DAIRY AND SOY PROTEINS: ACID AND HEAT INDUCED INTERACTIONS

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ABSTRACT

Based on the high consumer demand for high protein foods, there is some interest and potential for the creation of soy protein fortified dairy products. However, studies that evaluated combinations of milk and soy proteins highlighted the incompatibility between these classes of proteins. The differences in structure and properties between milk and soy proteins can potentially lead to significant inhomogeneities in mixed systems, which is highly undesirable for the manufacture of high quality food products. The few studies that exist to date on milk protein-soy protein mixtures were conducted in low to moderate concentration liquid systems, and were focused primarily on the effect of temperature on the protein interactions. Very little research is available on solid milk-soy protein systems.

In the first part of this thesis work, a study was conducted to evaluate the effect of pH and temperature, two major factors that affect protein conformation, on the structure and texture of a milk-soy protein product. Mixtures of skim milk and soy protein isolate, at a total protein concentration of 4.7% and a 3:1 (w/w) milk protein to soy protein ratio, were used to prepare a cheese-like product using a hybrid cheese making and tofu making process. Four pH levels (4.6, 4.9, 5.2, 5.5) and four temperatures (65 °C, 75 °C, 85 °C and 95°C) were used. The structure, texture, color and moisture content of the resulting products were evaluated. For all samples, the structure appeared as a network of aggregated proteins. Large protein aggregates and large spacing between aggregates were observed in the pH 4.6 samples, while the pH 5.5 samples had a more homogenous structure. Samples treated at 95 °C had denser aggregates with smaller spacing than the samples in the 65 °C groups. Hardness and elasticity of the product significantly increased as pH decreased and processing temperature increased ($p<0.05$). Additionally, a significant synergistic effect of pH and temperature on the structure and mechanical properties of

the mixed soy protein-milk protein systems was observed. Significant darkening occurred for the products with higher pH and higher temperature ($p<0.05$).

The findings of this study can be used as a basis for developing a high concentration milk-soy protein network with uniform structure, based on known properties of these proteins.

The second part of this work focuses on the development of a shelf stable string cheese product. Conventionally, cheese products (except process cheese) are stored at refrigeration temperature to ensure food safety and quality. To develop a shelf stable string cheese product, two main parameters of product needed to be achieved and maintained under an anaerobic environment: low water activity (a_w <0.93) and stringiness. The work was conducted in two phases: I) development and testing of prototypes at bench-top scale; II) formulation optimization at pilot scale and shelf life study. The microbiological and physical quality of the cheese product was monitored during storage, both under refrigeration and room temperature conditions. Ultimately, a successful prototype was developed at pilot scale, and is ready for adoption at commercial scale.

BIOGRAPHICAL SKETCH

Jiai Zhang was born in Beijing, China. She finished her Bachelors degree in the field of Food Science at China Agricultural University and Purdue University. Afterwards, she came to Cornell University to obtain a Masters of Science degree in Food Science.

Her interest in agribusiness dates back to 2008, when a big scandal that involved infant formula adulterated with melamine erupted. Her strongest desire became to improve public life and wellbeing by ensuring food quality and security. She also realized the importance of access to technology and having a global vision, which are both necessary to facilitate any businesses career in the future, since she started to understand that advanced technologies and strong logistic supply linked the world and made it flat and small. Therefore, she decided to go across the Pacific Ocean to immerse myself into a new world, with diverse cultures, world-class training programs and learning opportunities. She will join Bunge as a global commodity trader, and in this role she will help move commodities around the world by promoting global trading and ultimately feeding the world.

To my parents for their love, support, patience and encouragement

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

CCP = colloidal calcium phosphate

ANOVA = analysis of variance

CN = casein

MCN = micellar casein

a_w = water activity

CHAPTER 1

INTRODUCTION

1.1 DEMAND FOR HIGH PROTEIN FOODS

According to the Food and Agricultural Organization (FAO), more than 843 million people worldwide suffer from hungry nutrient deficiencies (Wu, Fanzo, Miller, Pingali, Post, Seiner & Thalacker-Mercer, 2014). Since the population is estimated to grow from the current 7.2 billion to 9.6 billion by 2050, preventing malnutrition will become even a greater challenge (Wu et al., 2014). In this context, the demand of more nutrient dense foods, e.g. high protein foods, will rise tremendously, since protein is essential for human growth and health. However, increasing protein consumption, especially animal protein, raises concerns on sustainability, such as increased greenhouse gas emissions, overutilization of water and generation of environmental pollution (Wu et al., 2014). To reduce the environmental footprint, there is a critical need to develop new approaches to meet the critical protein consumption worldwide, i.e. to expand the consumption of plant based proteins, which are viewed as a more sustainable protein source than animal based proteins.

1.2 OPPORTUNITIES FOR MILK-SOY PRODUCTS

In recent years, consumers started to consume less animal proteins, such as meat and dairy, and more plant based proteins (Roesch & Corredig, 2005). Soy proteins are the most commonly used plant-based proteins, due to their demonstrated health benefits, as well as various functionalities in food products. Soy proteins are often used in food products such as infant formulas or meat analogues for their texture enhancing, emulsification, or moisture binding properties (MarketsandMarkets: Global Soy Protein Market Worth $9.09B by 2017,

2017). Additionally, soy proteins are added to food products to increase their protein content. Furthermore, compared to animal proteins, farming practices for obtaining soy proteins are considered more sustainable because growing soybeans has less negative impacts on the environment. This allowed marketing campaigns to build a sustainable brand image for soy products (Soy food and beverages US, 2011).

Medical and clinical data also demonstrated that soy proteins have many health benefits, such as lowering risk factors for coronary heart disease, blood pressure, triglycerides, certain cancers, etc. In 1999, the Food and Drug Administration (FDA) allowed food manufactures to claim health benefits for soy protein foods with a minimum amount of 6.25g of soy protein per serving. Soy based products that incorporate soy proteins as ingredients include soymilk, energy bars, pasta products and soy yogurt (Roesch & Corredig, 2005). Foods containing soy proteins gained a strong consumption preference in U.S. diet in 2000, with total sales reaching $4 billion in 2005, and an average annual growth rate of 2.1% (Moon, Balasubramanian, & Rimal, 2011). However, after several years of steady growth in the early 2000s, the growth of soy protein foods slowed in 2008 and declined by approximately 14% between 2008-2010. A possible explanation is that soy foods lost the competition with dairy products due to an unpleasant taste (Soy food and beverages US, 2011).

Besides having high sensory acceptability, dairy products are rich in nutrients, such as essential amino acids, vitamins and minerals (Wu et al., 2014). It is also well known that milk proteins have many health benefits, such as cancer protection and lowering the risk of heart disease. Similar to soy proteins, dairy proteins have many functionalities in food applications, such as foaming, emulsifying and gelation (Wu et al., 2014). However, production of dairy

proteins have a more significant environmental footprint than plant-based proteins. Additionally, they are more expensive and have higher infrastructure requirements (Wu et al., 2014).

Since soy proteins and milk proteins have complementary benefits including nutritional value, functionality and desirable taste, there is a high market potential of products that incorporate both soy proteins and milk proteins (Comfort, 2002; Roesch & Corredig, 2005; Beliciu, 2011).

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CHAPTER 2

RESEARCH OBJECTIVES

Based on the demand for high protein foods and the potential of creating novel products by combining soy and milk proteins, the following research objectives were formulated:

Objective I

Investigate the effect of pH and temperature on the physico-chemical properties of a milk-soy protein network

Objective II

Develop a shelf stable cheese product, with or without the incorporation of soy proteins

CHAPTER 3

BACKGROUND ON MILK AND SOY PROTEINS

3.1 THE CASEIN MICELLES COMPOSITION, STRUCTURE AND FUNCTIONALITIES

Milk proteins consist of two major types of proteins: caseins and whey proteins. Caseins are phosphoproteins that precipitate at pH 4.6 at 20 ºC, and represent approximately 82% of the true proteins in milk (Holt, Davies, & Law, 1986). The major four classes of caseins are α_{s1}-CN, α_{s2}-CN, β-caseins and κ-caseins.

Unlike other proteins that have organized repeating structure (e.g., α-helix or β-sheet), caseins have a relatively random structure, due to the high proline content in their primary structure, whose ring structure prevents extensive secondary structure formation (Fox, 2003). Additionally, all caseins lack intramolecular disulfide bonds. Because of the random structure and lacking of intramolecular disulfide bonds, caseins are very stable to high heat (Holt, 1992).

Whey proteins represent approximately 18% of the milk proteins. The two main components of whey proteins are β-lactoglobulin (β-Lg) and α-lactalbumin (α-La). Whey proteins contain ~52% β-Lg, with a molecular weight of 18,300 Da, and 20% α-La, with a molecular weight of 14,200 Da. The isoelectric pHs of β-Lg and α-La are 5.3 and 5.1 respectively (Brownlow, Cabral, Cooper, Flower, Yerdall, Polikarpov, North, & Sawyer, 1997). Unlike caseins, whey proteins are globular and highly structured, and contain a large proportion of β-structures and α-helices. Whey proteins also have a relatively high content of intramolecular disulfide bonds, which are easy to be denatured upon heating (Fox, 2003). When temperature reaches 60-65 ºC, β-Lg molecules start to dissociate from their original dimers formation. The buried thiol groups are exposed, existing intramolecular disulfide bonds are

cleaved and new intermolecular disulfide bridges are formed, until no free thiol groups are left. These disulfide bonds lead to the formation of irreversible aggregates (Verheul, Pederson, Roefs, & de Kruif, 1998, 1999; Cairoli, Iametti & Bonomi, 1994; Iametti, Cairoli, de Gregory, & Bonomi, 1996; Boye, Alli, Ismail, & Gibbs, 1995). α-La only forms aggregates when heat is applied in an acidic environment (Morr & Ha, 1993). When milk is heated to 90 ºC for 10 min, ~80% of the whey proteins (mainly alpha-lactalbumin and beta-lactoglobulin) are denatured and attach to κ-casein by disulfide bonding (Fox, 2003). Heat denaturation also promotes intermolecular hydrogen-bonded β-sheet structures (Nagano, Mori, & Nishinari, 1994).

pH also affects unfolding of whey proteins by influencing the balance of polar and non-polar residues of proteins (Boye et al., 1995). Tanford, Bunville, & Nozaki (1959) concluded that higher pH promotes β-Lg unfolding because of deprotonation/ ionization and increased exposure of the thiol group, whereas at lower pH thiol groups are buried in the β-Lg dimers (McSwiney, Singh & Campanella, 1994). However, when heat exceeds 82 ºC, whey proteins completely denature under acidic environment (pH of 2-4) (Boye et al., 1995). Additionally, pH also affects electrostatic interactions between whey proteins (Philips, Whitehead, & Kinsella, 1994). With decreasing pH, electrostatic repulsion of whey proteins decreases, resulting in random aggregates by increased physical interactions such as hydrophobic and van der Waals interactions (Elofsson, Dejmek, Paulsson, & Burling, 1997).

Casein micelles: composition, structure and properties

In milk, ~95% of caseins exist as colloidal particles known as casein micelles. Casein micelles are spherical with an average diameter ~150 nm and an average mass of ~10^8 Da (Fox, 2003). Casein micelles are large protein complexes, composed of milk salts and the four types of

caseins (α_{s1}, α_{s2}, β and κ-caseins) (Swaisgood, 2003). These caseins associate with each other by different types of bonding forces, in the ratio of α_{s1}:α_{s2}:β:κ of 4:1:4:1 (Liu & Guo, 2008). Approximately 1/3 of the casein micelle volume is occupied by caseins, and about 2/3 by hydration or porous structure. Colloidal calcium phosphate (CCP) represents ~7% of casein micelles; other minerals in the micelle structure are Mg, citrate and other species (Holt et al., 1986).

Caseins consist of hydrophobic and hydrophilic amino acid residues (Liu & Guo, 2008). However, different caseins have different characters. α_{s1}-CN contains a hydrophobic domain and a polar domain with 8 phosphorus groups (Swaisgood, 2003). Due to hydrophobic interaction, α_{s1}-CN exhibits self-association into dimer, tetramers and hexamers (Swaisgood, 2003). As temperature increases, self-association of α_{s1}-CN gets stronger. The association property of α_{s1}-CN is also strongly correlated with the pH and ionic strength of the solution. Compared to α_{s1}-CN, α_{s2}-CN is more hydrophilic; it contains 10 to 13 phosphate groups and it has inter-and intramolecular disulfide bonds. Due to the highly charged structure, caused by the phosphoryl and carboxyl groups, and low hydrophobicity, α_{s2}-CN is less self-associated than α_{s1}-CN. α_{s2}-CN are also the most sensitive caseins to calcium. β-caseins contain the most hydrophobic groups and prolyl residues among all the caseins. Since β-caseins only have one anionic cluster with 5 phosphate groups, they are less sensitive to ionic strength compared to α_{s1}-CN and α_{s2}-CN. Instead, temperature is a major factor that affect self-association of β-caseins due to their high hydrophobicity. When temperature increases, self-association of β-caseins increases because of the stronger hydrophobic interaction. As temperature is lowered, β-caseins dissociate from casein micelles. κ-caseins are amphipathic, with only one phosphate group. However, κ-caseins have some unique characteristics that make them different from other caseins. κ-caseins remain

soluble in the presence of calcium and stabilize the micelle from precipitating by forming the surface layer of the casein micelles and binding calcium (Fox, 2003). Another interesting fact is that κ-caseins create a so-called "hairy" layer on the surface of casein micelle, which creates steric repulsion between micelles. Additionally, κ-caseins contain some free sulfhydryl groups and disulfide bonds, which contribute to their association with whey proteins during heat treatment (Swaisgood, 2003; Fox, 2003; Dalgleish, 1990; Walstra, 1990; Beaulieu, Pouliot, & Pouliot, 1999).

Milk salts, especially calcium phosphate and calcium citrate found in casein micelles, are also important in maintaining casein micelles integrity (Gaucheron, 2005).

There are different models of casein micelles presented in the literature. Of these, the model of Schmidt and the one of Holt and Horne are the most used (Figure 1). In the Schmidt model, sub-micelles are linked by micellar calcium phosphate, whereas subunits are not considered in the Holt and Horne model (Schmidt, 1982; Holt & Horne, 1996). These latter authors propose caseins as rheomorphic proteins, and calcium phosphate is being mainly bound to phosphoserine, glutamate and aspartate (Gaucheron, 2005). Nevertheless, both models propose that micellar calcium phosphate is an integral part of the casein micelles.

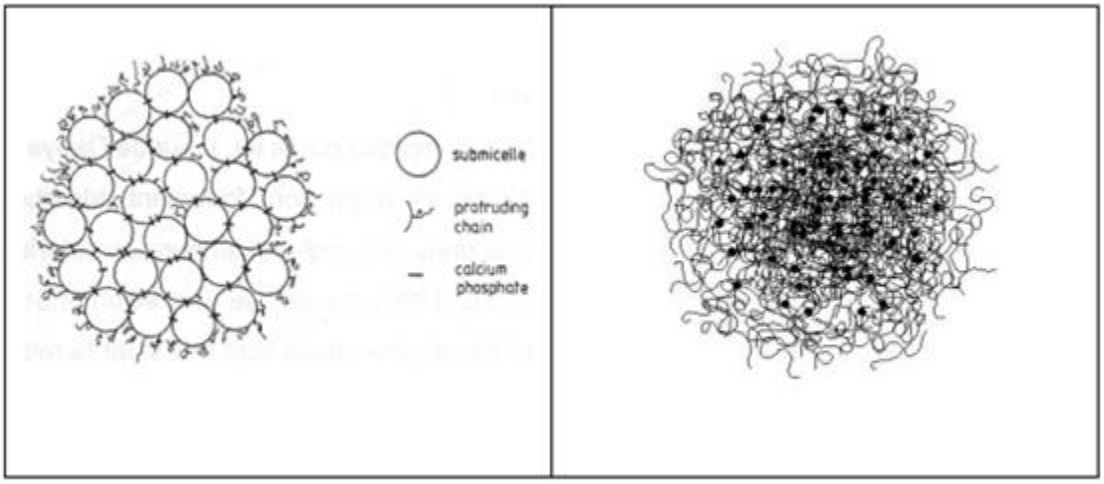

Figure 1. Models of casein micelles. Left: Model of Schmidt; Right: Model of Holt and Horne (Gaucheron, 2005).

There are three types of bonding forces that are very important for the structural integrity of the casein micelles. As mentioned previously, caseins (especially α_{s1}, β and κ) are highly hydrophobic, so hydrophobic interactions play a very important role for the casein micelle structure. Hydrophobic interactions strengthen with increasing temperature, which also strengthens micelles' structure de Kruif & Holt, 2003). The second type of bonding forces holding micelles together are protein-protein electrostatic interactions, which occur between negatively charged carboxylic acid groups and positively charged amino groups within casein molecules (Swaisgood, 2003). Protein-calcium electrostatic interactions represent another major bonding force, which occur between calcium ions (casein micelles contain approximately 20 mM calcium), and the negatively charged carboxyl and phosphate groups in the casein molecules (Gaucheron, 2005). Due to the character of these three major bonding forces, the interactions within casein micelles are affected by changes in pH and temperature (de Kruif & Holt 2003).

There are other two interactions within casein micelles, disulfide bonds and hydrogen bonds, but these forces are not as important as the previous three forces for micelle structure formation (de Kruif & Holt, 2003).

Electrostatic repulsion and steric repulsion are the two forces that stabilize casein micelles and prevent them from aggregation (Holt, 1992). Casein micelles repel each other due to a net negative surface charge of-20 mV to-30 mV at the native pH of milk. Additionally, since κ-caseins are positioned at the outside of casein micelles surface, they sterically hinder casein micelles from aggregation and coagulation (Swaisgood, 2003). During cheese making, decreasing the pH and/ or removal of a part of κ – casein molecules are the two methods that are commonly used to allow casein micelles to aggregate (Guinee, 2003). After reducing the repulsions, ionic calcium is required to create a link between casein micelles, which leads to the formation of cheese curds. Most of the serum proteins are drained out of cheese curds.

Factors that affect structure of casein micelle and the milk system

Environmental factors such as pH, temperature and salts affect casein micelle structure and stability (Figure 2).

Acid-induced changes to casein micelles in milk are irreversible. As pH is decreased, the acido-basic groups (organic and inorganic phosphate, citrate, carboxylic residues, etc.) in milk become more protonated. Due to the acidic environment, micellar calcium phosphate and citrate associated to casein micelles are dissolved. Consequently, caseins dissociate from casein micelles. When pH approaches to 5.2, the micellar calcium phosphate (inorganic) is completely dissolved whereas calcium is totally solubilized at pH 3.5 (Gaucheron, 2005). Gaucheron suggests that there is a positive correlation between solubilized calcium and solubilized inorganic

phosphate when pH decreases from 6.8 to 5.2. This result indicates that calcium ions have two types of associations, with both inorganic phosphate and organic phosphate (phosphoserine residues in casein molecules). Therefore, casein dissociation depends on the pH of milk.

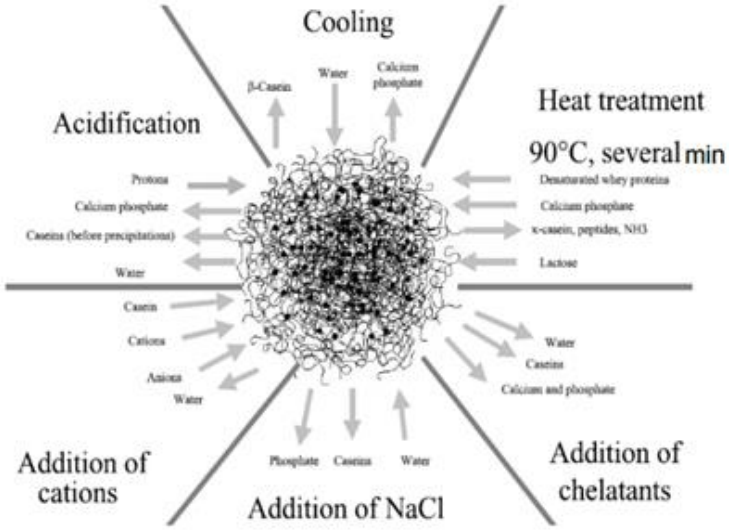

Figure 2. Effect of environmental factors on casein micelles (Gaucheron, 2005).

In fact, micelles do not dissociate when micellar calcium phosphate is dissolved, because of the reduced negative charge (Rollema, 1992). As shown in Figure 3, there is a positive correlation between pH and casein micelles size in the pH range 6 to 12, due to the increasing negative charges and electrostatic repulsion between caseins resulting in a loose structure (Liu, 2008). Casein micelles are the most compact at pH 5.5. Caseins precipitate from solution when pH approaches the casein isoelectric point (p*I*) of 4.6 (Fox, 2003).

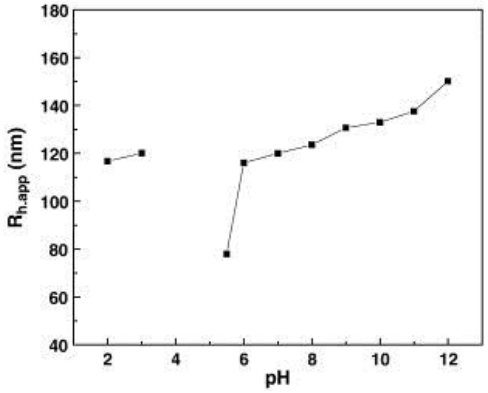

Figure 3. Relationship between casein micelle size and pH values (Liu, 2008)

Casein micelles are very stable to heat treatment. However, different extent of heat can cause various changes in milk. The reversibility of these changes is also depended on heating intensity (Gaucheron, 2005). Up to 90 ºC, changes in milk are mostly reversible (except whey protein denaturation), whereas at higher temperature most changes are irreversible (O'Connell and Fox, 2003). Major changes in milk upon heating include κ-casein dissociation, aggregation of casein micelles, dephosphorylation of caseins and decreased pH (Fox, 2003).

According to O'Connell and Fox (2003), when temperature increases, calcium previously associated with citrate precipitates as calcium phosphate, resulting in free citrate. Citrate then dissociates κ–caseins and solubilizes micellar calcium phosphate, which is important for casein micelle integrity. At the same time, high heat increases the net negative charge on the casein micelles. Consequently, micellar calcium phosphate links inside the micelles are not strong enough to maintain casein micelle integrity.

Additionally, high heat leads to dephosphorylation of caseins (hydrolysis of organic phosphate from phosphoserine), disrupting the native micellar structure, which depends on micellar calcium phosphate linkages (O'Connell and Fox, 2003). When temperature increases to 140 ºC, κ–caseins and α_{s2}-CN are likely to aggregate, presumably because of the presence of two

cysteine residues in each casein. The aggregation of κ–caseins depends on the presence of salts in milk. For instance, the higher concentration of NaCl, the more heat liable κ–caseins are (O'Connell and Fox, 2003).

During high heat treatment, pH of the milk system decreases and one of the main causes is lactose degradation. Lactose undergoes pyrolysis and is converted into organic acids, such as formic acid, acetic acid and acid (O'Connell and Fox, 2003). Another reason for pH decreasing is that primary phosphate ($(H_2PO4)_2^{-1}$) and secondary phosphate (HPO_4^{-2}) associate with calcium and precipitate as tertiary calcium phosphate (PO_4^{-3}), releasing H^+. Thus, heat-induced dephosphorylation of casein contributes to acidification of milk (O'Connell and Fox, 2003).

Conversely, the solubility of micellar calcium phosphate increases as the temperature decreases. About 10% of calcium and inorganic phosphate diffuse in a cooling milk, and β-caseins exit the micelles due to decreased hydrophobic interactions (Davies and Law, 1983; Gaucheron, 2005). This process is reversible and casein micelles can be reconstituted by warming.

Effect of pH on casein micelles: implications for cheese structure and quality

Many studies focused on the effect of pH on cheese texture (Pastorino, Hansen, & McMahon, 2003; Guinee, Feeney, Auty, & Fox, 2002; McMahon, Paulson, & Oberg, 2005; Marchesseau, Gastaldi, & Lagaude, 1997). It is well known that pH is a major factor that affects cheese texture by reducing electrostatic repulsion and increasing calcium solubilization (Pastorino et al., 2003; Lawrence, Gilles, & Creamer, 1983). However, cheese texture responds differently to decreasing pH in different pH ranges.

The correlation between casein micelles interactions and pH is not linear, due to the competing effects of calcium solubilization and acid precipitation (McMahon et al., 2005). When pH decreases from 5.5 to 5.0, calcium solubilization increases, resulting in reduced protein interactions and casein hydration, although electrostatic repulsion decreases between proteins (Pastorino et al., 2003). However, when pH decreases further to below 5.0, protein interactions increase because acid precipitation of the caseins overcomes the opposing effect of calcium solubilization, leading in an increase in protein interactions and a stronger protein network (Pastorino et al., 2003). While both calcium solubilization and acid precipitation are important factors that affect cheese texture, which factor is more predominant in depends on the pH of cheese. According to McMahon et al. (2005), at pH ranging from 5.3 to 5.6, calcium content has a major and more direct effect on cheese texture compared to the pH of cheese.

Microstructure of cheese also changes with pH. At pH 5.2, larger aggregates are formed compared to those formed at lower pH (Pastorino et al., 2003). When pH decreases below 5.0, protein aggregates are more heterogeneous, which decreases the structural uniformity of the matrix. Protein matrix is also denser at pH 5.2 compared to pH 5.7 (Pastorino et al., 2003). The increased protein density and stronger linkages result in a harder cheese texture (McMahon et al., 2005). When the protein network is weaker, it has a much more homogenous structure, with no visible folds or pockets (McMahon et al., 2005).

3.2 SOY PROTEINS: COMPOSITION, STRUCTURE AND FUNCTIONALITY

Soy proteins: composition and structure

Soy proteins are primarily represented by four types of globular proteins (90%): 2s (15%), 7S (34%), 11S (41.9%) and 15s (9.1%) globulins (Berli, Deiber, & Añón, 1999;

Shimoyamada, Tsushima, Tsuzuki, Asao, & Yamauchi, 2008; Beliciu & Moraru, 2011). Glycinin and polymers of glycinin are the main fraction of 11S and 15S, whereas β-conglycinin is the major component of 7S (Wolf, 1970; Beliciu & Moraru, 2011). Although 7S also contains γ-conglycinin, lipoxygenases, α-amylases and hemagglutinins (Nielsen, 1985), β-conglycinin and glycinin represent ~80% of the total protein in soy (Beliciu & Moraru, 2011). Therefore, these two components significantly contribute to properties of soy proteins. β-conglycinin has a molecular mass of 150-200 kDa and is a trimeric protein, mainly consisting of α, α' and β subunits (Utsumi, Matsumura, & Mori, 1997; Berli et al., 1999). Glycinin has a molecular mass of 300-380 kDa and consists of six subunits. Each of the subunit consists of acidic and basic polypeptides (Yuan, Velev, Chen, Campbell, Kaler, & Lenhoff, 2002). Both glycinin nad conglycinin have complex quaternary structure, which can be easily denatured by environmental conditions (Hermansson, 1985).

Factors that affect structure of soy proteins

pH plays an important role in soy protein structure. As the pH approaches the isoelectric point (p*I*) (4.5) (Puppo, Lupano, & Añón, 1995), soy proteins precipitate because of decreased electrostatic repulsion (Yuan et al., 2002). When pH decreases, it also promotes protein denaturation and formation of covalent (disulfide bonds) at lower temperatures (Renkema, Lakemond, Jongh, Gruppen, & Vliet, 2000; Yuan et al., 2002). According to Hermansson (1985), two types of networks are formed under different pHs. When pH is lower, coarse gel is formed by random aggregation of proteins, resulting in thick strands. When pH is higher, a fine-stranded network is formed by moderately unfolded proteins, which attach to each other as a "string of beads" (Hermansson, 1985).

The other factor that significantly affects properties of soy proteins is heat treatment. When heat is applied to soy proteins, hydrophobic areas that were buried in the native conformation become exposed to the solvent because thermal treatment weakens the bonds that maintain the secondary and tertiary protein structure (Berli et al., 1999). This leads to the aggregation of partially unfolded protein molecules. Once a critical concentration of ~7% w/w is reached, the aggregation will lead to network formation (Puppo & Añón, 1998; Berli et al., 1999; Beliciu & Moraru, 2011). Different proteins denature at various temperatures, and form different types of protein networks (Babajimopoulos, Damodaran, Rizvi, & Kinsella, 1983; Beliciu & Moraru, 2011). Glycinin starts to denature around 72 ºC and has a peak denaturation temperature of 92 ºC, whereas β-conglycinin thermally denatures between 70 ºC to 80 ºC (Shimoyamada et al., 2008; German, Damodaran, & Kinsella, 1982; Kitabatake, Tahara, & Doi, 1990). Glycinin forms gel via disulfide bonds, hydrophobic interactions and hydrogen bonds (Mori, Nakamura, & Utsumi, 1982; Shimoyamada et al., 2008). Hydrophobic interactions and hydrogen bonds are the important forces for gel formation by β-conglycinin. Without disulfide bond formation, β-conglycinin form a less rigid gel compared to glycinin (Nagano, Motohiko, Kohyama, & Nishinari, 1992). In contrast with hydrophobic interactions and hydrogen bonds, disulfide bonds are irreversible (Puppo, 1998).

Application based on soy proteins properties: Tofu

In making tofu, there are three stages involved: denaturation, aggregation and gelation (Catsimpoolas & Meyer, 1970; Schmidt, 1982; Aguilera, 1995). The denaturation process makes proteins unfold and expose their hydrophobic groups, which increases surface hydrophobicity gradually when temperature increases to 70 ºC-80 ºC (Liu, Chang, Li, & Tatsumi, 2004).

Therefore, hydrophobic interaction and hydrogen bonds are two forces that lead to the initial formation aggregates by clustering of partially denatured proteins. Once temperature exceeds 90 ºC, glycinin denatures into acid and basic polypeptides, and a gel forms through disulfide bonds (Shimoyamada et al., 2008). According to Ren (2009), the majority of aggregates are linked by disulfide bonds between basic and acidic polypeptides of glycinin, as well as hydrophobic interactions and hydrogen bonds among subunits of conglycinin and glycinin. Hydrophobicity increases dramatically when higher temperatures are applied (90 ºC to 100 ºC). When temperature decreases, denatured proteins, such as glycinin subunits, become associated or partially refolded because of their decreased surface hydrophobicity (Shimoyamada et al., 2008).

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CHAPTER 4

EFFECT OF PH AND TEMPERATURE ON THE STRUCTURE AND TEXTURE OF A MILK-SOY PRODUCT[1]

Abstract:

In this study, the effect of pH and temperature on the structure and texture a milk-soy protein product was investigated. Mixtures of skim milk and soy protein isolate, at a total protein concentration of 4.7% and a 3:1 (w/w) milk protein to soy protein ratio, were used to prepare a cheese-like product using a hybrid cheese making and tofu making process. Four pH levels (4.6, 4.9, 5.2, 5.5) and four temperatures (65 °C, 75 °C, 85 °C and 95°C) were used. The structure, texture, color and moisture content of the resulting products was evaluated. Hardness and elasticity of the product significantly increased as pH decreased and processing temperature increased ($p<0.05$). For all samples, the structure appeared as a network of aggregated proteins. Large protein aggregates and large spacing between aggregates were observed in the pH 4.6 samples, while the pH 5.5 samples had a more homogenous structure. Samples treated at 95 °C had denser aggregates with smaller spacing than the 65 °C groups. Significant darkening ($p<0.05$) was observed for the products with higher pH treated at higher temperature, due to Maillard reaction. These findings can be used as a basis for developing milk-soy cheese-like products.

[1] *This chapter will be submitted to publication under the title:*

"Effect of pH and temperature on the structure and texture of a milk -soy product". Authors: Jiai Zhang, Juan D. López, and Carmen I. Moraru

4.1. Introduction

In recent years, consumers became increasingly interested in plant based proteins (Roesch & Corredig, 2005). After the U.S. Food and Drug Administration (FDA) allowed manufactures to claim health benefits for foods with a minimum amount of 6.25 g of soy protein per serving, foods containing soy proteins saw a strong growth in consumption in the U.S. (Moon, Balasubramanian, & Rimal, 2011). In this context, there is interest and potential for the creation of soy protein fortified dairy products (Beliciu & Moraru, 2011; Comfort & Howell, 2002; Roesch & Corredig, 2005). Some examples of soy protein fortified dairy products include yogurts, coffee creamers and whipped toppings (Comfort & Howell, 2002). However, studies that evaluated combinations of milk and soy proteins highlighted the incompatibility between these classes of proteins, particularly between casein and soy proteins, mostly due to their different structure and properties (Beliciu & Moraru, 2011; 2013).

Milk contains two major proteins: caseins, which represent approximately 82% of the true protein in milk, and whey (serum) proteins. Caseins are phosphoproteins that precipitate at pH 4.6 and 20 ºC (Holt, Davies, & Law, 1986), and have a relatively random structure due to the high content of ring-structured proline in their primary structure (Fox, 2003). In milk, about 95% of caseins exist as colloidal casein micelles, with an average diameter of about 150 nm (Fox, 2003). Casein micelles are large protein complexes, composed of α_{s1}, α_{s2}, β and κ-caseins, held together by hydrophobic bonds and ionic bonds facilitated by colloidal calcium phosphate (Swaisgood, 2003). κ-caseins are preferentially located on the outside of casein micelles and, due to their negatively charged side groups, create both steric and electrostatic repulsion between micelles. κ-caseins contain some free sulfhydryl groups and disulfide bonds, which mediate the formation of casein-whey protein complexes during heat treatment (Swaisgood, 2003; Fox,

2003; Dalgleish, 1990; Beaulieu, Pouliot, & Pouliot, 1999). Casein micelles are very stable to high heat (Holt, 1992), but precipitate as pH reaches an isoelectric point of 4.6 (Lucey & Singh, 2003).

Whey proteins represent about 18% of the milk proteins, and are mostly represented by *β*-lactoglobulin (*β*-Lg), with a molecular weight of 18,300 Da, and *α*-lactalbumin (*α*-La), with a molecular weight of 14,200 Da. The p*I* values of *β*-Lg and α-La are 5.3 and 5.1 respectively (Brownlow, Cabral, Cooper, Flower, Yerdall, Polikarpov, North, & Sawyer, 1997). Unlike casein micelles, whey proteins are globular and highly structured. They are also sensitive to heat because of a relatively high content of intramolecular disulfide bonds, which can easily denature upon heating (Fox, 2003). *β*-Lg starts to denature at 60-65 ºC (Iametti, Cairoli, de Gregory, & Bonomi, 1996; Verheul, Pederson, Roefs, & de Kruif, 1999). When milk is heated to 90 ºC for 10 min, about 80% of the whey proteins can be denatured and attach to κ-casein via disulfide bonds (Fox, 2003). Similar to casein micelles, pH affects whey protein interactions by changing electrostatic interactions (Philips, Whitehead, & Kinsella, 1994).

Soy proteins are primarily globular proteins (90%), of four different types: 2S (15%), 7S (34%), 11S (41.9%) and 15S (9.1%) (Berli, Deiber, & Añón, 1999; Shimoyamada, Tsushima, Tsuzuki, Asao, & Yamauchi, 2008). Glycinin and polymers of glycinin are the main fraction of 11S and 15S, whereas β-conglycinin is the major component of 7S (Wolf, 1970). β-conglycinin and glycinin represent ~80% of the total protein in soy (Beliciu & Moraru, 2011), and thus these two components significantly contribute to the overall properties of soy proteins. β-conglycinin, with a molecular mass of 150-200 kDa, is a trimeric protein mainly consisting of α, α' and β subunits (Utsumi, Matsumura, & Mori, 1997; Berli et al., 1999) while glycinin, with a molecular mass of 300-380 kDa consists of six subunits (Yuan, Velev, Chen, Campbell, Kaler, & Lenhoff,

2002). Both glycinin and conglycinin have complex quaternary structure, which can be easily denatured by environmental conditions (Hermansson, 1985). As pH approaches the p*I* of 4.5, soy proteins precipitate (Puppo, Lupano, & Añón, 1995; Yuan et al., 2002). When pH decreases, besides decreasing the electrostatic repulsion among soy proteins, it also promotes protein denaturation and formation of covalent bonds at lower temperatures (Renkema, Lakemond, Jongh, Gruppen, & Vliet, 2000; Yuan et al., 2002). Heat treatment affects soy proteins significantly. Upon heating, the bonds that maintain their secondary and tertiary structure weaken, and the hydrophobic areas buried in the native conformation become exposed to the solvent (Berli et al., 1999). This facilitates aggregation of partially unfolded protein molecules. Glycinin has a denaturation temperature of 92 ºC, whereas β-conglycinin thermally denatures between 70 ºC to 80 ºC (Shimoyamada et al., 2008; German, Damodaran, & Kinsella, 1982; Kitabatake, Tahara, & Doi, 1990). Aggregation of glycinin occurs mainly via disulfide bonds, hydrophobic interactions and hydrogen bonds, while β-conglycinin creates a network by hydrophobic interactions and hydrogen bonds (Mori, Nakamura, & Utsumi, 1982; Shimoyamada et al., 2008).

The differences in structure and properties between milk and soy proteins can lead to significant inhomogeneities in mixed systems, which is highly undesirable for food product manufacture. The few studies that exist to date on milk protein-soy protein mixtures were conducted in low to moderate concentration liquid systems, and focused primarily on the effect of temperature on the protein interactions (Beliciu & Moraru, 2011, 2013). Very little research is available on solid milk-soy protein systems (Roesch & Corredig, 2005). To fill this knowledge gap, the objective of this study was to investigate the effect of pH and temperature on the microstructure and texture of a solid milk-soy protein matrix, obtained using a hybrid cheese

making – tofu making process. The findings of this study can be used as a basis for the development of mixed or dairy-soy food products.

4.2. Materials and Methods:

4.2.1. Materials

Soy protein isolate (The Sausage Maker, Inc; Buffalo, N.Y.) was used as a source of soy proteins. The soy protein isolate contained 87.75 g / 100 g (dry weight) protein, 4 g / 100 g (dry weight) fat, 4 g /100 g ash (dry weight) ash and 4.42 g /100 g (dry weight) moisture. Commercial, HTST pasteurized skim milk was obtained from Cornell Dairy (Ithaca, NY). A mixture of acetic acid (distilled white vinegar, 5% acidity, Heinz; Pittsburgh, PA) and lactic acid (88%, LD Carlson Company; Kent, OH) at a ratio of 4:1, v/v, were used to acidify the samples. Japanese Nigari, consisting of Magnesium Chloride (Handy Pantry, Japan) was used as a coagulant in tofu making. Granulated iodized salt (Morton; Silver Springs, NY) was also used.

4.2.2. Milk-soy protein product manufacture

In order to obtain a 3:1 (w/w) ratio of milk protein to soy protein, 21 g soy protein isolate was added to 1450 mL skim milk, which resulted in a total protein concentration of the mix of 4.7 g/ 100g. Two heating steps were conducted. The first heating step consisted in preheating the liquid mixture to 55 ºC on a hot plate, followed by heating to 85 ºC with stirring in the heating bowl of a KitchenAid mixer (Benton Harbor, MI), for 30 min. A Japanese Nigari solution of 0.05 mol/L concentration was obtained by dissolving 7.13 g Nigari in 65 mL hot water. The Nigari solution was added to the milk-soy protein mixture, at 85 ºC. The mixture was immediately acidified to the desired pH level (5.5, 5.2, 4.9 and 4.6) by adding a mixture of acetic acid and lactic acid, in a ratio of 4:1. This acid mixture is used commercially for the manufacture of

cheese by direct acidification. The acidified mixture was held at 55 ºC for 20 min to induce aggregation and coagulation. The obtained curds were drained by gravity using a tofu maker (Yamako Tofu Maker Kit; Japan). Salt (1.5 g / 100g w/w) was added to the curds and mixed well. After that, a second heating step was conducted, at four temperature levels. To achieve this, the curds were immersed in hot brine (2.5 g salt/ 100g w/w) at different temperatures (65 °C, 75 °C, 85 °C and 95°C), for 10 min. After this heat treatment, samples were drained, cooled and equilibrated for one day under refrigeration. After one day, measurements of texture, color, microstructure and moisture content and pH were conducted for all the samples. The study was repeated three times.

4.2.3. Sample characterization

Hardness and elasticity of samples were measured using a texture analyzer (TAXT.Plus, Texture Technologies Corp., Scarsdale, N.Y.). Samples were cut into 25 mm × 25 mm pieces of 15 mm thickness. A penetration test was conducted, using a 5 kg load cell, a stainless steel cylinder probe of 6 mm diameter, at a speed of 1 mm/sec and a penetration distance of 5 mm, at 20 °C.

Color was measured using a Minolta CR-400/410 Chroma-meter (Minolta, Japan) in the Hunter (L, a, b) color system. Calibration was conducted immediately before the measurements with a white standard tile with illuminant D65 (Y = 93.3, x = 0.3161 and y = 0.3328). The moisture content of the samples was determined using an IR-30 Moisture Analyzer (Denver Instrument Company, Bohemia, NY). The samples' pH was measured using a pH meter equipped with a stainless steel probe (HACH COMPANY, Loveland, CO). Two-point calibration (at pH 7.00 and pH 4.01) was performed immediately before measurements.

The texture, color, moisture and pH measurements were conducted in triplicate.

4.2.4. Scanning Electron Microscopy (SEM)

The microstructure of the samples processed under extreme conditions were visualized using a Zeiss LEO 1550 field emission SEM (Carl Zeiss Microscopy, Jena, Germany), using the procedure described by Feng, Cheng, Wang, Hsu, Feliz, Borca-Tasciuc, Worobo, & Moraru (2014). Cheese samples were sliced into 2 mm x 2 mm thin pieces (thickness < 1 mm), which were mounted horizontally onto microscopic coverslips. Samples were rinsed in cacodylate buffer three times, 5 min each time, then subjected to a secondary fixation using 1% (w/v) osmium tetroxide in cacodylate buffer, for 1 h. The fixated samples were rinsed in cacodylate buffer three times, then dehydrated using ethanol solutions of 25% (v/v), 50%, 70%, 95%, 100% and 100% for 10 min each, followed by critical point-drying with carbon dioxide. Dried surfaces were coated with gold-palladium alloy. Images were acquired using the SmartSEM software AT 500 x magnification (Carl Zeiss Microscopy, Jena, Germany).

4.2.5. Statistical analysis of data

Means were calculated from triplicate values of the measured parameters and analyzed using the software JMP Pro 12 (Cary, NC). Two-way ANOVA with an interaction effect was conducted to determine significant differences in experimental results ($p<0.05$). The Tukey-Kramer HSD (honestly significant difference) test was conducted to compare the differences between means.

4.3. Results and Discussion

4.3.1. Microstructure of the milk-soy products

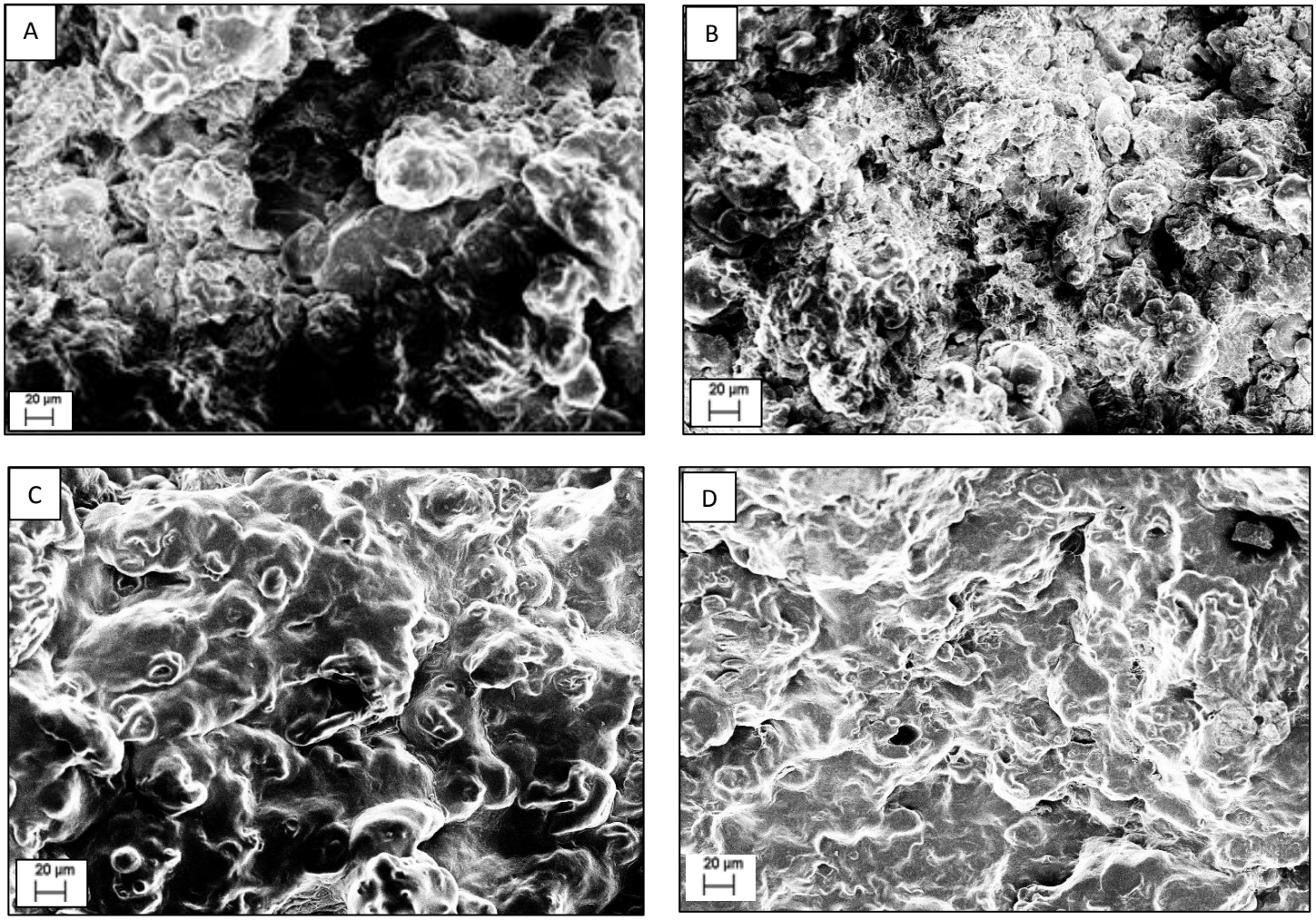


Figure 4.1. Micrographs of samples processed at A: pH 4.6 & 65 °C; B: pH 4.6 & 95 °C; C: pH 5.5 & 65 °C; D: pH 5.5 and 95 °C

The microstructure of the milk-soy products was evaluated by SEM for four extreme conditions, namely the combination of pH 4.6 and 5.5 with temperatures of 65 °C and 95 °C. As seen in Figure 4.1, the pH 4.6 samples featured a highly aggregated structure, with significant pockets between aggregates, whereas the pH 5.5 samples had a more homogenous, continuous structure. Additionally, when comparing Fig. 4.1.A and Fig. 4.1.C to Fig. 4.1.B and Fig. 4.1.D, respectively, it can be observed that samples treated at 95 °C had a more dense protein structure, with smaller spacing between protein aggregates, than the 65 °C group. Direct visual observation of the samples also confirmed that the lower pH samples had a denser structure, and appeared coarser and stiffer than the pH 5.5 samples, which had a smoother, more uniform, softer structure.

The microstructure of the mixed milk-soy protein products obtained here is similar to the microstructure of soy protein networks reported before (Puppo et al., 1995; Puppo & Añón, 1998). According to Hermansson (1985), the type of soy protein network formed depends on pH. At low pH, a coarse gel is formed by random aggregation of proteins into thick strands. At higher pH, a fine-stranded network is formed by moderately unfolded proteins, which attach to each other as a "string of beads" (Hermansson, 1985).

In acidified milk protein networks (cheese), the network structure also becomes more heterogeneous with decreasing pH, at pH below 5.0, due to stronger protein-protein (mainly casein) interactions (McMahon, 2005). According to McMahon (2005), the strength of the casein network depends mostly on calcium content rather than pH value in less acidic environments, at pH above 5.0. This complex correlation between pH and casein-casein interactions is due to the competing effect of calcium solubilization and acid precipitation

(Pastorino, Hansen, & McMahon, 2003). When pH decreases from 5.5 to 5.0, calcium solubilization increases, which reduces protein interactions and increases casein hydration, although electrostatic repulsion decreases between proteins (Pastorino et al., 2003). However, when pH decreases below 5.0, protein interactions strengthen because acid precipitation of the caseins overcomes the opposing effect of calcium solubilization, leading to a stronger protein network (Pastorino et al., 2003).

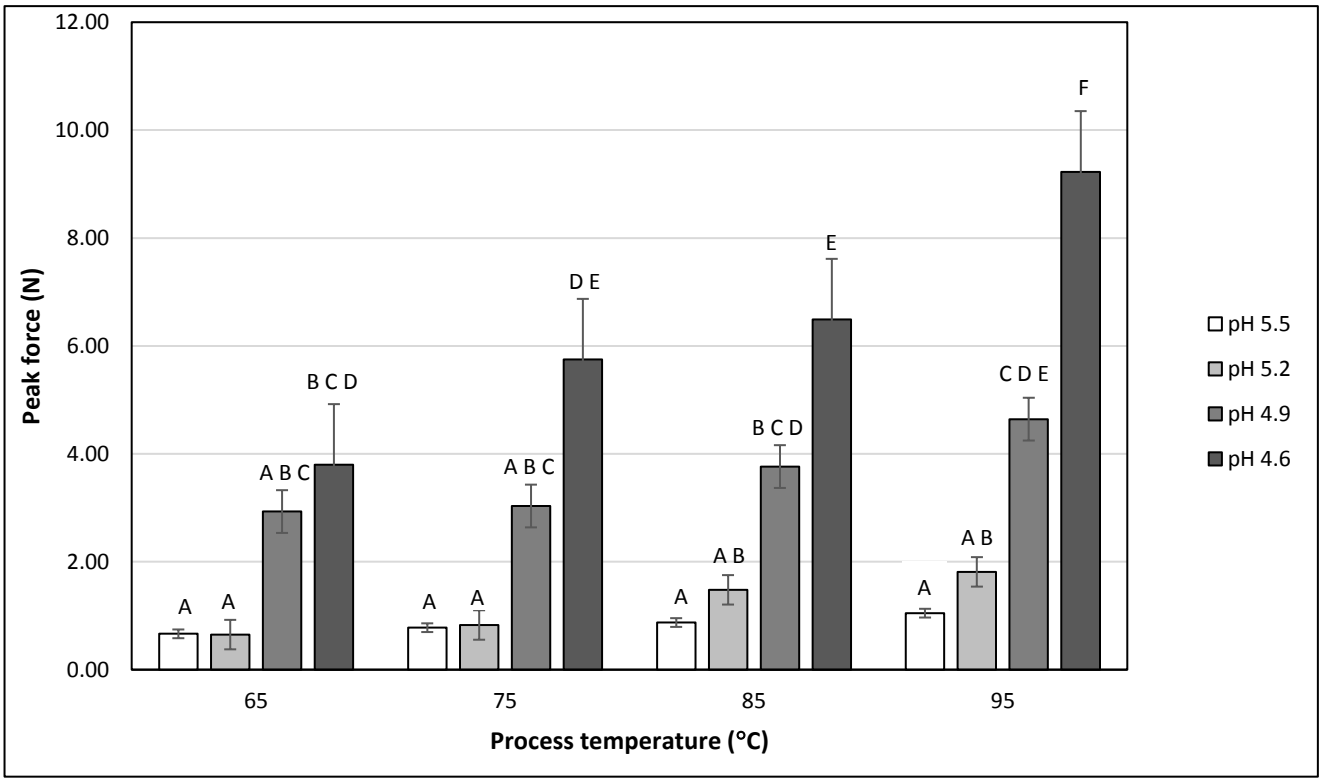


Figure 4.2. Effect of pH and temperature on the peak force of the milk-soy protein network

4.3.2. Texture of the milk-soy protein products

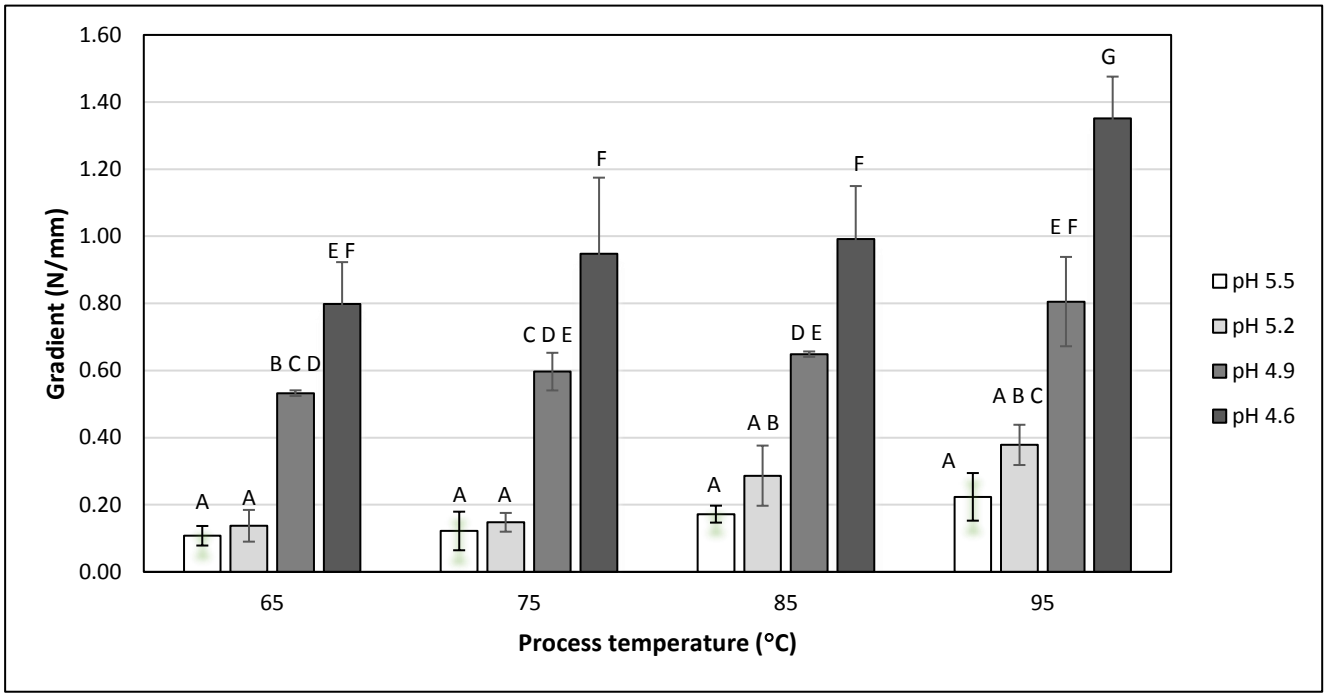


Figure 4.3. Effect of pH and temperature on the elasticity of the milk-soy protein network

Instrumental texture analyses confirmed the visual and structural observations described above. Sample hardness was expressed by the peak force of the force-distance curves, and elasticity was expressed by the gradient of the initial linear portion of the force-distance curves. Both hardness and elasticity of the milk-soy protein products increased when pH decreased and process temperature increased. As seen in Fig.4.2 and Fig. 4.3, samples in the high pH group (5.5 and 5.2) had similar mechanical properties, but some differences existed between the samples in the low pH group (4.6 and 4.9). For samples of pH 4.6, peak force values ranged between 3.80 N and 9.23 N when process temperatures increased from 65 °C to 95 °C, respectively. For samples treated at 95 °C, the peak force values ranged between 1.05 N (at pH 5.5) to 9.23 N (at pH 4.6). Samples produced at lowest pH (4.6) and highest temperature (95 ºC) had the highest hardness (9.23 N) and elasticity (1.30 N/mm). Statistical analyses indicated significant differences in

hardness and elasticity among samples ($p<0.05$), especially for the lower pH (pH 4.6 and 4.9) and higher temperature (95 °C) levels.

Additionally, pH and temperature had a significant synergistic effect on the mixed protein network texture ($p<0.05$). Lowering pH decreases surface charge of proteins, thus decreasing electrostatic repulsion among soy proteins, caseins and whey proteins present in this complex system. When the net surface charge of protein molecules decreases due to protonation, protein molecules come closer together, which promotes intermolecular disulfide interchange reactions and hydrophobic interactions (Liu & Guo, 2008; Puppo & Añón, 1998). Consequently, a stronger protein network and a more rigid structure are formed. Additionally, decreasing pH promotes casein dissociation, denaturation of globular (soy and whey) proteins, as well as intermolecular bonds and insoluble aggregates formation in soy proteins (Mauri & Añón, 2006; Nagano et al., 1994; Ryan, McEvoy, Duignan, Crowley, Fenelon, O'Callaghan, & FitzGerald, 2008; Gaucheron, 2005; Catsimpoolas & Meyer, 1970; Nagano et al., 1994; Renkema et al., 2000; Mohamed & Xu, 2003; O'Connell & Fox, 2003). It is also worth noting that samples produced at lower pH levels (4.6 and 4.9) have more distinct results on texture because these two pHs are near p*I*s of major proteins in the system.

Heat treatment is the other critical factor that affects protein structure significantly. In this work, heat treatment was applied in two stages: 1) an initial heat treatment of the liquid protein mixture at 85 °C, followed by 2) a heat treatment of the protein aggregates (curds) at four temperature levels.

The first heating step was used to promote both mixing and denaturation of the globular (whey and soy) proteins (Nagano et al., 1994; Fox, 2003; Liu et al., 2004; Ryan et al., 2008). Additionally, this treatment favored the formation of a casein-whey protein complex, by

disulfide bonding of the denatured β-lactoglobulin and the κ-casein on the outside of casein micelles (Fox, 2003). The second heat treatment was primarily used to promote a stronger protein network, by promoting stronger hydrophobic interactions for both milk proteins and soy proteins (Holt, 1992; Shimoyamada et al., 2008). It has been shown before that at temperatures between 75 °C to 90 °C, hydrophobic interactions, hydrogen bonds and electrostatic interactions between glycinin and β-conglycinin are major forces to create the network by clustering partially denatured proteins (Nagano, Motohiko, Kohyama, & Nishinari, 1992; Babajimopoulos, et al., 1983; Catsimpoolas & Meyer, 1970; Berli et al., 1999; Liu et al., 2004). Based on previous research, it is hypothesized that this secondary heating, particularly at 85 °C and 95 °C, resulted in additional globular protein unfolding and exposure of – SH, S-S, and hydrophobic amino acid side chains (Shimoyamada et al., 2008; Ren, Tang, Zhang, & Guo, 2009; Ren et al., 2009).

Overall, it can be assumed that when the milk-soy curds were subjected to heating temperatures of 65 °C to 85 °C, network formation was dominated by hydrophobic interactions, hydrogen bonds and electrostatic interactions, which are physical bonds that result in a weak protein network. When the product was heated at 95 °C, covalent disulfide bonds likely formed, mostly by soy proteins, but also possibly between soy proteins and whey proteins, as well as between caseins and whey proteins (Roesch & Corredig, 2005). These bonds are irreversible and strong (Renkema et al., 2000), which explains why samples treated at 95 °C had a stronger structure than those treated at lower temperatures.

The observed synergistic effect of pH and temperature on the mixed protein system can also be explained based on previous research. Nagano et al. (1994) stated that both pH and temperature significantly affect texture due to protein denaturation and bonds formation, while Ryan et al. (2008) have shown that when pH approaches p*I*, proteins are less stable to heat.

Furthermore, some studies showed that decreased pH favors formation of covalent (disulfide) and noncovalent (hydrophobic, van der Waals) interactions in soy proteins at lower temperatures (Ryan et al., 2008b; Yuan et al., 2002; Hermansson, 1978).

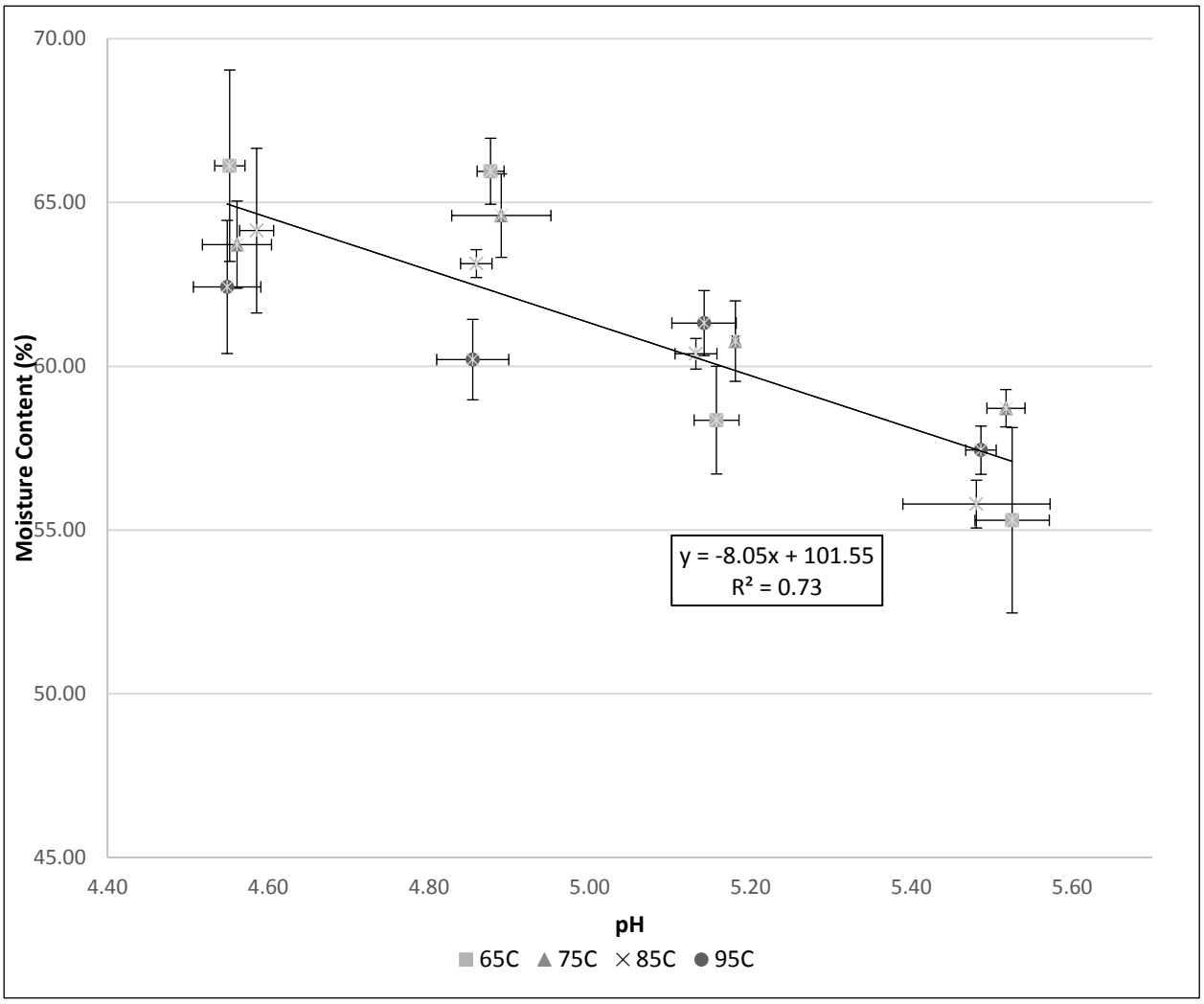


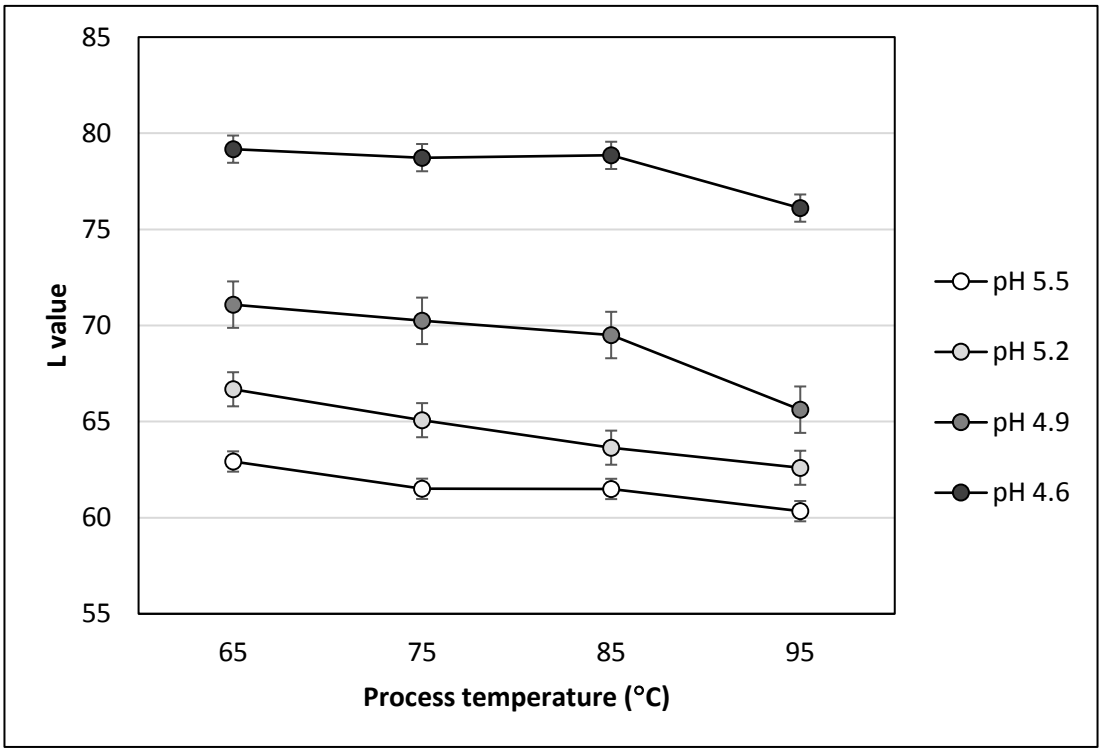
Figure 4.4 pH vs. moisture content for the milk-soy samples

An additional factor that could have affected the mechanical properties of the mixed product is moisture content. There was a concern that samples treated at the highest temperatures may have dehydrated more compared to those treated at lower temperatures, which could have resulted in higher hardness and elasticity. However, no correlation was found between process

temperature and moisture content. Somewhat surprisingly though, a statistically significant correlation was found between sample pH and moisture content ($p<0.05$). Specifically, as pH decreased, moisture content increased (Figure 4.4). This result can be explained by the significant synergistic effect between pH and temperature ($p<0.05$). A higher degree of protein denaturation in the lower pH samples resulted in higher exposure to free water of carbonyl and amino groups upon heating (Puppo & Añón, 1998). This could have facilitated the formation of hydrogen bonds between the denatured protein chains and water, thus increasing the amount of moisture retained in the protein network.

4.3.3. Effect of temperature and pH on the color of the milk-soy products

Figure 4.5. Effect of pH and process temperature on luminosity (L) of the milk-soy protein network



The L color parameter differed significantly among the different pH and temperature levels ($p<0.05$), with samples produced at highest pH and highest temperature being the darkest

(lowest L value) (Figure 4.5). This can be attributed to Maillard browning, which is known to be favored by higher temperature and more alkaline environment (Martins, Jongen, & Boekel, 2001; O'Connell and Fox, 2003).

4.3.4. Hypothesized milk-soy protein network formation

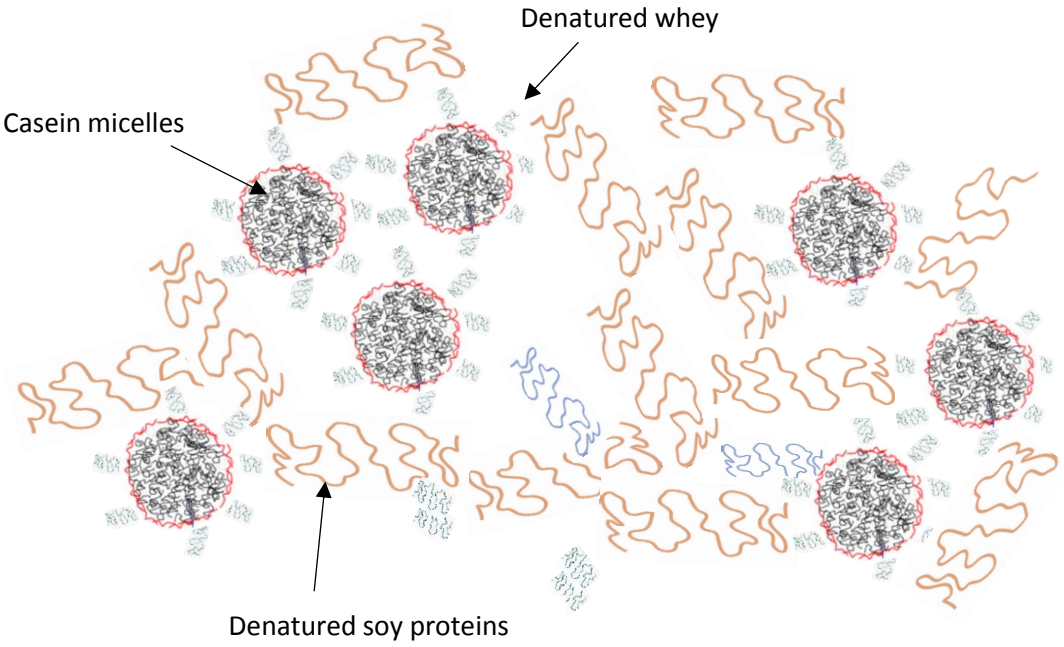


Figure 4.6. Hypothesized mixed soy protein – milk protein network structure formed under low pH and high temperature conditions

Based on the data presented above and previous research, it can be hypothesized that the protein network was mainly formed based on casein-whey protein interactions, soy-soy proteins interactions and soy-whey protein interactions. A schematic representation of this proposed structure is shown in Fig.4.6. During the first heating step, whey proteins attached to casein micelles, while soy proteins were partially denatured and interacted with each other. During the second heating step, particularly when temperature exceeded 95 °C, whey and soy proteins likely formed network stabilized primarily by disulfide, covalent bonds. Beliciu & Moraru's (2011)

suggested that heating did not result in direct interactions between casein and soy proteins. Under the heating and acidification conditions used in the current study, it appears that whey proteins acted as binder between soy proteins and casein micelles, which can alleviate the problem of incompatibility and immiscibility between soy proteins and casein.

4.4.Conclusions

By manipulating pH and temperature, two of the factors that have a significant impact on protein structure and properties, it is possible to create relatively homogeneous mixed milk protein – soy protein products that have characteristics between a cheese product and tofu. Specifically, lower pH values favored formation of a stronger protein network, with a somewhat inhomogeneous microstructure and large pockets; higher temperatures resulted in a denser protein network. Samples produced at lowest pH (4.6) and highest temperature (95 °C) had the highest hardness and elasticity, presumably due to strong covalent and noncovalent protein-protein interactions. Additionally, there was a significant synergistic effect of pH and temperature on the structure and texture of the mixed soy protein – milk protein systems. The findings of this study can be used as a basis for the development of mixed soy-dairy cheese-like products of desired structure and textural properties.

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CHAPTER 5

DEVELOPMENT OF A SHELF STABLE CHEESE PRODUCT[2]

5.1. Abstract

A shelf stable, vacuum packed string cheese product was developed at pilot scale, by adapting a traditional string cheese process. In order to prevent growth of *Clostridium botulinum*, the water activity of the product was maintained below 0.93, the pH below 5.2, and the antimicrobial nisin was incorporated into the cheese product. To lower water activity, salt (NaCl) and a humectant, glycerol, were incorporated into the cheese product. Parameters of traditional string cheese processing were adapted to further decrease water activity. To improve stringiness of the cheese product, stretching of the curd in a mechanical Mozzarella stretcher was conducted at high temperature. A natamycin surface dip was applied before packing to control the growth of yeasts and molds. The Additional recommendations were made to ensure safety of cheese product, especially the use of hygienic processing conditions to prevent contamination with *Staphylococcus aureus*. The microbiological and physical quality of the cheese product were monitored during storage, both under refrigeration and room temperature conditions.

5.2. Introduction

A shelf stable cheese product with taste and texture similar to string cheese was developed, as described in this paper.

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[2] *The work described in this chapter was part of a project funded by Yili Co. A patent application will be submitted for this work and product.*

Conventionally, cheese products-except process cheese-are stored at refrigeration temperatures to ensure food safety and quality. For string cheese, refrigerated storage is also important for maintaining the stringy texture. The main objective of the project was to develop a shelf stable cheese product with stringy texture. The approach to achieve this goal was to control the water activity (a_w <0.93) and pH value (~5.15) of the product, by using certain approved food additives (humectants and antimicrobials) and manipulating processing parameters used in traditional Mozzarella production (Gustavo, 2008; FDA, 2015; Cliver, 1990; FDA, 2015).
The work was conducted in two phases: I) Development and testing of prototypes at bench-top scale; II) Formulation optimization at pilot scale and shelf life study. The main technical challenge associated with the development of a shelf stable cheese product with a stringy texture was to achieve the texture while ensuring shelf life stability. Systematic work has been conducted at both lab and pilot plant scale to develop and optimize several formulations and processing procedures. All the solutions developed were designed so that they can be implemented at industrial scale. The final composition (%protein, %fat, %moisture content) of the product was determined. The following parameters of the developed prototypes were tested weekly for three months (12 weeks), both under refrigeration and room temperature: water activity, pH, stringy texture and product spoilage (by visual observation), microbiological parameters (coliforms, yeasts and molds).

5.3. Materials and methodology

5.3.1. Materials

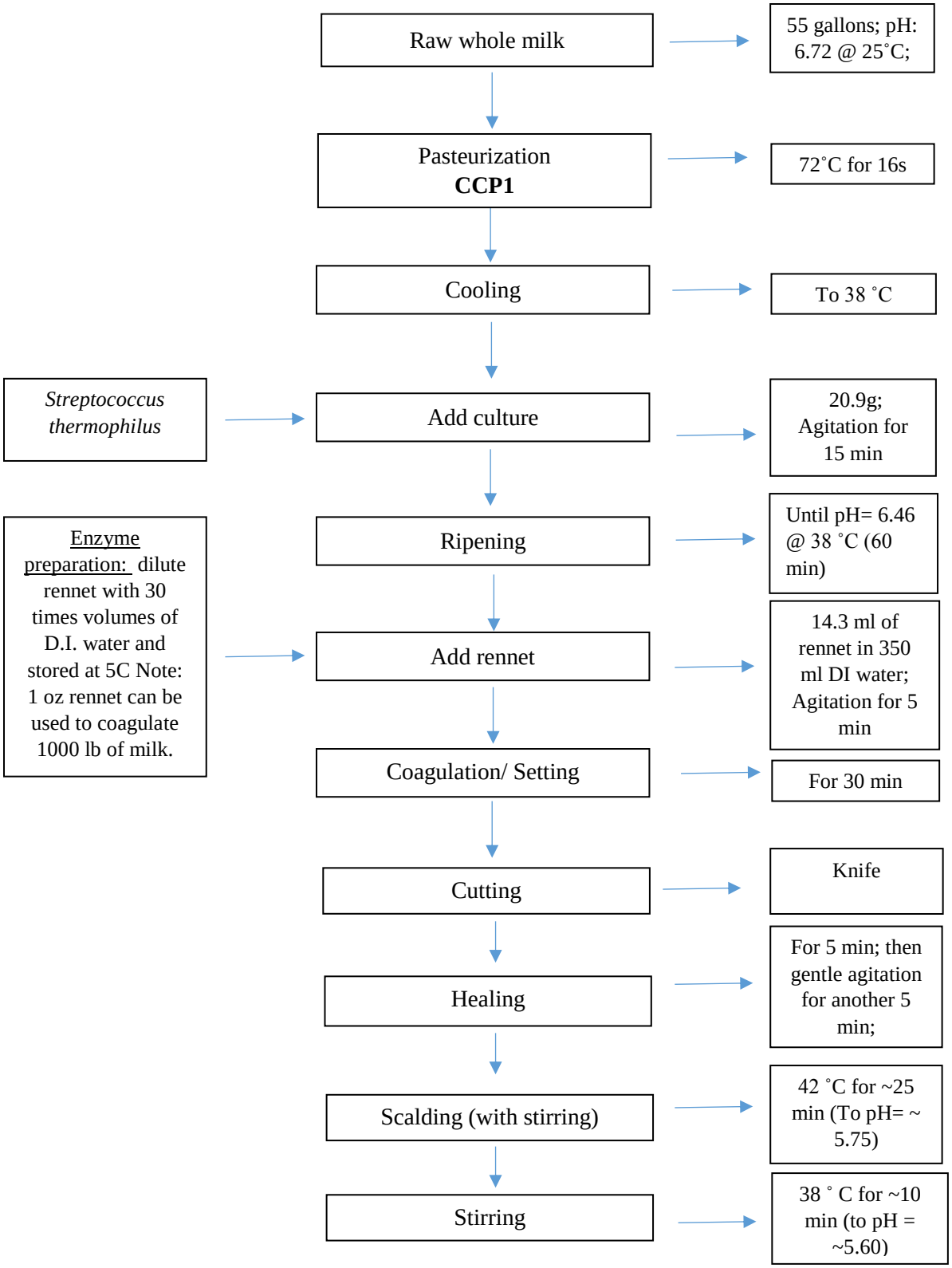
Raw, unhomogenized whole milk (Butter fat 3.93%; protein 3.12%; Standard plate count 1000 cfu /ml; Somatic cell count 220,000; pH 6.72; Cornell Dairy, Ithaca, NY) was used to make

shelf stable string cheese. Rennet (Liquid, CHY-MAX Plus, Chr. Hansen; Milwaukee, WI) and starter culture (Streptococcus *thermophiles*, Frozen DVS, ST-M5; Chr. Hansen; Milwaukee, WI) were added to coagulate milk and form cheese curds. Salt (NaCl; Morton; Silver Springs, NY) and glycerol (Essential Depot; Sebring, FL) were used to decrease water activity of final product. Nisin (niprosin$^{(TM)}$; ProFood International, Inc; Naperville, IL) and natamycin (natachloride$^{(TM)}$; ProFood International, Inc; Naperville, IL) were added to prevent growth of Clostridium *botulinum* and yeast and molds respectively.

5.3.2.1. Shelf stable string cheese production

55 gallons of raw whole milk was pasteurized at 72˚C for 16s. Pasteurized milk was cooled down to 38 ˚C in a cheese vat (Kusel equipment company, Watertown, WI). 20.9g of starter culture was added to milk at 38 ˚C with agitation for 15 minutes. Cultured milk ripened for approximate 60 minutes until pH reached 6.46. After ripening, 14.3ml of rennet was diluted in 350 ml DI water and was added into milk with agitation for 5 minutes. Then milk was set to coagulate for 30 minutes. Upon the ripening and coagulation, cheese vat was covered by plastic lid to keep the temperature at 38 ˚C. Coagulated milk was cut by a knife when a clear cut can be achieved. After cutting, milk was healed for 5 minutes to maintain fat content in the system and gentle agitation was conducted for another 5 minutes. After agitation, scalding was applied to the cut coagulated milk at 42 ˚C for approximate 25 minutes with stirring until pH of repelled whey reached 5.75. When pH was 5.75, temperature of cheese curds was dropped back to 38 ˚C in the cheese vat. Constant stirring was conducted at 38 ˚C for approximate 10 minutes. When pH of repelled whey reached 5.60, we started to drain the whey from cheese vat with stirring. Draining took about 20 minutes. Curds were weighed when the pH of the curds reached 5.35. After weighing, 7% salt was added to cheese curds with stirring at 38 ˚C. Salting takes approximate 15

minutes. Final pH of cheese curds were around 5.10. Curds were drained further with cheese cloth and were transferred to a steam kettle (FT100 Kettle, Direct Steam; Groen; Chicago, IL). The curds were heated up to 55 ˚C in the steam kettle. 0.085% Nisin and 8% glycerol were dissolved in 300ml DI water, heated to 60 ˚C and added to heated curds prior stretching. Mozzarella stretcher (double extruder) with a die (cone shape with two diameters at 2 cm and 5cm of each end) was set at 85 ˚C at half speed at the beginning of production. A gearmotor (FAF40D14BD780N4; SEW – EURODRIVE, INC.; Bruchsal, Germany) was installed behind the stretcher as power to push cheese curds through the die. Once products were coming out of the die, speed of the stretcher was slowed down. Natamycin dip was made at concentration 10g/4L. Cheese products were dipped in Natamycin dip and were dried on stainless steel perforated trays covered by cheesecloth. Samples were vacuum packed (C450, Packaging machine; MULTIVAC) after drying. See process flow chart below (Figure 5.1).


Raw whole milk
55 gallons; pH: 6.72 @ 25˚C;
Pasteurization
CCP1
72˚C for 16s
Cooling
To 38 ˚C
Streptococcus thermophilus
Add culture
20.9g; Agitation for 15 min
Ripening
Until pH= 6.46 @ 38 ˚C (60 min)
Enzyme preparation: dilute rennet with 30 times volumes of D.I. water and stored at 5C Note: 1 oz rennet can be used to coagulate 1000 lb of milk.
Add rennet
14.3 ml of rennet in 350 ml DI water; Agitation for 5 min
Coagulation/ Setting
For 30 min
Cutting
Knife
Healing
For 5 min; then gentle agitation for another 5 min;
Scalding (with stirring)
42 ˚C for ~25 min (To pH= ~ 5.75)
Stirring
38 ˚ C for ~10 min (to pH = ~5.60)

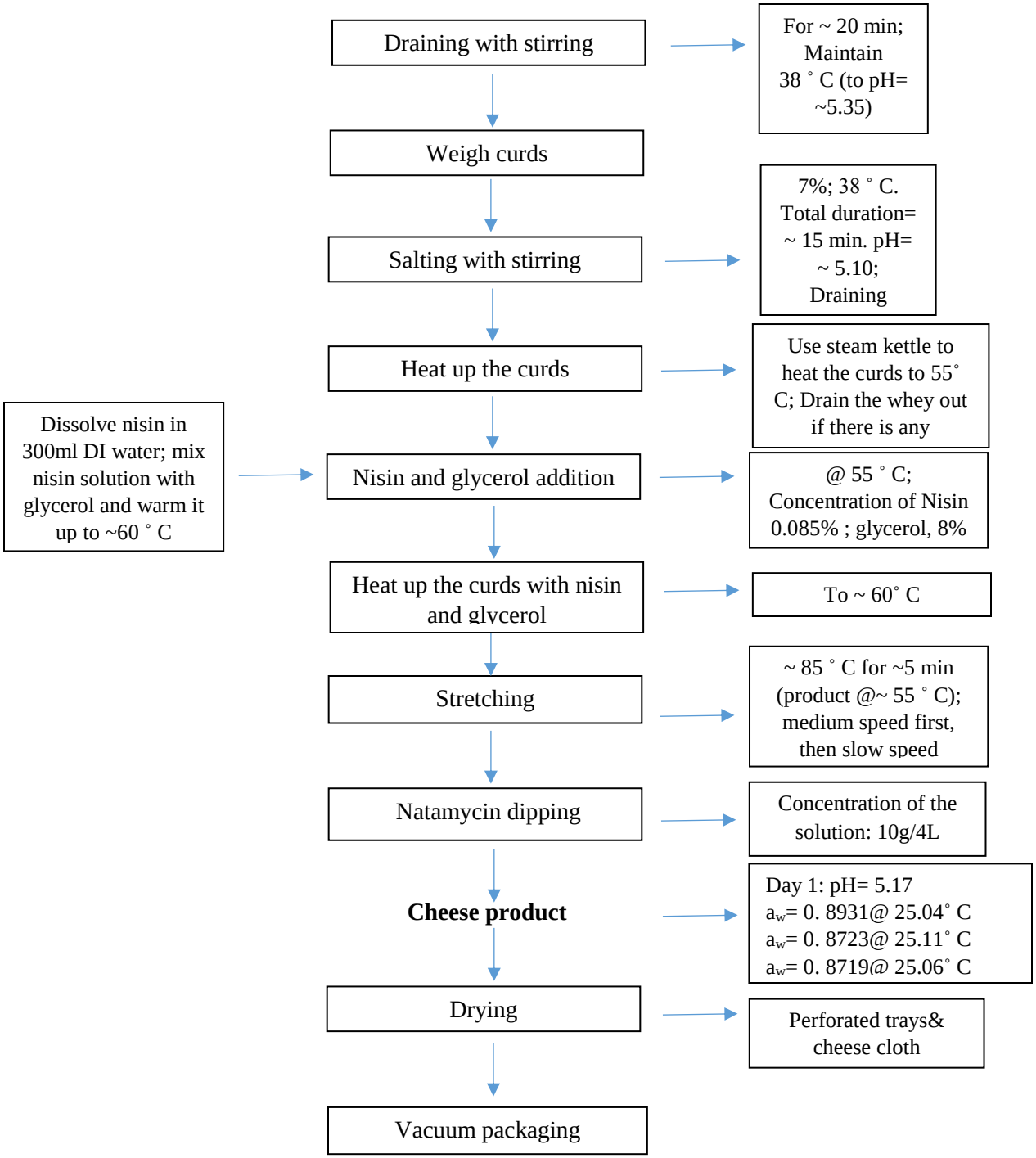


Figure 5.1. Process Flow Diagram at Pilot Plant

5.3.2.2. Details on processing procedures, results and features of process cheese product

Ingredients:

Cheese curds (obtained from production on April 18th), sodium phosphate (emulsifying salt), glycerol and nisin.

Process procedures:

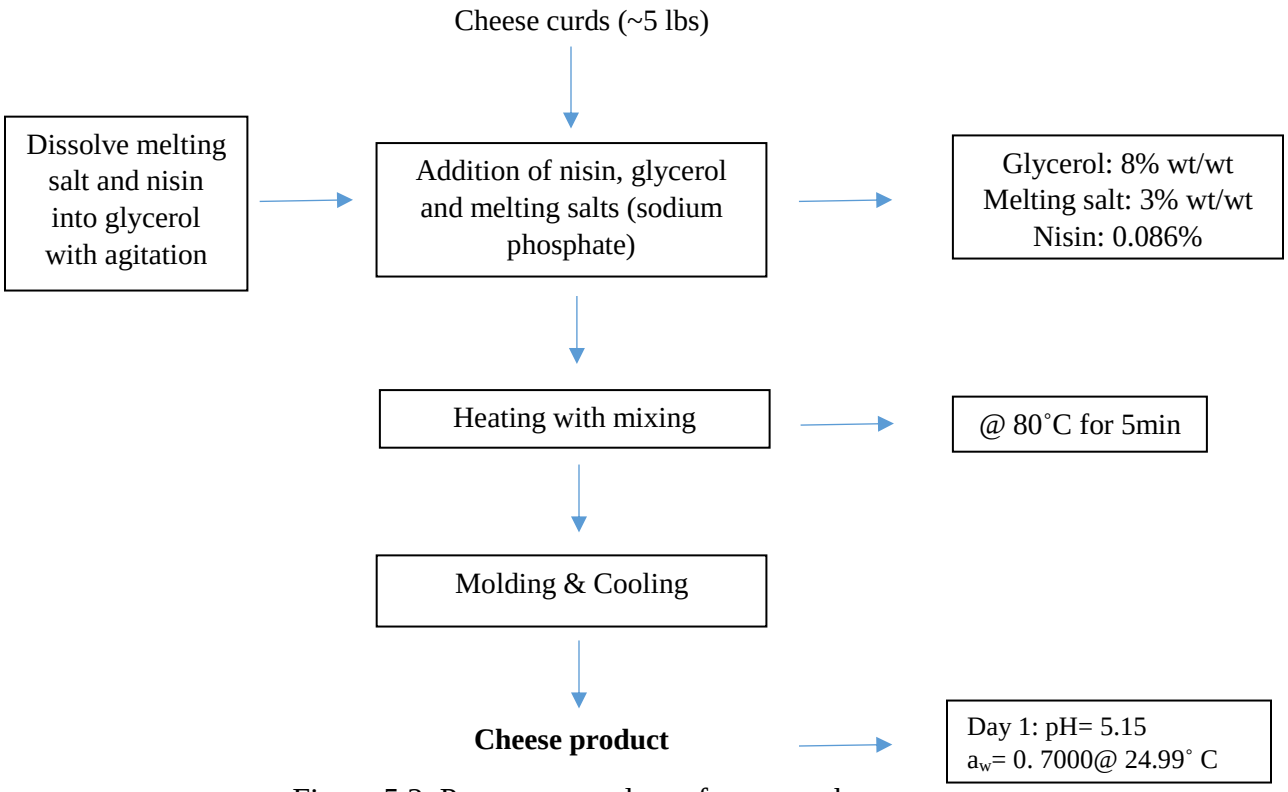


Figure 5.2. Process procedure of process cheese

Note: The amount of sodium phosphate and salt addition depend on baseline of cheese curds, such as salt content and pH

5.3.3. Cheese composition

Moisture, dry matter, salt and calcium content of cheese product were determined for product composition at Dairy One, Inc. in Ithaca, NY.

5.3.4. Microbiological tests

Coliform test, yeasts and molds test and spoilage evaluation were conducted by Cornell Dairy. Spoilage evaluation was conducted by visual observation of cheese product in appearance and molding. Cheese samples from 2 separate packages were diluted by sterile phosphate buffer at the ratio 1:5 (25 g of cheese samples in total into 100 ml sterile phosphate buffer) in a stomacher bag. Sterile 0.1moL/L NaOH was used to bring pH of sample to 7.0± 0.2. Stomacher (Seward Stomacher 400 circulator, Islandia, NY) was used to ready the cheese for plating. 1 ml of the prepared samples was pipetted with a sterile pipet onto 2 coliform plates (coliform count plate, 3M Petrifilm ^{TM}, St. Paul, MN) and 2 yeast and mold plates (Yeast and mold count plate, 3M Petrifilm ^{TM}, St. Paul, MN) respectively for each sample each week. Coliform plates were incubated at 32±1 °C for 24 hours and counted. Yeast and mold plates were kept at room temperature (~25 °C) for 10 days and counted. An average of the two plates were recorded.

5.3.5. Physical quality tests

Water activity (a_w) was measured by water activity meter with accuracy ±0.003 (Dew Point Water Activity Meter 4TE; AQUALAB). Calibration was performed immediately before measurements by measuring NaCl 6.0 mol/kg in H_2O at 0.760. The samples' pH was measured using a pH meter equipped with a stainless steel probe (PHW77-SS, HACH COMPANY, Loveland, CO). Two-point calibration (pH 7.00 and pH 4.01) was performed immediately before measurements. Stringiness was evaluated according to methodology in Oberg's paper (Oberg, et al., 2015). All the measurements were conducted in duplicate.

5.4. Results

The target low water activity (a_w<0.93) was achieved by optimizing the salt content and adding glycerol (a humectant) to the cheese, as both of these ingredients can lower water activity by binding free water. A slightly elevated salt content also helps decrease the activity of the starter culture, thus slowing down "cheese ripening effects" that could deteriorate stringy texture during storage, particularly at room temperature. The addition of salt and glycerol also added some positive flavor notes to the product. To control the moisture content and water activity of the product, we promoted the expulsion of whey from cheese curds by increasing the temperature and extending the draining time. The increased temperature and draining time also resulted in cheese curds with a significantly lower pH before salting (pH=5.30, which is close to the final pH for conventional fresh cheese curds). This also favors shelf stability because a pH of 5.15 was obtained after salting, and this value remained almost consistent throughout shelf life.

To achieve and maximize the desirable stringy texture, we controlled the stretching conditions in the Mozzarella stretcher (high temperature and slow stretching), which helped achieve the desired stringy texture of the product. To prevent mold and yeasts growth, we vacuum packaged the product, which created an anaerobic environment. Additionally, antimicrobials nisin and natamycin were added to inhibit the growth of *Listeria, Clostridium* and molds, respectively.

5.4.1. Product texture and appearance

The cheese product presented clear stringy, fibrous structure, as shown below:

Figure 5.3. Stringy structure (top, middle) and vacuum-sealed samples for shelf life (bottom)

5.4.2. Product composition

Table 5.1. Results of product composition

Measurements	Results (%)
Moisture	32.7
Dry Matter	67.3
Salt (NaCl)	3.33 (4.94 on dry basis)
Calcium	0.58 (0.86 on dry basis)

5.4.3. Product shelf life evaluation

The product was stored both under refrigeration (4 °C) and at room temperature (~25 °C), and evaluated weekly for: microbiological quality (coliforms, yeasts and molds), product spoilage (changes in appearance and molding) and stringy texture. The shelf life data for 12 weeks of storage is presented in Table 5.2 below.

Table 5.2. Microbiological, visual and textural quality of cheese samples during 12 weeks of shelf life study

Weeks of storage/ Date	Storage temp (^{o}C)	Coliforms (cfu/g)	Yeasts &Molds (cfu/g)	Spoilage	Texture (stringiness)	a_w	pH
Week 0 1/20/17	4 °C	<5	<5	None	Stringy	0.881	5.17
	25 °C						
Week 1 1/27/17	4 °C	<5	<5	None	Stringy	0.929	5.11
	25 °C	<5	<5	None	Stringy	0.920	5.21
Week 2 2/2/17	4 °C	<5	<5	None	Stringy	0.924	5.12
	25 °C	<5	<5	None	Stringy	0.917	5.12
Week 3 2/9/17	4 °C	<5	<5	None	Stringy	0.913	5.11
	25 °C	<5	<5	None	Stringy	0.909	5.11
Week 4 2/16/17	4 °C	<5	<5	None	Stringy	0.904	5.10
	25 °C	<5	<5	None	Stringy	0.904	5.12
Week 5 2/24/17	4 °C	<5	<5	None	Stringy	0.912	5.18
	25 °C	<5	<5	None	Stringy	0.906	5.16
Week 6 3/3/17	4 °C	<5	<5	None	Stringy	0.911	5.17
	25 °C	<5	<5	None	Stringy	0.913	5.17
Week 7 3/10/17	4 °C	<5	<5	None	Stringy	0.916	5.04
	25 °C	<5	<5	None	Stringy	0.899	5.05
Week 8 3/17/17	4 °C	<5	<5	None	Not Stringy	0.917	5.02
	25 °C	<5	<5	None	Stringy	0.890	5.06
Week 9 3/24/17	4 °C	<5	<5	None	Not Stringy	0.926	5.01
	25 °C	<5	<5	None	Stringy	0.909	5.04
Week 10 4/2/17	4 °C	<5	<5	None	Stringy	0.912	5.25
	25 °C	<5	<5	None	Stringy	0.912	5.26
Week 11 4/8/17	4 °C	<5	<5	None	Not Stringy	0.928	5.06
	25 °C	<5	<5	None	Stringy	0.903	5.13
Week 12 4/15/17	4 °C	<5	<5	None	Stringy	0.896	5.11
	25 °C	<5	<5	None	Stringy	0.863	5.15

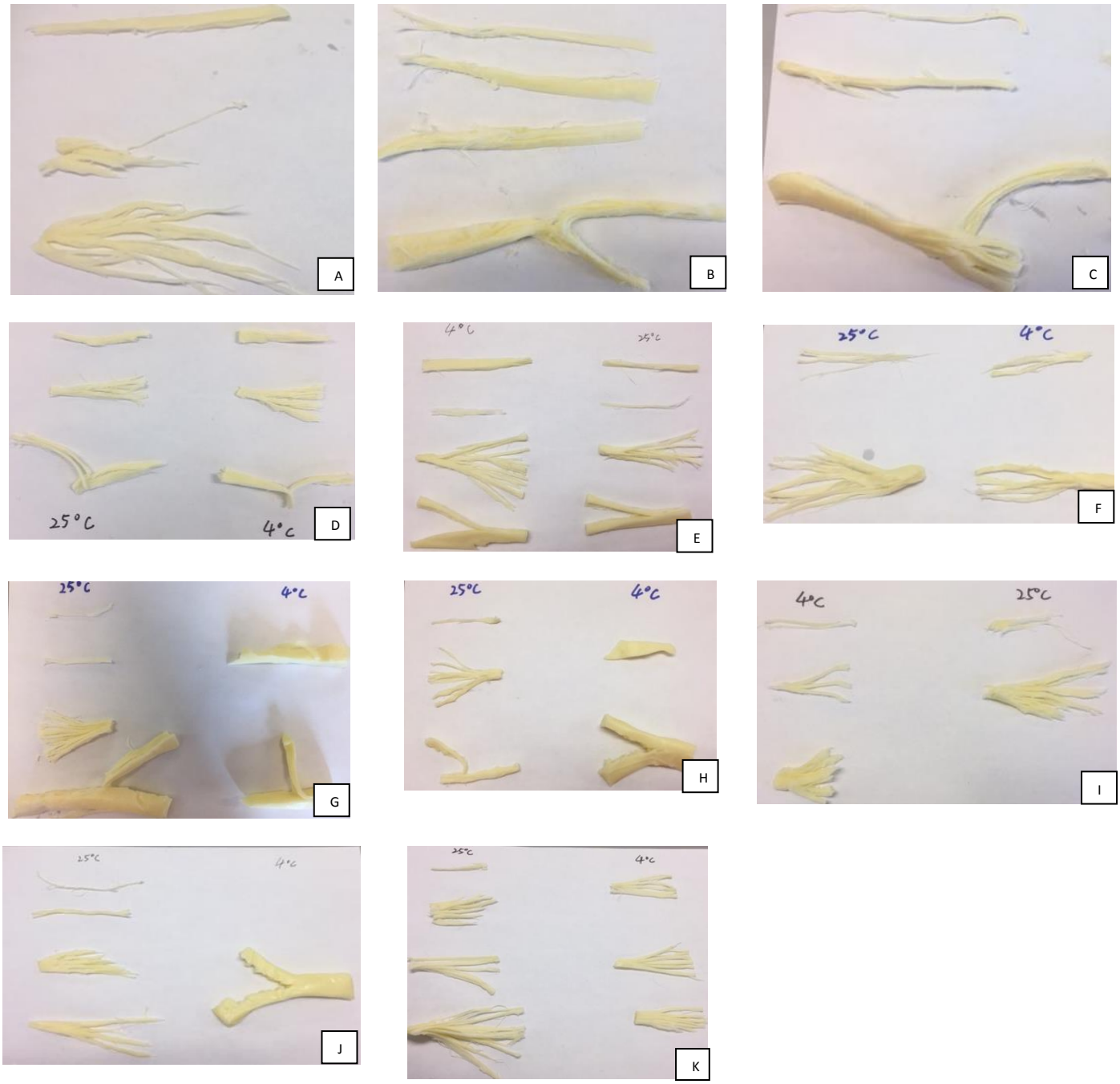


Figure 5.4. Stringiness of samples stored at room temperature (~25 °C) in February, March and April. A. 2/2/17. B. 2/10/17. C. 2/16/17. D. 2/24/17. E. 3/3/17. F. 3/10/17. G. 3/17/17. H. 3/24/17. I. 4/2/17. J. 4/8/17. K. 4/15/17.

For 12 weeks, it can be concluded that the product stored at ~25 °C has met both safety and quality standards regarding to the microbiological and physico-chemical results. Although a few samples lost stringiness during shelf life study, it could be due to the inhomogeneity during production. Reducing water activity is a critical point for this product because it reduces the rate of enzymatic activity, which helps maintain the stringiness texture, and also controls pathogens growth.

5.5. Conclusions and recommendations

We were able to successfully develop a shelf stable cheese product with a stringy texture, thus meeting the project objectives.

For the successful commercial production of these products, it is imperative that the processor: 1) uses high quality milk; 2) follows precisely Cornell's formulations, procedures and recommendations; 3) implements strict plant sanitation and good manufacturing practices (GMPs) during cheese production. The products and processes were designed with the assumption of maintaining strict sanitary conditions during the manufacturing process. Pathogenic and spoilage microorganisms can be introduced into the product if such conditions are not strictly observed. Of particular concern is contamination with *Staphylococcus aureus*, which could be transmitted via human contact, but could also be present in milk originating from cows with mastitis. Sterilization of the stretcher, minimization of human contact with the product after the stretching step and the use of clean, sanitary packaging materials are critical for minimizing the risk of post-processing microbial contamination. Other suggestions to improve safety include creation of an aseptic environment after stretching by creating positive pressure or by using UV light.

Table 5.3. Microbiological, visual and textural quality of process cheese samples during shelf life

Weeks of storage/ Date	Storage temp (^{o}C)	Coliforms (cfu/g)	Yeasts &Molds (cfu/g)	Spoilage (changes in appearance, molding)	a_w	pH
Week 0 1/20/17	4 °C	<5	<5	None	0.886	4.84
	25 °C					
Week 1 1/27/17	4 °C	<5	<5	None	0.887	4.86
	25 °C	<5	<5	None	0.886	4.84
Week 2 2/2/17	4 °C	<5	<5	None	0.873	4.84
	25 °C	<5	<5	None	0.870	4.81
Week 3 2/9/17	4 °C	<5	<5	None	0.844	4.82
	25 °C	<5	<5	None	0.825	4.83
Week 4 2/16/17	4 °C	<5	<5	None	0.862	4.81
	25 °C	<5	<5	None	0.845	4.82
Week 5 2/24/17	4 °C	<5	<5	None	0.871	4.87
	25 °C	<5	<5	None	0.866	4.91
Week 6 3/3/17	4 °C	<5	<5	None	0.858	4.96
	25 °C	<5	<5	None	0.856	4.76
Week 7 3/10/17	4 °C	<5	<5	None	0.855	4.74
	25 °C	<5	<5	None	0.859	4.73
Week 8 3/17/17	4 °C	<5	<5	None	0.838	4.69
	25 °C	<5	<5	None	0.833	4.68
Week 9 3/24/17	4 °C	<5	<5	None	0.879	4.60
	25 °C	<5	<5	None	0.864	4.64
Week 10 4/2/17	4 °C	<5	<5	None	0.853	4.66
	25 °C	<5	<5	None	0.840	4.75
Week 11 4/8/17	4 °C	<5	<5	None	0.842	4.68
	25 °C	<5	<5	None	0.854	4.74
Week 12 4/15/17	4 °C	<5	<5	None	0.834	4.69
	25 °C	<5	<5	None	0.839	4.73

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CHAPTER 6

CONCLUSIONS

The first part of this research focused on developing a milk and soy protein product. Specifically, the effect of two major factors that affect protein conformation, pH and temperature, was investigated. One of the main findings is that hardness and elasticity of the product significantly increased as pH decreased and processing temperature increased ($p<0.05$). Additionally, there is a significant synergistic effect of pH and temperature on the mixture of soy proteins and milk. Furthermore, large protein aggregates and large spacing between aggregates were observed in the pH 4.6 samples, while the pH 5.5 samples had a more homogenous structure. Samples treated at 95 °C had a denser aggregates with smaller spacing than the 65 °C groups. These findings can be used as a basis for developing milk-soy cheese-like products.

The second part of this thesis focused on developing a shelf stable cheese product with string texture. The approach to achieve this goal was to control the water activity (a_w <0.93) and pH values (~5.15) of the product, by using certain approved food additives (humectants and antimicrobials) and manipulating processing parameters of traditional Mozzarella production. Samples successfully achieved 12-week shelf life with string texture at both refrigerated temperature and room temperature.

The main conclusion is that a good understanding of food protein structure and properties and of the factors that control them allow the creation of high protein food products of desired texture and properties.