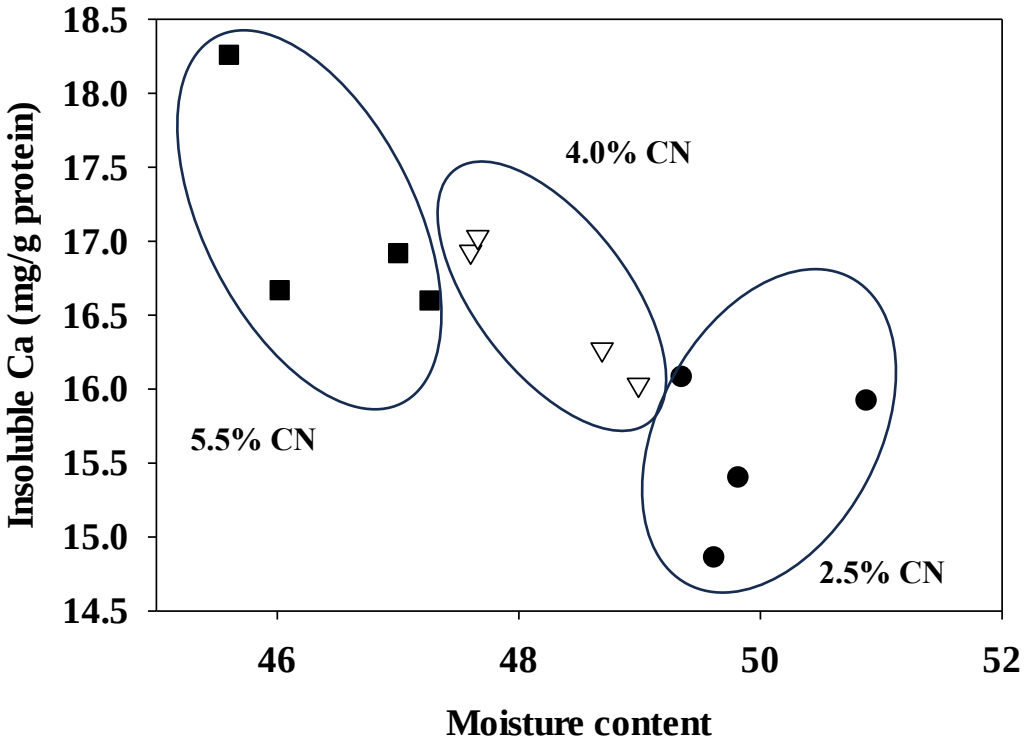


Figure 6.6. Impact of moisture content on the insoluble calcium content in LMPS Mozzarella cheeses at 2 d timepoint made from milk with varying casein content. All milk were PA to pH 6.00 using lactic acid and pH at whey drainage were similar varying between 5.70-5.87, with milling pH at 5.10.

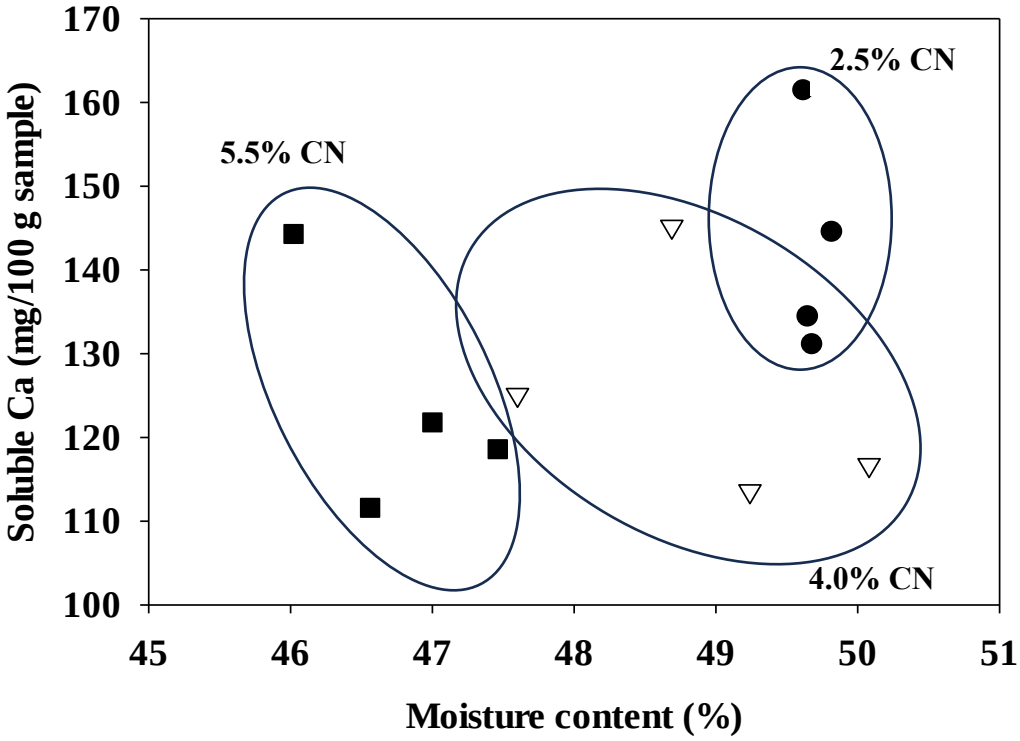


Figure 6.7. Impact of moisture content on the soluble calcium content in LMPS Mozzarella cheeses at 2 d timepoint made from milk with varying casein content. All milk were pre-acidified to pH 6.00 using lactic acid and pH at whey drainage were similar varying between 5.70-5.87, with milling pH at 5.40.

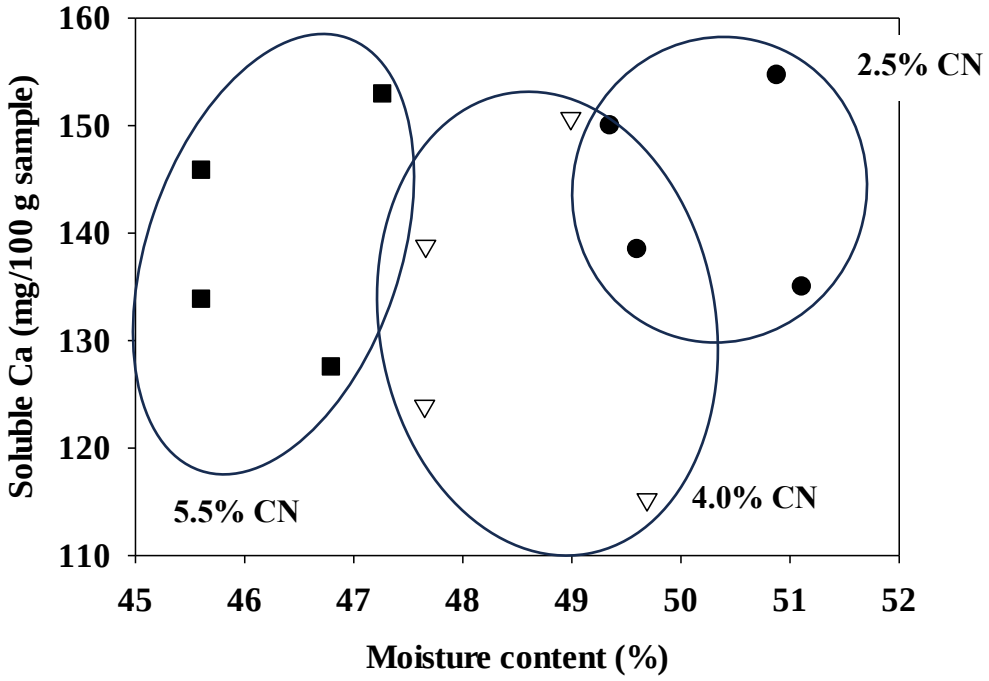


Figure 6.8. Impact of moisture content on the soluble calcium content in LMPS Mozzarella cheeses at 2 d timepoint made from milk with varying casein content. All milk were pre-acidified to pH 6.00 using lactic acid and pH at whey drainage were similar varying between 5.70-5.87, with milling pH at 5.40.

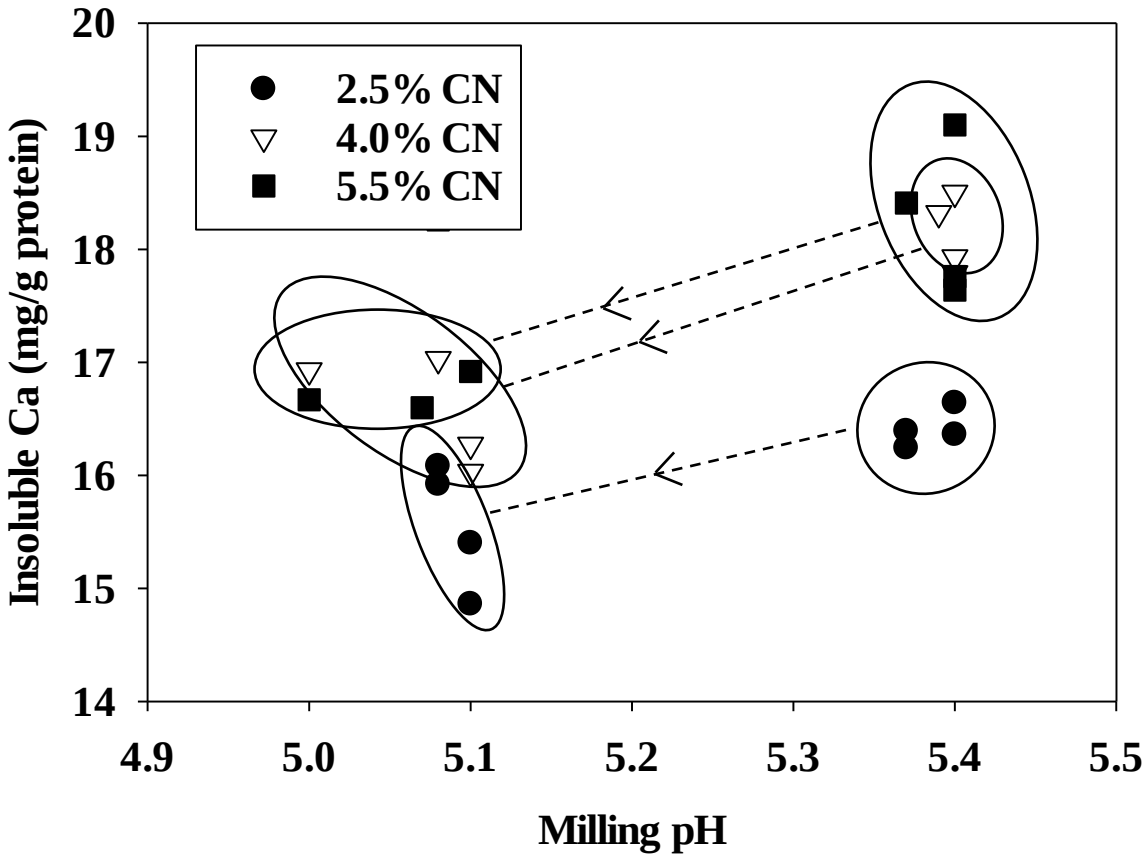


Figure 6.9. Impact of milling pH on the insoluble calcium content in LMPS Mozzarella cheeses at 2 d timepoint. Cheeses were made from milk with varying casein content. All milk samples were PA to pH 6.00 using lactic acid and pH at whey drainage were similar varying between 5.70-5.87.

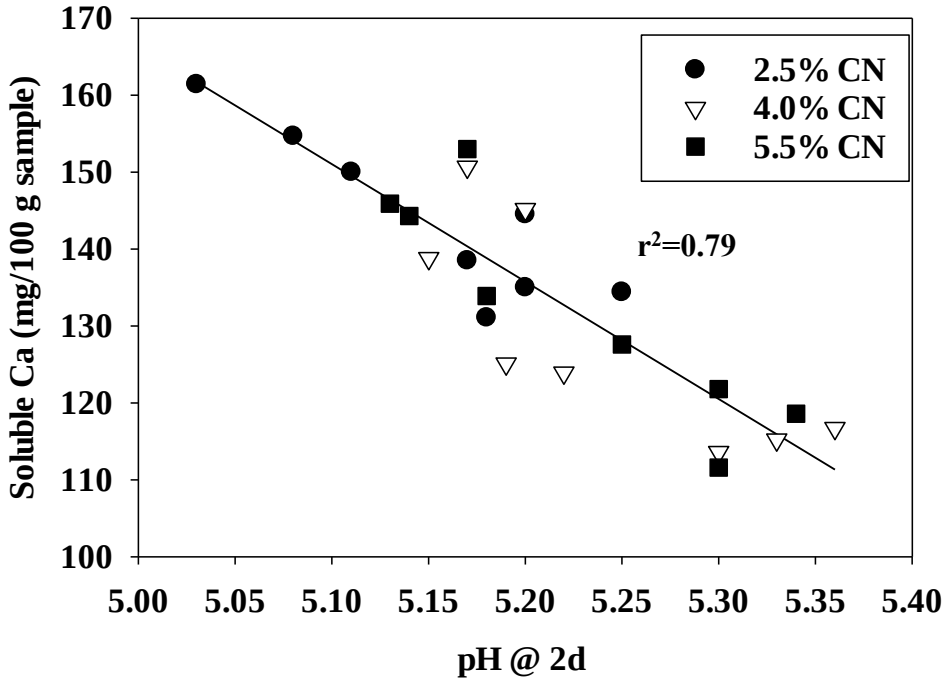


Figure 6.10. Impact of cheese pH on the soluble calcium content in LMPS Mozzarella cheeses at 2 d time point. Cheeses were made from milk with varying casein content. All milk samples were PA to pH 6.00 using lactic acid and pH at whey drainage were similar varying between 5.70-5.87.

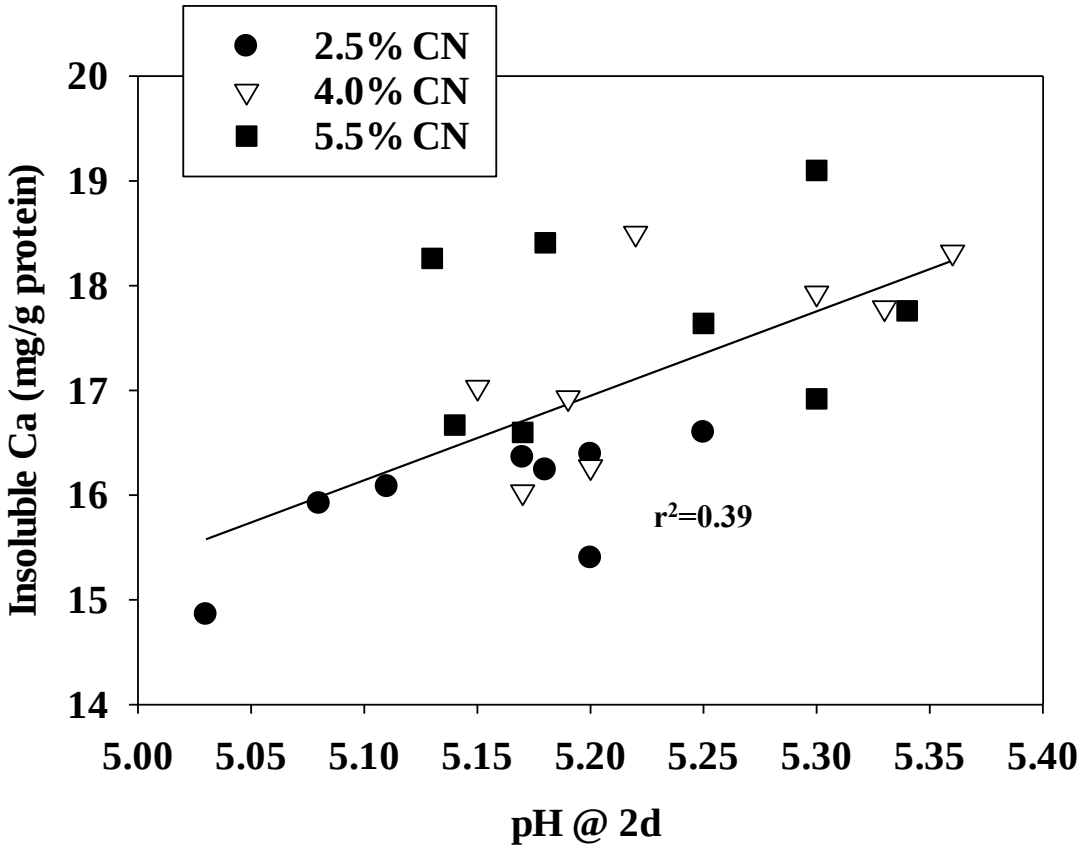


Figure 6.11. Impact of cheese pH on the insoluble calcium content in LMPS Mozzarella cheeses at 2 d timepoint. Cheeses were made from milk with varying casein content. All milk samples were PA to pH 6.00 using lactic acid and pH at whey drainage were similar varying between 5.70-5.87.

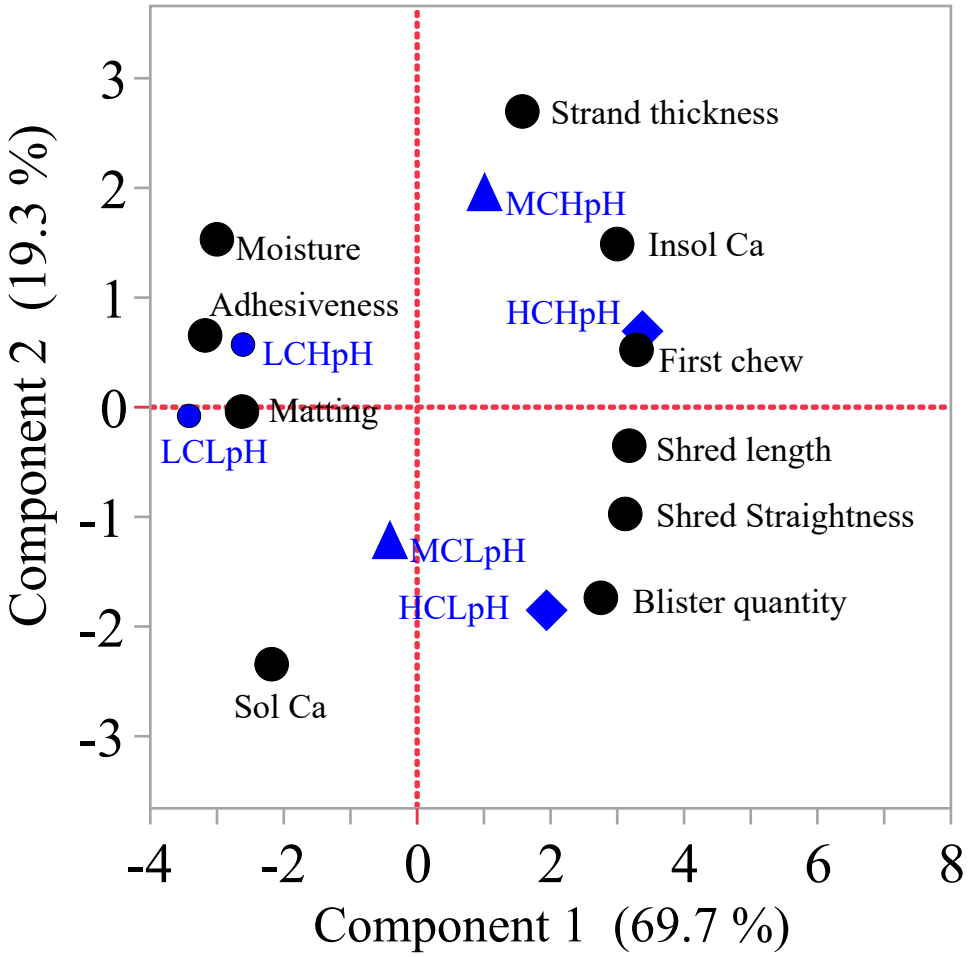


Figure 6.12. Biplot showing the impact of compositional parameters on cheese functionality between different LMPS Mozzarella cheese samples made from milk with 2.5 (low casein- LC), 4.0 (medium casein MC), and 5.5% (high casein-HC) casein content milled at pH 5.40 (high pH-HpH) and pH 5.10 (low pH- LpH).

Table 6.1. The change in the amount of insoluble calcium content with respect to changing a certain manufacturing variable during manufacture of LMPS Mozzarella

Manufacturing Variable	Change in Insoluble Calcium (mg/g protein)
Decreasing pH at whey drainage (by 0.2 units)	Decreases by approx. 1 mg
Starter Culture vs PA to pH 6.40	Decreases by 0.5 to 1 mg when PA with acids except citric
Starter Culture vs PA to pH 6.00	Decreases by 1 to 2 mg when PA with acids except citric
Acid type	1 to 2 mg lower when PA to pH 6.40 with citric but decreases by > 4 mg when PA to pH 6.00 with citric compared to control cheese
Casein content in milk	2 to 3 mg higher in the cheese made from milk with 5.5% casein compared to cheese made from milk with 2.5% casein
Decreasing milling pH (by 0.3 units)	Decreases by 1 to 1.5 mg in the final cheese
Salting method	2 to 3 mg lower when brined for 4 hours instead of pre-salting and brined for 1 hour

Table 6.2. Pairwise Pearson correlations between different variables measured in LMPS Mozzarella cheeses made from milk with varying casein and milled at different pH values

Variable	**by Variable**	**Correlation (r^2)**	**Significance (*P*-value)**
Insol Ca	Sol Ca	-0.9150	0.0105*
First chew	Insol Ca	0.9312	0.0069*
Blister quantity	Moisture	-0.9300	0.0072*
Adhesiveness	Moisture	0.9513	0.0035*
Shred length	Moisture	-0.8303	0.0408*
Shred length	Insol Ca	0.8326	0.0397*
Shred Straightness	Moisture	-0.9205	0.0092*
Shred Straightness	Adhesiveness	-0.8547	0.0301*

Table 6.3 Impact of varying insoluble calcium content in LMPS Mozzarella cheese on functional performance, tested as melted cheese on pizza (n=4).

	Control-SC cheese	**5.5% CN- milled at pH 5.40**	**4.0% CN - PA to pH 6.40 with Citric**	**4.0% CN - PA to pH 6.00 with Citric**
Moisture,%	48.4	49.2	48.5	49.1
Protein content,%	25.0	24.5	25.0	25.0
Total Ca, mg/g protein	25.0	22.6	23.3	19.9
Insol Ca, mg/g protein	18.8	17.8	17.3	14.3
Insoluble Ca (% total)	0.75	0.79	0.74	0.72
pH	5.19	5.27	5.12	5.04
Functionality at 2 wk (Sensory)				
Blister quantity	7.01	6.90	6.38	7.04
Blister color	11.27	11.32	11.07	10.78
Melt	14.9	14.60	14.98	14.90
Flow	0.41	0.05	1.20	0.11
Free oil release	5.97	5.42	6.23	6.21
Strand thickness	7.49	6.25	5.87	4.80
Strand length	14.05	13.50	16.57	13.97
First chew hardness	5.45	5.80	4.57	5.44
Chewiness	6.65	6.77	6.19	6.23
Cohesiveness	10.88	11.07	11.40	11.50
Functionality at 1 mo (Sensory)				
Blister quantity	8.21	8.07	7.97	8.37
Blister color	11.91	12.02	11.79	12.16
Melt	14.88	14.47	14.88	14.87
Flow	0.68	0.12	0.12	1.22
Free oil release	6.68	6.05	6.77	6.23
Strand thickness	5.88	6.07	6.00	4.92
Strand length	15.70	15.40	15.42	18.22
First chew hardness	5.18	5.22	5.29	3.65
Chewiness	6.45	6.60	6.54	5.23
Cohesiveness	11.49	11.37	11.62	12.59

Chapter 7. General Conclusions and Recommendations for Future Work

7.1 Overall learnings and conclusions from our research are as follows:

- The parameters of the water-soluble calcium (WSC) method including water temperature, centrifugation speed, and time were optimized to create a rapid in-process measurement of calcium losses during the manufacture of low-moisture part-skim Mozzarella.
- The developed WSC method was used to measure the impact of pH at whey drainage during the manufacture of LMPS Mozzarella from unconcentrated milk using mini-vats. The greatest loss in total and insoluble calcium during manufacture happened during the whey drainage step. Changing the pH at whey drainage by 0.2 units (curd pH) changed the total and insoluble calcium content in the final cheese by ≥ 1 mg/g protein. The total calcium content and insoluble calcium content in these cheeses were in the range of 26 - 28.5 mg/g protein and 22 - 24 mg/g protein, respectively. The moisture content in these cheeses was around 46%.
- Pre-acidifying the cheese milk to pH 6.40 and 6.20 using lactic acid in the cheese made from unconcentrated milk reduced the total calcium content in the final cheese by 0.8 and 2 mg/protein, respectively and insoluble calcium content by 0.5 and 2 mg/g protein, respectively as compared to control cheese (starter culture, no pre-acidification). The moisture content in these cheese were between 44 - 45% as enough ripening time was not given to the starter cultures before rennet addition which significantly reduced the rate of acidification in these cheeses; the time taken to reach the target milling pH was therefore longer which resulted in cheeses attaining lower moisture content. A correction in ripening time was applied in all our future cheese-making experiments. In our benchtop experiment, we observed that a slower rate of pre-acidification (60 min vs 20 min) resulted in approx.

0.5 mg lower insoluble calcium content in the milk dispersion. We therefore kept the rate of pre-acidification constant with longer pre-acidification time in our pilot plant cheese-making trials.

- Pre-acidifying the concentrated cheese milk (4% casein) to pH 6.40 and 6.00 using acetic and carbonic acid lowered the total and insoluble calcium content in the final cheeses by 0.5-1 mg/g protein and 1-2 mg/g protein, respectively, as compared to control cheese. When the cheese milk was pre-acidified with citric acid to pH 6.40 and 6.00, the reduction in total and insoluble calcium were approx. 2 and 5 mg/g protein, respectively. Significantly more calcium was dissolved during the cheese manufacturing process when citric acid was used due to its calcium chelating ability which partly allowed the cheeses to reach a lower pH at whey drainage due to reduced buffering (loss of phosphate with greater solubilization of calcium phosphate in cheese milk).
- Cheeses made from milk with 5.5% casein had significantly lower levels of insoluble calcium dissolved during cheese manufacture as compared to cheese made from 2.5% casein milk, possibly due to increased buffering in the former. Although the cheese made from high casein milk dissolved less insoluble calcium, we were able to achieve the optimum amount of insoluble calcium in the final cheese samples with moisture that met the requirements for LMPS Mozzarella, i.e., 18 mg/g protein, which allowed this cheese to have good functional performance. The cheese made from milk with 5.5% casein shredded well even at the 3 mo timepoint as it was less adhesive due to its reduced moisture (approx. 46%).
- Lowering the milling pH by 0.3 pH units lowered the insoluble calcium content in the final cheeses by ≥ 1 mg/g protein, although the total calcium content did not change.

- Cheeses that were pre-salted and brined for less time had a slightly higher insoluble calcium content in the final cheese as compared to cheeses that were not pre-salted and brined for 4 hours. This could probably be because as the cheese was brined for longer time, more CCP was dissolved provided the serum phase in the cheese was not already saturated. The brine entering the cheese could also probably dilute the serum phase of the cheese reducing calcium saturation and allowing more CCP to dissolve.
- The impact of changes in insoluble calcium content on the functionality of the cheese was observed as follows: If the cheeses had a difference of approx. 0.5-1 mg insoluble Ca/g protein, we did not see any obvious difference in cheese functional performance. Some differences or trends in functionality were observed if the difference in insoluble calcium content was between 1-2 mg/g protein, but the most obvious difference in cheese functional performance were observed when the difference is greater than 2 mg insoluble Ca/g protein.
- The percent insoluble calcium as a percent of total calcium varied in our cheeses and the range was between 69-82% at 2 d of storage. For most of the cheese made from milk with 4% casein with moistures around 49% (brined), the percent insoluble calcium was between 73-76%; only when citric acid was used for pre-acidification to pH 6.00 did the percent insoluble calcium become reduced to 69-72%. For cheeses that had moisture around 46%, the percent insoluble calcium varied between 79-82%.

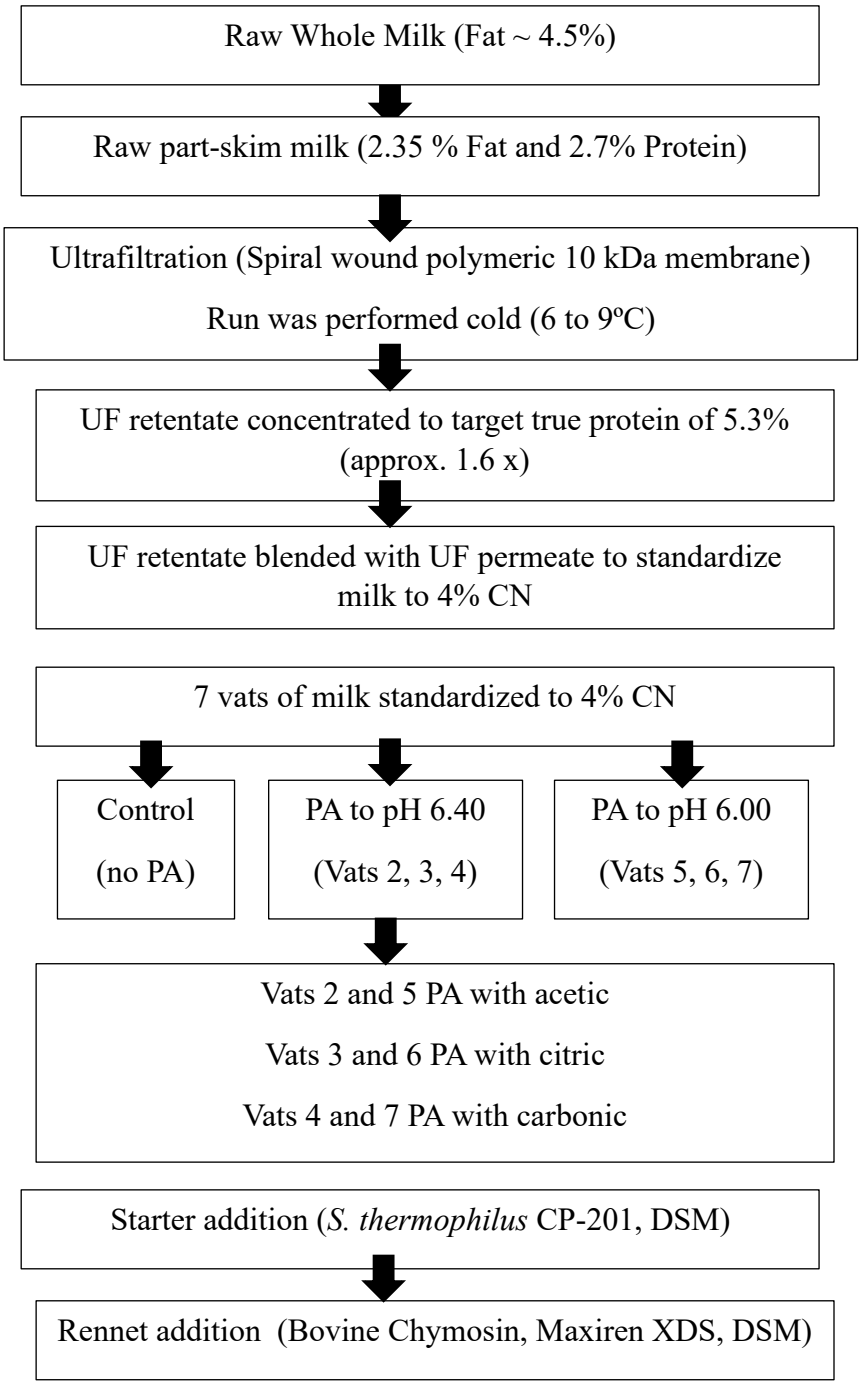
We were able to quantify the total and insoluble calcium losses during cheese manufacture as a result of different variables. There are a few factors that we did not address in our research study; this is discussed in the next section.

7.2 Recommendations for Future Work

- The rate of acid development during cheese manufacture influences the solubilization of insoluble calcium, i.e., faster rate of acidification results in cheese with higher insoluble calcium because there was not enough time for the solubilization to occur. A lot of commercial Mozzarella manufacturers use various starter cultures with different rates of acidification. A comparison of thermophilic starters and a mix of thermophilic and mesophilic starters can be studied to understand the impact of the rate of acidification on calcium losses during manufacture.
- In our research, we observed that the salting method (pre-salting along with brining vs brining only) caused a significant impact on the final total and insoluble calcium content in cheese. The reason for this could likely be due to differences in moisture loss during stretching or shifts in calcium equilibrium when brined for a longer time. We cannot specify the reason for this difference with certainty as we did not measure the moisture loss that happened in the curds with and without pre-salting during stretching and we also did not measure the calcium losses that happened between stretching and brining. Pressed block cheese (no stretching and pre-salted and brined) has been of interest in recent years as cheese manufacturers can use their existing Cheddar equipment to manufacture Mozzarella cheese. Skipping the stretching step can also influence the calcium balances towards the end of manufacture. Therefore, measuring the calcium losses in these steps can provide further useful information on how the cheese-making process can be altered to meet the necessary insoluble calcium requirements for optimal cheese functional performance.

- In our study, the moisture content in the cheeses made from milk with 5.5% casein was approx. 46-47%. Commercial LMPS Mozzarella typically has a moisture content between 47-49%. One of the reasons that we saw increased moisture loss in the cheese made with 5.5% casein could be due to faster aggregation (9 min from rennet addition) resulting in a stiffer curd being formed that expelled more moisture. Pre-acidifying the cheese milk to a lower pH and increasing the temperature of the cheese milk at rennet addition results in faster aggregation of casein micelles. Experiments with different pre-acidification pH values and set temperatures on gelation profile and curd firmness attained when using milk with high casein (>5.0% casein) can be investigated. This study can help us understand and adjust these parameters for the optimum gel firmness to be reached and to reduce significant moisture losses for cheese made from high casein milk.
- In our cheeses that were PA with carbonic acid, we noticed that the rate of acidification between curds after whey drain and milling was slower as compared to cheeses pre-acidified using acetic or citric acid. We don't know if this slower rate of acidification was due to slightly higher protein content in the curds (as a result of fat loss due to excess foaming during our technique for CO_2 incorporation) or due to the inhibitory effects of carbonic acid on starter cultures. If we can create a better method to pre-acidify the milk using carbonic acid without any foaming issues, the count of starter cultures during manufacture along with the rate of acidification can be measured to study the impact of carbonic acid on the growth of starter cultures.

Appendices



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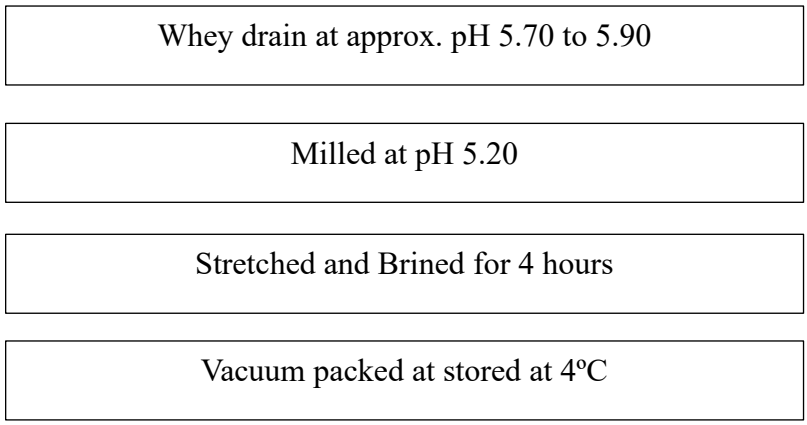
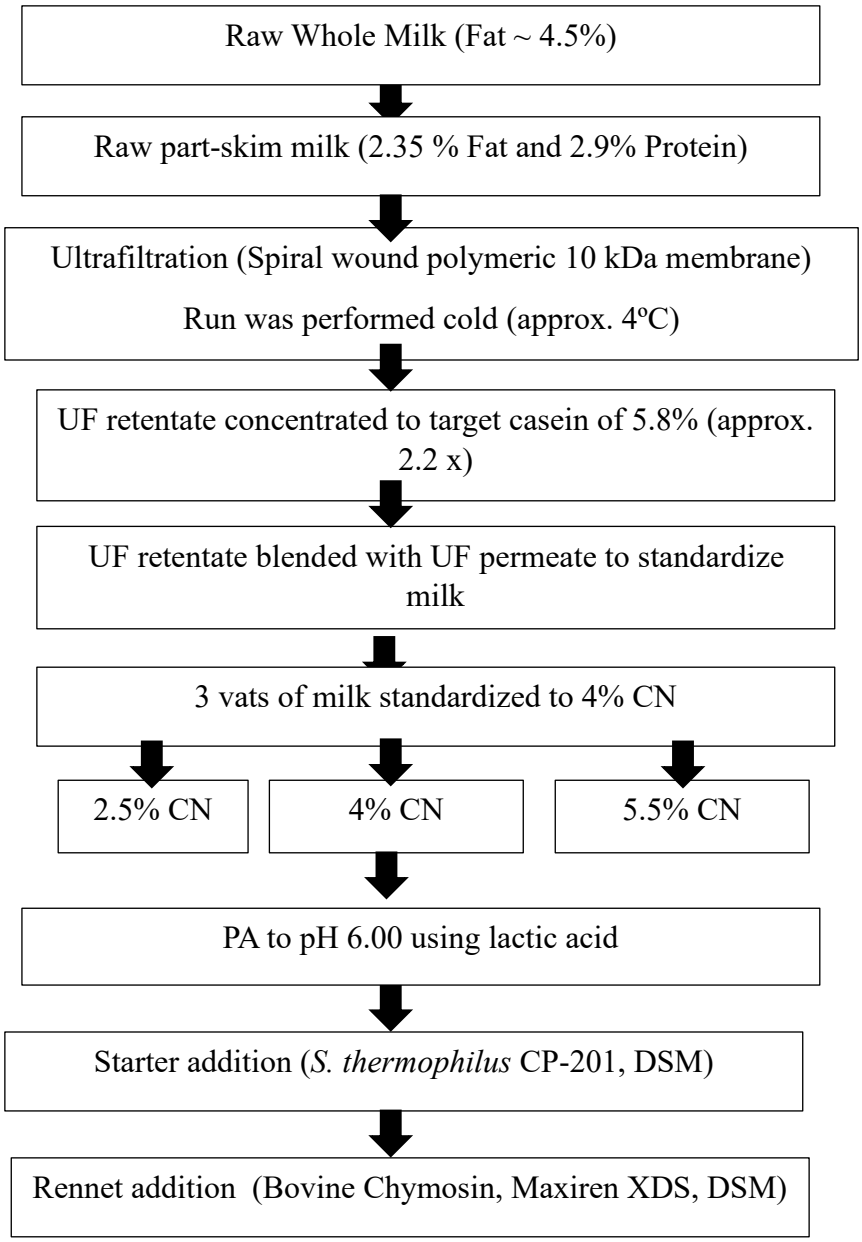
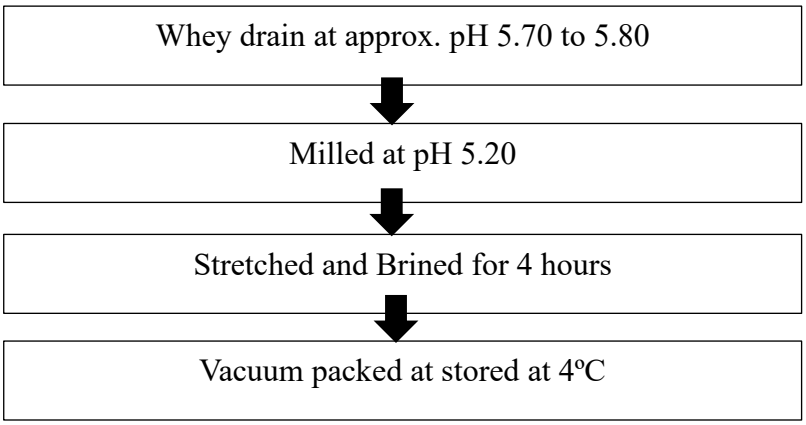


Appendix 1. Schematic representation of LMPS Mozzarella manufacturing process from 4% CN milk using different acidification methods (cultured vs pre-acidified) and various acid types (acetic, citric, and carbonic)


Raw Whole Milk (Fat ~ 4.5%)
Raw part-skim milk (2.35 % Fat and 2.9% Protein)
Ultrafiltration (Spiral wound polymeric 10 kDa membrane)
Run was performed cold (approx. 4ºC)
UF retentate concentrated to target casein of 5.8% (approx. 2.2 x)
UF retentate blended with UF permeate to standardize milk
3 vats of milk standardized to 4% CN
2.5% CN
4% CN
5.5% CN
PA to pH 6.00 using lactic acid
Starter addition (S. thermophilus CP-201, DSM)
Rennet addition (Bovine Chymosin, Maxiren XDS, DSM)

(Continued)



Appendix 2. Schematic representation of LMPS Mozzarella manufacturing process from milk with 2.5, 4.0, and 5.5% CN content and milled at varying pH values (pH 5.40 and pH 5.10)

Appendix 3. Moisture content in the samples during manufacturing of LMPS Mozzarella made from milk with 4.0% CN using different acidification methods (cultured vs pre-acidified) and various acid types (acetic, citric, and carbonic) (n=4)

Cheese samples	Milk	Curds after whey drain	Milled curds	Cheese @ 2w
Control	85 ± 0.3	63.7 ± 0.3	54.2 ± 0.8	48.4 ± 1.3
pH 6.40 Acetic	85 ± 0.3	64.4 ± 4.0	57.0 ± 2.5	48.2 ± 0.7
pH 6.40 Citric	85 ± 0.3	64.3 ± 4.9	56.4 ± 2.3	48.5 ± 0.6
pH 6.40 Carbonic	85 ± 0.3	63.6 ± 3.7	55.6 ± 0.3	48.0 ± 0.2
pH 6.00 Acetic	85 ± 0.3	63.8 ± 5.7	57.6 ± 2.3	49.2 ± 1.9
pH 6.00 Citric	85 ± 0.3	62.7 ± 4.4	56.8 ± 1.7	49.1 ± 0.5
pH 6.00 Carbonic	85 ± 0.3	61.1 ± 1.4	57.0 ± 1.0	48.7 ± 1.2

Appendix 4. Moisture content in the samples during manufacturing of LMPS Mozzarella made from milk with milk with 2.5, 4.0, and 5.5% CN content and milled at varying pH values (pH 5.40 and pH 5.10) (n=4)

Cheese samples	Milk	Curds after whey drain	Milled curds	Cheese @ 2w
2.5% CN milled at pH 5.40	88.9 ± 0.1	62.6 ± 4.9	56.1 ± 1.1	50.0 ± 0.7
2.5% CN milled at pH 5.10	88.9 ± 0.1	62.6 ± 4.9	54.5 ± 1.6	49.9 ± 0.7
4.0% CN milled at pH 5.40	86.0 ± 0.4	60.5 ± 3.0	54.6 ± 1.2	49.2 ± 1.1
4.0% CN milled at pH 5.10	86.0 ± 0.4	60.5 ± 3.0	53.4 ± 1.1	48.2 ± 0.7
5.5% CN milled at pH 5.40	82.4 ± 0.1	55.6 ± 0.8	51.3 ± 0.9	46.6 ± 0.8
5.5% CN milled at pH 5.10	82.4 ± 0.1	55.6 ± 0.8	50.7 ± 1.2	46.5 ± 0.8