Maximizing yogurt firmness as functions of thermal denaturation and milk solids nonfat concentration

By

Emily Joy Donald

B.S., Texas A&M University, 2018

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Animal Sciences and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2021

Approved by:
Major Professor
Dr. Karen A. Schmidt

Abstract

Gel formation and quality in yogurts may be manipulated by varying yogurt mix heating temperature or altering milk solids nonfat (MSNF) concentration, which impacts the gel structure through the denaturation and aggregation of milk proteins. The overall objective of this research was to produce yogurts with maximum firmness through mix heating treatment and MSNF concentration. During the phase one study, yogurts were produced from mixes with 16% MSNF heated at 70, 78, 86 or 95 ^{o}C for 30 min. As process temperature increased from 70 to 78 ^{o}C, the resultant yogurts exhibited an increasingly firm texture. Yogurt mix heat treatments of 78, 86 and 95 ^{o}C resulted in firmness that did not significantly differ; however, yogurts produced from mixes heated at 95 ^{o}C exhibited decreased quality, ascertained by graininess, which increased. Variations in texture, correlating to protein denaturation and aggregation, were visually observed in microscopic images and microfluidic gel electrophoresis. In the phase two study, variations in yogurts were generated by manipulating milk solids nonfat (MSNF) concentration (9 and 12%) and mix heating temperatures (70, 75, or 85 ^{o}C for 30 min) to produce the firmest yogurt. Yogurt mixes, manufactured from nonfat dry milk, were analyzed using nitrogen fractionation, polyacrylamide gel electrophoresis and fluorescence of Maillard products and soluble tryptophan (FAST) index. Similar to the previous study, combining mix analyses with yogurt's textural, rheological and quality testing, the relationship between MSNF contents, heating temperature, protein denaturation and gel strength was investigated. The combination of 12% MSNF and 85 ^{o}C produced yogurts with the greatest firmness, storage and loss moduli directly correlating with FAST index. Yogurts produced with 12% MSNF exhibited the lowest syneresis and highest water holding capacities, while mix degree of denaturation increased due to heating temperature, independent of MSNF concentration. Meanwhile, greater MSNF concentration and heating

temperature translated to a denser protein gel network, more resistant to compression and shear. Increased MSNF correlates with increased protein content, resulting in additional denaturation and aggregation upon heating, translating to a denser protein gel network. With additional knowledge of the relationship between gel formation, MSNF and heating allows for conditions to be optimized to yield yogurts with maximum firmness. Further research to determine how MSNF concentration affects denaturation and aggregation may potentially lead to the production of firmer yogurts.

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Acknowledgements

I want to acknowledge and thank my major advisor, Dr. Karen Schmidt, for her advice and support over the course of my research. Her contribution to guiding me through the many challenges and unexpected turns of the last two years is greatly appreciated. I would also express my thanks to my committee members, Dr. Jayendra Amamcharla and Dr. Kelly Getty.

I am also extremely thankful to my lab mates, friends, faculty and staff at Kansas State University. Their willingness to assist in the lab or empathize over the many new experiences we have faced has been invaluable.

Lastly, I am extremely grateful to my family, Drs. Ben and Susan Donald, Tyler and Erin Hervey, Fran Giroux, Luna, and Kelsi Hooker who have supported me more than I can express.

Chapter 1 – Introduction

Yogurt is recognized worldwide as a product made from thickened fermented milk and is a commonly consumed dairy food throughout the world, however, types vary based on regional traditions and preferences (Remeuf, Mohammed, Sodini, & Tissier, 2003; Fisberg & Machado, 2015). In the U.S., nonfat yogurt is defined as the product resulting from the culturing of milk with *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* into a product that contains no more than 0.5% milkfat and not less than 8.25% MSNF with a titratable acidity (TA) of at least 0.9%, expressed as lactic acid (21 CFR § 131.206). Yogurt texture is critical to consumer acceptability of the product (Mahomud et al., 2016) and more stable gels correlate to improved textural characteristics (Akalin, Unal, Dinkci, & Hayaloglu, 2012). Methods to improve yogurt texture, a part of quality, include adjusting the heat treatment of the yogurt mix prior to inoculation and fermentation and increasing the MSNF content, which ultimately increases the protein content (Karam, Gaiani, Hosri, Burgain, & Scher, 2012).

Heat treatment of the yogurt mix improves quality due to the thermal denaturation of milk proteins (whey) which form aggregates with casein (Zhao et al., 2016) and are fundamentally important to the yogurt gel structure (Lucey et al., 1997, 1998; Morand, Guyomarc'h, Pezennec, & Famelart, 2011). Gel formation during yogurt production is a result of the interactions between casein and denatured whey proteins as the pH drops during fermentation (Akalin et al., 2012). Yogurt structure is formed as the number of interactions increases, allowing for the formation of chains, or bridges, between κ-casein, β-Lactoglobulin (β-Lg) and α-Lactalbumin (α-La) (Lee & Lucey, 2002). The protein chains form a chain matrix, trapping and creating the yogurt gel structure (Das, Choudhary, & Thompson-Witrick, 2019). The

composition of the three-dimensional structure translates into yogurt texture, ideally creamy and smooth, yet firm (Lesme et al., 2019; Aryana & Olson, 2017).

The heating of yogurt mixes is significant as the heat susceptibility of proteins vary (Wijayanti, Bansal, & Deeth, 2014). Hydrophobic bonding and/or disulfide linkages form between milk proteins, specifically, β-Lg, α-La and κ-casein, during the heating of yogurt mix (Donato & Guyomarc'h, 2009). At 70 to 74 ^{o}C, reversible denaturation of β-Lg begins, and aggregates of β-Lg/β-Lg and/or β-Lg/κ-casein form mainly through hydrophobic bonds (Wijayanti et al., 2014). However, when mix is heated $\geq$ 75 ^{o}C, denaturation is irreversible and aggregation occurs via disulfide interactions, resulting in stronger bonds (Wijayanti et al., 2014). Further, when the mix reaches > 80 ^{o}C, the aggregation rate of β-Lg/κ-casein increases, resulting in a more hydrophilic casein micelle complex (Mahomud, Katsuna, Zhang, & Nishizu, 2016). At the same time, heating at > 90 ^{o}C results in α-La denaturation, forming α-La/β-Lg aggregates as well as with the casein micelle (Vasbinder, Alting, & de Kruif, 2003). Thus, the heating temperature of the mix controls how proteins denature, interact and aggregate, which during fermentation will affect the formation and structure of the chain matrix (Mahomud et al., 2016).

The concentration of MSNF directly affects yogurt firmness as a function of protein content in the mix (Akalin et al., 2012) as a greater percentage of protein aggregation occurs when more protein is present (Marafon et al., 2011). Damin, Alcântara, Nunes, and Oliveira (2009) reported that increasing the MSNF from 8.25 to 16% (~3 to 6% protein) maximizes yogurt firmness while Law and Leaver (1997) found that increasing the protein concentration (~3.3 to 4.1%) was proven to increase whey protein denaturation slightly (~75 to 80%) when the mix was heated at 80 ^{o}C for 20 min. The response of increased denaturation in yogurt mix that contained increased protein content produces a yogurt gel with a higher density of interacting

proteins, forming a strong protein network (Marafon et al., 2011). To an extent, greater protein concentration and aggregation will produce a greater number of κ-casein, β-Lg and α-La chains, resulting in firmer yogurts (Lee & Lucey, 2010). Moreover, increased MSNF improves other yogurt gel quality characteristics such as reduced syneresis and enhanced water holding capacity (WHC) (Akalin et al., 2012).

The objective of this research was to study the relationships between heat treatment, MSNF content and texture to evaluate the potential to produce yogurts with the greatest firmness possible by controlling the protein interactions. Firmness and rheological properties of yogurts were analyzed to study the relationship of the mix heating temperature and MSNF concentration on gel structure. Heated yogurt mixes were analyzed for protein denaturation using three different methods – (a) nitrogen determination to calculate the degree of denaturation (DD), (b) images from reducing and non-reducing sodium dodecyl sulfate (SDS) and native polyacrylamide gel electrophoresis (PAGE) visualized denaturation and (c) fluorescence spectroscopy, which was used to calculate the fluorescence of advanced Maillard products and soluble tryptophan (FAST) index (Singh & Amamcharla, 2021).

Chapter 2 – Literature Review

2.1 – Yogurt

Yogurt is a fermented dairy food popularized by high amounts of protein, calcium and other essential vitamins and nutrients (Wang, Livingston, Fox, Meigs, & Jacques, 2013). From a historical perspective, producing yogurt was a preservation method used to extend the shelf life of milk which spoiled quickly (Fisberg & Machado, 2015). Although many different names for yogurt have existed throughout history and the world, they all refer to milk fermented with lactic acid bacteria (Tamime & Robinson, 1999). In the United States, yogurt consumption has more than doubled in the last twenty years, from 2.77 kg per person in 1999 to 6.08 kg per person in 2019 (USDA, 2021).

2.2 – Yogurt Manufacturing

2.2.1 - Formulation

Optional ingredients may be added to yogurt for flavor, to alter texture, or enhance nutritional content which may include sweeteners, stabilizers, additional dairy ingredients (such as nonfat dry milk (NFDM) or whey protein concentrate), vitamins, fruits or food colors (21 CFR § 131.200b; 21 CFR § 131.200d). Yogurts produced in the United States are traditionally made from bovine milk, but goat, sheep or camel milks are commonly used in other parts of the world (Das et al., 2019). Many different types or styles of yogurt exist. The two main styles include yogurts classified by physical state which are stirred-style and set-style yogurt (Gharibzahedi & Chronakis, 2018). Additional classifications can be based on fat and/or protein content (Meletharayil, Patel, & Huppetz, 2015; Jørgensen, Abrahamsen, Rukke, Hoffmann, Johansen, & Skeie, 2019).

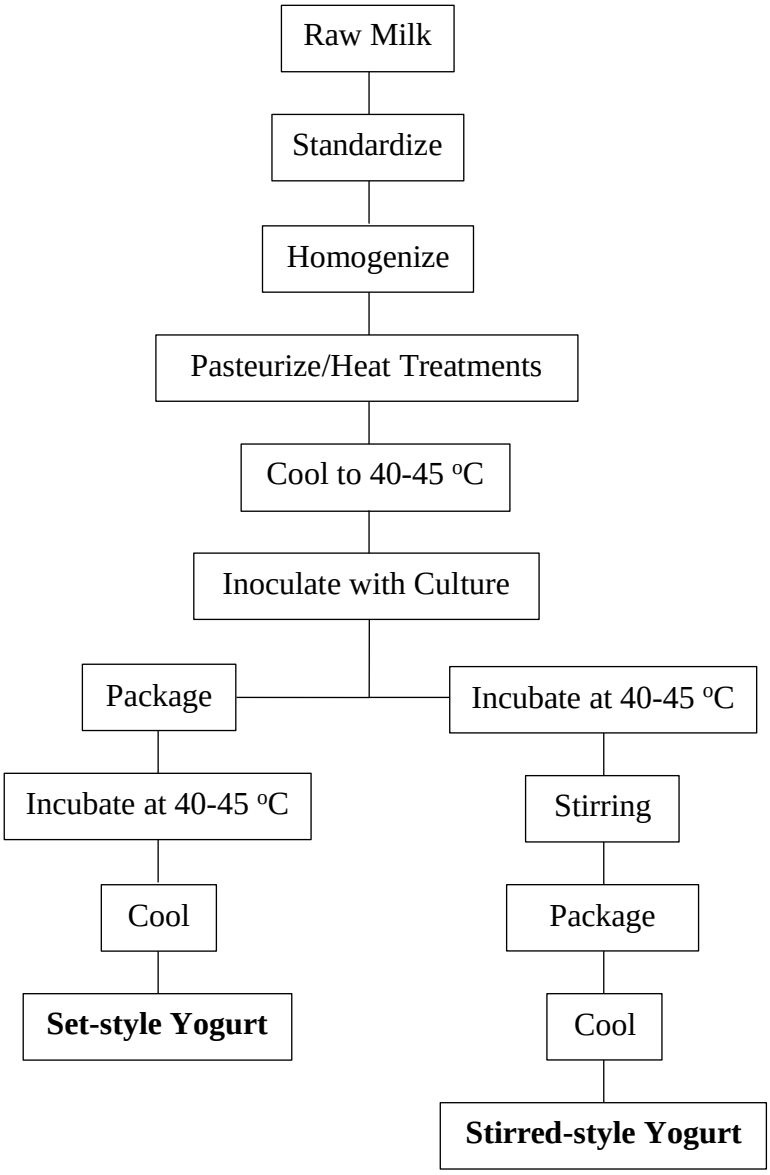


Figure 1: Yogurt processing steps.

Source: Lee and Lucey, 2010

2.3 – Yogurt Processing

2.3.1 – Yogurt Mix

Yogurt mix, which includes all ingredients before the addition of the culture, consists predominately of milk. Commercial yogurts must have at least 2.7% milk protein while most

vary in protein content from 4.5 to 8% globally (Jørgensen et al., 2019). It is common practice to increase the protein content, and therefore the MSNF, in yogurt mix with the addition of dried dairy powders (Damin et al., 2009). Ingredients that can be added to increase the solids content of yogurt mix include NFDM, whey protein concentrate (WPC) or other concentrated dairy products (Table 1) (Chandan & O'Rell, 2006). Although the minimum MSNF is 8.25% (21 CFR § 131.200a, 2009), yogurts with the best consistencies range in MSNF from 14 to 16% (Tamime & Robinson, 1999). Benefits of increasing protein contents in yogurt mixes include producing yogurts with thicker, creamier textures which corresponds with consumer interests (Jørgensen et al., 2019). Increasing milk protein contents and MSNF in mixes beyond 8% and 16%, respectively, result in yogurts with undesirable texture attributes, including graininess, whey separation, or syneresis, and reduced gel firmness (Damin et al., 2009; Tamime & Robinson, 1999; Delikanli & Ozcan, 2016; Jørgensen et al., 2019). Nevertheless, yogurts containing milkfat are reported to have enhanced creaminess and firmness despite the results summarized by Jørgensen et al. (2019) that 80% of consumers purchased low or non-fat, high protein yogurts. Sweeteners, natural and artificial, and stabilizers, pectins, carrageenan or neutral gums such as xanthan and guar gums, are frequently incorporated into the yogurt mix to satisfy consumer demands and improve sensory and textural properties (Tamime & Robinson, 1999; Soukoulis, Panagiotidis, Koureli, & Tzia, 2007).

Table 1: Composition of various dairy-based powders used in yogurt mixes.

Component	% in NFDM	% in WPI	% in WPC35
Lactose	53.5	< 1.0	48-52
Protein	34.0	90-92	34-36
Ash	7.9	2.5-3.5	6.5-8
Moisture	3.8	4-5	3-4.5
Fat	0.8	< 1.0	3-4.5

Source: DairyAmerica, 2020; Lucey et al., 2006

Abbreviations are NFDM, Nonfat dry milk; WPI, Whey protein isolate; WPC35, Whey protein concentrate with ~35% protein.

2.3.2 – Standardization

The first step in yogurt processing is the standardization of dairy mixes for yogurts (Fig. 1). As previously stated, fat and protein contents may be adjusted in order to meet regulations or preferences for various types of yogurts. In many countries, yogurts have regulated minimums for MSNF and protein contents as well as maximum fat contents (Lee & Lucey, 2010). If optional stabilizers (0.2-2% depending on the stabilizer used) or sweeteners (≤5%) are incorporated, additions occur during standardization (Soukoulis et al., 2007; Lee & Lucey, 2010).

2.3.3 – Homogenization

Yogurt mix is homogenized first at pressures of 10 to 20 MPa followed by pressures of 5 MPa at 55 to 65 ^{o}C in order to reduce the fat globule size, from 0.001 to 0.01 nm to < 2 nm and

suspend milk fat globules in solution (Lee & Lucey, 2010). Homogenization of yogurt mix prevents fat separation during fermentation and results in some serum protein denaturation, promoting protein-protein, whey-whey and whey-casein, interactions as a result of protein denaturation (Tamime & Robinson, 1999).

2.3.4 – Pasteurization and Heat Treatment

Various temperature and time combinations exist for yogurt mix pasteurization, ranging from ~65 ^{o}C for up to 40 min to 150 ^{o}C for a few seconds. These different heating conditions result in varying heat-induced changes and decreased microbial counts (Tamime & Robinson, 1999). Additional heat treatments beyond pasteurization temperatures and times typically range from 80 to 85 ^{o}C for 30 min to 90 to 95 ^{o}C for 5 to 30 min and are commonly used to promote whey protein denaturation, which is beneficial to yogurt quality (Soukoulis et al., 2007).

2.3.5 – Inoculation and Fermentation

Yogurt mix is cooled to ~40 to 45 ^{o}C and a 1:1 ratio of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* is added to the mix (Chandan, 1999). Yogurt mix is incubated at ~40 ^{o}C until the pH drops from pH 6.9 to pH 4.0 to 4.5 with gelation initiating at pH 5.2-5.4 (Lee & Lucey, 2010). The fermentation step is completed once the pH $\leq$ 4.6 and the TA, measured as percent lactic acid, reaches $\geq$ 0.9% (21CFR131.200, 2020).

2.4 – Milk proteins

The fermentable base ingredient of yogurt is milk which is produced by all mammals. While each species' milk is unique, they are all comprised of the same basic components. Milk

consists of water, sugar, lipids, minerals and proteins. Proteins constitute around 2-4% of bovine milk and can be categorized into two types: casein and whey proteins (Table 2) (O'Mahony & Fox, 2014). Casein predominates, consisting of ~80% of the protein component, while whey proteins account for the other ~20% (Berry et al., 2014).

Table 2: Components in bovine milk.

Component		% of milk
Water		87.3
Lactose		4.60
Fat		3.90
Protein		3.30
	Casein proteins	2.60
	Whey proteins	0.70
Minerals		0.70
Organic Acids		0.20

Adapted from Patel and Patel, 2015

When producing yogurt, each milk component contributes to the quality of the yogurt; however, protein is especially important due to its effect on the structure and thickness of the coagulum (Tamime & Robinson, 1999). To better understand the role of protein in yogurt, the unique characteristics of each protein must be examined. The most common method to separate casein and whey protein is isoelectric precipitation, which differentiates insoluble proteins by acidifying milk to pH 4.6 at 20 ^{o}C, resulting in casein aggregation and precipitation (Rowland,

1938). The remaining supernatant, or the soluble protein component, contains the whey proteins (Anema, 2014). Other protein separation methods include ultracentrifugation, salting-out, filtration (ultra- or micro- or gel) and ethanol precipitation (O'Mahony & Fox, 2014).

2.4.1 – Casein

Caseins are suspended in milk as casein micelles, which consist of protein (~93% of the mass) and calcium phosphate (remaining ~7%) (de Kruif & Huppertz, 2012; Eigel, Butler, Ernstrom, Farrell, Harwalkar, Jenness, & Whitney, 1984). Caseins consist of four different families: α_{s1}-casein, α_{s2}-casein, β-casein and κ-caseins (Table 3). The first family of caseins, α_{s1}-casein, represents ~40% of the total fraction of casein in bovine milk (Farrell et al., 2004). All components of α_{s1}-casein have the same amino acid (AA) sequence, contain 199 AAs, and are single chain polypeptides. The second family, α_{s2}-caseins, contribute ~10% of the casein fraction, are hydrophilic in nature and contain 207 AAs. The next family of caseins are β-caseins, which contain 240 AAs in length and represent ~35% of the casein micelle. α_{s1}-Casein, α_{s2}-casein, and β-caseins are extremely phosphorylated allowing them to bind to calcium ions; thus, they are critical junctions of the calcium phosphate fraction in the micelle (Dalgleish, 2011). The remaining 15% of the casein families, κ-caseins, consist of 169 AAs, with a hydrophilic end, AA 106-169. Within each family of casein, many variations exist due to the differences in milk produced by various breeds of cattle (Farrell et al., 2004).

Table 3: Composition of casein micelles in bovine milk.

Proteins	Concentration (g/L) in milk	Amino Acids	% (W/W) Mass	Isoelectric Point
α_{s1}-caseins	12-15	199	40	4.44-4.76
β-caseins	9-11	209	35	4.44-4.76
α_{s2}-caseins	3-4	207	10	4.44-4.76
κ-caseins	2-4	169	15	5.45-5.77

Adapted from Farrell, Jimenes-Flores, Bleck, Brown, Butler, Creamer, Hicks, Hollar, Ng-Kwai-Hang, Swaisgood, 2004.

2.4.2 – Casein Micelle

The casein micelle is a dynamic structure composed of aggregates of the four different caseins and calcium phosphate. Proteins are arranged spherically with α_{s1}-casein, α_{s2}-casein, and β-casein oriented into the inner portion (Donato & Guyomarc'h, 2009). The majority of the κ-caseins are located on the surface of the micelle and extend into the surrounding solution acting as a hairy layer that provide stabilization under normal conditions (Dalgleish, 2011). The formation of the spherical micelle is attributed to hydrophobic and electrostatic repulsive interactions. Overall, β-caseins are the most hydrophobic (Horne, 2017), are loosely bound and can dissociate/re-associate from the micelle (Anema, 2014). Casein micelles are typically heat and pressure stable and may be held at 100 ^{o}C for 24 hours while exhibiting no thermally induced denaturation (O'Mahony & Fox, 2014). Likewise, to denature casein micelles, pressures greater than 250 MPa, more than 10 times the pressure of homogenization, must be used (Huppertz, Fox, & Kelly, 2004). But, during fermentation of the yogurt mix, the pH of milk

decreases from ~6.7 to 4.6, destabilizing the casein micelles which then become susceptible to aggregation (Horne, 2017). The interactions that may result in aggregation begin as the pH decreases, from 6.7 to 5.1, reducing the negative charge on the casein macro peptide (Horne, 2014). The change in pH causes the highly negative surface charge on the casein micelle to decrease to zero net charge as the pH nears the isoelectric point, pH 4.6 (Lucey, 2004).

2.4.3 – Whey proteins

Whey proteins are a group of globular soluble proteins remaining after the casein fraction has been precipitated and removed at pH 4.6 (Madureira, Pereira, Gomes, Pintado, & Malcata, 2007), therefore, whey proteins are soluble at pH 4.6 at 20 ^{o}C (Farrell et al., 2004). Whey proteins are considered highly beneficial to the diet and are frequently used as ingredients in bakery products, health foods and other specialty products (de Wit, 1998). Typically, whey proteins are obtained as a co-product from cheese manufacturing; however, the whey removed during cheese making is known as sweet whey while whey from yogurt is acid whey. Acid whey has limited applications in the food industry when compared with sweet whey as acid whey has less protein and lactose but more calcium, phosphorus and lactic acid (Chandrapala, Duke, Gray, Weeks, Pamer & Vasiljevic, 2015). Whey has six major protein fractions (Table 4) (Eigel et al., 1984). Identified by AA sequence, the main whey proteins include β-Lg, α-La, immunoglobulins, bovine serum albumin (BSA), lactoferrin and lactoperoxidase (Table 4) (Farrell et al., 2004). Other whey proteins present in smaller amounts may include milk fat globule proteins, transferrin, β_2-microglobulin, or proteolytic products (Berry et al., 2014). Like casein proteins, different variants exist based on species and genetics (Eigel et al., 1984). Unlike casein proteins, whey proteins are highly susceptible to thermal denaturation. Whey proteins

begin denaturing at ~70 ºC with irreversible denaturation occurring at temperatures of ~80 to 90 ºC (Anema, 2014).

Table 4: Composition of whey proteins in bovine milk.

Proteins	Concentration (g/L) in milk	% Mass	Number of Amino Acids	Isoelectric Point
β-Lactoglobulin	3.2	~58	162	5.13
α-Lactalbumin	1.2	~20	123	4.2-4.5
Bovine Serum Albumin	0.4	~5-10	583	4.7-4.9
Immunoglobulins	0.7	~10-15	500+	5.5-6.8
Lactoferrin	0.2	~1-2	689	8.81
Lactoperoxidase	0.03	~0.5	612	_

Adapted from: Eigel, Butler, Ernstrom, Farrell, Harwalkar, Jenness, Whitney, 1984; Farrell, Jimenes-Flores, Bleck, Brown, Butler, Creamer, Hicks, Hollar, Ng-Kwai-Hang, Swaisgood, 2004; Madureira, Pereira, Gomes, Pintado, & Malcata, 2007; and de Wit, 1998

Bovine milk has high amounts of β-Lg when compared with milk from other mammals (Felfoul, Lopez, Gaucheron, Attia, & Ayadi, 2015). The β-Lg contain 162 AAs and are one of the most important proteins in acid milk gels. At pH 5.2-7, globular β-Lg has low solubility and is stable in the quaternary structure (Madureira et al., 2007). Rich in cysteine, β-Lg can bind with many small molecules. Upon heating to 70 ºC, denaturation and association via covalent and hydrophobic interactions and disulfide interchange reactions occur at a neutral pH. On the other

hand, α-La, contain 123 AAs and is globular in structure stabilized by disulfide bonds and has the ability to bind calcium (Farrell et al., 2004). In milk, α-La is more heat stable due to the lack of free sulfhydryl groups in the secondary structure; however, at ≥ 90 ^{o}C irreversible denaturation occurs (Wijayanti et al., 2014).

2.5 – Milk Protein Reactivity

Milk protein denaturation occurs during yogurt manufacture due to two main factors: heat treatment of the mix and acid development during fermentation (Akalin et al., 2012). Thus, casein and whey proteins are affected during yogurt production (Needs, Capella, Bland, Manoj, MacDougal, & Paul, 2000; Anema, & Li, 2003).

2.6 – Milk Protein Gels

2.6.1 – Thermally Induced Protein Denaturation

Beginning at ~70 ^{o}C, with increasing heating temperature and exposure, whey proteins thermally denature in the following order: immunoglobulins, serum albumin and lactoferrin, β-Lg and then α-La (Law & Leaver, 1997). When whey proteins denature, the compact globular conformation is lost and, as a result, internal amino acid side groups are exposed (Anema & Li, 2003). When β-Lg is exposed to < 75 ^{o}C, hydrophobic groups are exposed, and are available to interact and form hydrophobic bonds with other whey proteins and casein micelles (Law & Leaver, 1997). At temperatures > 75 ^{o}C, protein unfolding exposes free sulfhydryl groups and disulfide linkages begin to form protein complexes (Wijayanti et al., 2014). Denatured β-Lg is an especially important component in forming the microstructure in yogurt (Donato &

Guyomarc'h, 2009). During denaturation, β-Lg unfolds and becomes available to form protein complexes due to exposure and increased reactivity of free thiol groups (Anema & Li, 2003).

In contrast, α-La, is considerably more heat stable than β-Lg. At temperatures < 90 ^{o}C, denaturation is reversible while at temperatures > 90 ^{o}C, irreversible denaturation begins (Vasbinder et al., 2003). Irreversible denaturation gives way to disulfide bond breakage exposing free sulfhydryl groups which then may aggregate with other protein groups and, in turn, result in the formation of weak gels (Wijayanti et al., 2014). Factors that affect the rate of denaturation in whey proteins include heating temperature and the concentration of proteins (Law & Leaver, 1997). At lower temperatures, longer times are required to achieve the same level of denaturation. Temperatures and times of 95 ^{o}C for 256 seconds, 110 ^{o}C for 180 seconds, and 130 ^{o}C for 80 seconds all denature whey proteins to ~99% (Kücükcetin, 2008). Law and Leaver (1997) determined that when the concentration of protein in skim milk was doubled, the rate at which the proteins denatured increased with an increase in MSNF content from ~70% to ~90%.

2.6.2 – Acid Induced Protein Denaturation

During the formation of acid milk gels, the pH drops from ~6.7 to 4.6 due to lactic acid formation. Proteins in milk are subjected to changes in charge due to the acidification (Lucey, 2004). The surface charge on the casein micelle reduces with the decreasing pH and results in decreased electrostatic repulsion (O'Mahony & Fox, 2014). As the pH approaches the isoelectric point of casein, pH 4.6, casein micelles become susceptible to hydrophobic interactions with the protein κ-casein (Lucey, 2004). Casein micelles aggregate as a result of the reduction in net charge and begin forming a three-dimensional network (Lucey et al., 2006). Denatured whey

proteins are also affected by acid production as they become less soluble when the pH reaches the isoelectric point of β-Lg, pH ~5.3 (Lucey et al., 2006).

2.6.3 – Protein Complex Formation

In summary, the heat and/or acid induced denaturation results in the formation of protein complexes, including but not limited to β-Lg, α-La and κ-casein (Lucey et al., 1998). Denatured whey proteins attach to κ-casein on the micelle surface and behave as appendages allowing for further interactions during acidification. Potential reactions that may occur are temperature dependent as previously stated (Lucey et al, 1997).

2.7 – Yogurt Quality

Yogurt should also be firm, lack syneresis and have a high water holding capacity (WHC) (Kroger, 1975; Amatayakul, Halmos, Sherkat, & Shah, 2006). Factors affecting texture include fortification of yogurt mix, stabilizer use, fat content, heat treatments, starter cultures, incubation temperatures, pH, cooling and handling the product after manufacturing (Lucey, 2014).The quality of yogurts is measured by subjective tests, such as sensory analysis, and by objective or laboratory tests, such as chemical, physical and microbiology assays (Kroger, 1975). From a sensory aspect, lactic acid, which is responsible for the flavor, and small amounts of aromatic compounds including acetaldehyde, diacetyl and acetic acids, (Kroger, 1975), should be detected in yogurt imparting a distinctive aroma, and an astringent, tart or sour taste (Tomaschunas, Hinrichs, Kōhn, & Busch-Stockfisch, 2012). Texture, a main aspect of sensory analysis, is highly important in yogurt as consumers expect yogurt to be thick and smooth (Amatayakul et al., 2006). Laboratory tests to determine quality include testing for and analyzing

texture, including firmness and adhesiveness, rheology, WHC, color, syneresis, graininess, as well as imaging to view the microstructure of yogurt.

2.7.1 – Yogurt Texture

The texture of yogurt may be characterized by using a texture analyzer, which provides values for firmness, the maximum force measured by compression, adhesiveness, negative area under the axis, cohesiveness, the maximum negative force achieved when retracting the probe, work of cohesion, the area between firmness and cohesiveness peaks (Delikanli & Ozcan, 2016; Akalin et al., 2012). Firmness increases during yogurt gel formation e.g., pH 6.6-6.7 drops to pH ≤ 4.5 (Das et al., 2019). One method to increase the consistency and firmness of set-style yogurts includes increasing MSNF by evaporation, membrane filtration or adding additional milk powders (Jørgensen, Abrahamsen, Rukke, Johansen, & Skeie, 2017). Adding additional MSNF increases the percent of proteins in the yogurt, which reduces the area inside the gel without protein chains, trapping additional water and increasing the overall firmness of the gel (Tamime & Robinson, 1999). Delikanli and Ozcan (2016) tested yogurts with and without additional milk proteins and reported that firmness (0.33 to 0.42 N), adhesiveness (0.10 to 0.13 N mm) and springiness (0.94 to 0.96 mm) increased while cohesiveness (0.44 to 0.40) decreased significantly ($P < 0.05$) when comparing yogurt without milk protein concentrate (MPC) to the yogurt made with MPC. Denaturing at least 90% of the β-Lg through heat treatments also significantly increases the firmness. Tamime and Robinson (1999) reported that by heating yogurt mix at 70, 78, 86 and 95 ^{o}C for 30 min, the viscosity, measured using a Posthumus funnel (9.0 seconds 70 ^{o}C to 18.7 seconds at 95 ^{o}C), and thickness (3.0 cm 70 ^{o}C to 1.2 cm at 95 ^{o}C),

measured with a falling sphere, of yogurt significantly increased as the temperature increased (Tamime & Robinson, 1999).

2.7.2 – Water Holding Capacity

In yogurt, WHC refers to the amount of water held inside the gel matrix and the ability of the yogurt to hold on to that water (Akalin et al., 2012). As WHC increases, yogurts typically exhibit more stable gels, which correlate to a desirable texture and firmness, therefore, the conclusion is that high quality yogurts typically have high WHC (Tamime & Robinson, 1999). WHC can be manipulated by denaturing whey proteins or increasing MSNF, through the addition of dairy powders (Remeuf et al., 2003). Sodini, Montella, and Tong (2005) produced yogurts with skim milk powder (SMP) and various WPC. Yogurts with SMP had WHCs of 50.1% while yogurts with WPC had WHC of ~62.6% (Sodini et al., 2005) which they attributed to the additional denatured whey proteins in the WPC products. Remeuf et al. (2003) discovered that yogurts containing various protein additions had increased WHCs as the heating times of the mixes increased from 1 to 5 min at 90 ^{o}C (six samples all increased in WHC, varied from 84 to 92%). Yogurts with a greater WHC have greater numbers of protein interactions that were evenly distributed throughout the gel matrix trapping and holding water (Tamime & Robinson, 1999). Mahomud et al. (2016) observed a correlation between heat treatment and protein denaturation as well. When testing yogurts made from mixes that were heated (85 ^{o}C for 30 min) versus mixes that did not receive a heat treatment, yogurts made from non-heated mixes had WHCs of ~34% while yogurts made from heated mixes had average WHCs of ~55% (Majomud et al., 2017). Most methodologies for WHC in gels involve the centrifugation of the gel (yogurt

in this case) and weighing the expelled whey. Values are calculated as the difference in weight between the whey expelled and the remaining yogurt (Akalin et al., 2012).

2.7.3 – Color

Color is the first characteristic consumers notice about a product and will affect their perceptions of taste and acceptance (Giusti & Wrolstad, 2003). Whiteness of milk is affected by the flocculation of whey protein, aggregation of casein micelles and the state of calcium, e.g., soluble or insoluble (Tamime & Robinson, 1999). The size of the casein micelle affects the color that is reflected off the yogurt as well as the opacity (Needs et al., 2000). Using a colorimeter, color can be measured on a scale of L*, whiteness, a*, red/green, and b*, yellow/blue. Values for L* range from 0 (black) to 100 (white) while a* and b* values are measured in the positive or negative direction where positive is red and yellow and negative is green and blue, respectively (McClements, 2002a). The color of yogurt should be near bright, L* near 100, or near white, which can be calculated using the whiteness index (WI) from L*, a* and b* values (Vargas, Cháfer, Albors, Chiralt, & González-Martínez, 2008).

Yogurts made with and without the addition of dairy-based powders vary significantly in color. The colors, L* values of 91.48, a* -1.40, and b* 12.32, were obtained from control yogurts during an experiment completed by Delikanli and Ozcan (2016) and L* 88.31 to 91.69, a* -0.62 to -1.05 and b* values of 11.95 to 13.34, from supplemented (skim milk powder, sodium and calcium caseinates or milk protein concentrate) yogurts.

2.7.4 – Syneresis

Syneresis, or the formation of liquid on the surface of yogurt, is seen as an undesirable quality (Soukoulis et al., 2007). Syneresis is formed when the yogurt gel matrix shrinks and expels whey. This process, called "wheying off," is decreased when the MSNF is increased and as the pH decreases ($pH \leq 4.5$) (Das et al., 2019). Many methods to measure syneresis exist, including removing whey after centrifugation, straining or by siphoning free water from the surface of yogurts. Lucey, Munro and Singh (1998) reported the best method for accurately measuring syneresis was to measure the spontaneous whey expelled, whey siphoned off without any external forces. Nevertheless, all methods are based on the difference between the volume of removed whey from the total volume of the yogurt sample (Lucey et al., 1998). Lee and Lucey (2004) reported that syneresis increased in yogurts made from mixes heated at lower temperatures, as heating at 93 ^{o}C (0.66%) had significantly less syneresis than yogurts produced from mixes heated at 82.5 (1.07%) and 72 ^{o}C (1.18%).

2.7.5 – Graininess

Grains, small visible particles noticeable in the texture, are an undesirable quality in yogurt (Tomaschunas et al., 2012). Consumers want yogurt with a consistent texture throughout, which, depending on the type of yogurt, can become more or less apparent and result in negative product perception (Morell, Hernando, Llorca, & Fizman, 2015). Production of drinkable and stirred yogurt includes processing steps of mechanical shear which reduces the quantity of noticeable graininess. As a result, grains are more apparent in set-style yogurt. Various factors can increase the presence of grains in yogurt including heat treatments and incubation temperatures (Tomaschunas et al., 2012) and may be a result of denatured whey protein to casein

complex formation during production (Remeuf et al., 2003). Remeuf et al. (2003) determined that grains in yogurts range from ~50 to 250 in number depending on yogurt ingredient formulation in 1 g of yogurt. Graininess was concluded to increase from ~75 grains when heated at 90 ºC for 1 minute to ~250 grains when heated for 5 min at 90 ºC (Remeuf et al., 2003). Because grains are visible, image analysis is used to determine the presence or absence of grains (Kücükcetin, 2008).

2.7.6 – Rheology

Measuring rheological properties provides information pertaining to the flow and viscosity of yogurts. Obtaining the flow curves and apparent viscosity for acid gels may determine the variations created by heat treatment or MSNF concentration (Lucey, Teo, Munro, Singh, 1997). Flow behavior, characterized through thixotropic tests, models the shear stress of gels under varying shear rates (Paseephol, Small, & Sherkat, 2008). Curves from yogurts may be fitted to the Herschel-Bulkley model, $\sigma = \sigma_o + K\gamma^n$, in Microsoft Excel (Microsoft, Redmond, WA), which gives insight into rheological parameters including shear stress (σ), yield stress (σ_o), shear rate (γ) and consistency (K) and flow behavior (n) indices (Morrell et al., 2015). Morrell et al. (2015) reported that while texture of yogurts is perceived through shear and elongational flow in the mouth, apparent viscosity measured through flow behavior analysis may provide experimental insight into orally discerned thickness. Variations in flow behavior properties are presented in Table 5.

Table 5: Rheological properties of yogurts, produced with nonfat dry milk, with varying milk solids nonfat concentration and/or heating temperatures.

Temperature (°C)	Duration of heating (min)	MSNF (%)	Rheological properties					Reference
			σ_o (Pa)	K (Pa.s^n)	*n* (-)	G'(Pa)	G" (Pa)	
90	10	16	7.54	1.04	0.70	94.3	26.8	Paseephol et al., 2008
82-85	30	10	-	6.6	0.56	249	62	Morrell et al., 2015
90	5	14	-	2.35	0.55	~55	-	Purwandari et al., 2007
92	5	10.6	189	693	0.151	989	241.5	Guggisberg et al., 2007
85	30	10.7	48	-	-	143	-	Torres et al., 2018
90	5	10	-	46.7	0.12	342.8	91.5	Lesme et al., 2019
95	4.3	11	65.4	-	-	349.9	-	Kücükcetin, 2008
110	3	11	55.4	-	-	272.3	-	Kücükcetin, 2008
130	1.3	11	20.2	-	-	233.8	-	Kücükcetin, 2008

Abbreviations are MSNF, Milk solids nonfat; σ_o, yield stress; K, consistency coefficient; *n*, flow behavior index; G', storage modulus; G", loss modulus.

The storage modulus (*G'*) and loss modulus (G") are generated by rheological dynamic oscillation tests, which depict the viscoelastic properties of gels (Lucey, Teo, Munro, Singh, 1997). Lucey, Teo, Munro and Singh (1997) noticed that as yogurt mix heating time increased from 15 to 30 min, and at temperatures from 75 to 90 ^{o}C (in 5 ^{o}C increments), the resultant yogurts differed significantly in gelation time and *G'* values as seen below in Table 6. It was concluded that gelation times and *G'* values increased with increased heating time and temperature of the mix, which was a result of the denatured proteins. However, a decrease in *G'* values was observed in the mixes heated to the highest temperatures which was attributed to be a result of formation of large whey protein/casein aggregates which interrupted the formation of the gel during fermentation (Lucey et al., 1997). Later studies further concluded that these results were attributed to the amount of denatured β-Lg which, as amounts increased, produced higher *G'* values, 16 Pa in yogurts produced from unheated milk and 290 Pa in yogurts produced from heated milk (Lucey, Tamehana, Singh, Munro, 1998).

Table 6: Gelation time and storage modulus of yogurts produced from unheated and heated yogurt mixes.

	Gelation time	Storage modulus (G'), (Pa)	β-Lg Denaturation (%)	α-La Denaturation (%)
Unheated milk	22.3	15	-	-
75 ^{o}C for 15 min	22.3	15	18	18
75 ^{o}C for 30 min	10.7	55	20	21
80 ^{o}C for 15 min	9.9	190	48	46
80 ^{o}C for 30 min	7.3	370	77	69
85 ^{o}C for 15 min	8.2	315	86	70
85 ^{o}C for 30 min	5.3	455	95	81
90 ^{o}C for 15 min	5.2	450	95	83
90 ^{o}C for 30 min	4.6	360	100	100

Source: Lucey et al., 1997

β-Lg: β-Lactoglobulin; α-La: α-Lactalbumin

2.7.7 – Microstructure

The formation of chains throughout the yogurt gel result in a chain matrix, which traps water creating yogurts' microstructure. Microstructure may be analyzed using image analysis from images produced by a scanning electron microscope (SEM), transmission electron microscope (TEM) (Tamime & Robinson, 1999), or a confocal scanning laser microscope (CSLM). CSLM images target the protein gel network specifically and do not require as much

sample preparation when compared to other methods. As a result, CSLM is becoming one of the most popular methods of analyzing milk gels and dairy products (Tamime & Robinson, 1999; Skytte, Ghita, Whelan, Andersen, Møller, Dahl, & Larsen, 2015). Harte, Luedecke, Swanson, and Harbose-Canovas (2003) used TEM images to view the micelle structure in yogurts produced from raw milk, pressure treated (300 to 676 MPa) and thermally treated milk (85 ^{o}C for 30 min). From the images, it was concluded that, when compared to raw milk yogurts, pressure treating the milk resulted in yogurts with smaller, rounder and more evenly distributed micelles. The thermally treated milk produced yogurts with rounder micelles, yet they were more irregular in shape and had rougher edges than raw milks (Harte et al., 2003). Silva and Mahony (2017) used CSLM to visually show that gel softness correlates to a more open gel network with more pores when compared with the firmer gel that had a fine gel network with many small pores. This was also related to WHC as the gel with the more open network was significantly higher (Silva & O'Mahony, 2018).

2.8 – Protein Denaturation Analysis

2.8.1 – Protein Denaturation

Several methods exist to monitor thermal denaturation of protein including nitrogen fractionation analysis, gel electrophoresis and fluorescence spectroscopy (Oldfield, Singh, & Taylor, 2005; Singh and Amamcharla, 2021). Identifying protein denaturation and aggregation connects the effect of heat treatment and MSNF concentration to the formation and strength of the yogurt gels.

2.8.2 – Nitrogen Fractionation

Separating yogurt mixes into different nitrogen fractions allows for the calculation of the amount and type of protein present (O'Grady et al., 2001). Mixes can be separated into three different fractions, total nitrogen (TN), non-casein nitrogen (NCN) and non-protein nitrogen (NPN), and then analyzed via the Kjeldahl method to measure the nitrogen contents (Jorgensen et al., 2015). The TN fraction represents all nitrogen in the sample, including nitrogen associated with milk proteins, while the NPN designates nitrogen not associated with milk proteins, and the NCN fraction is the nitrogen in whey proteins (Outinen, Rantamaki, & Heino, 2010). Each fraction is multiplied by 6.38 to calculate the protein contents, which includes total protein (T), non-casein protein (NCP) and the non-protein (NP) (Jorgensen et al., 2015). These values can be used to calculate the true protein (TP), difference between T and NP, casein protein, T minus NCP and the protein soluble at pH 4.6 (whey protein) (SP), TP minus the casein protein. The SP is used to calculate the degree of whey protein denaturation (DD), a relative value comparing denaturation in heat treated and control mixes (Sodini et al., 2005).

2.8.3 – Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) separates proteins across an electric gel field based on net charge or molecular weight (Sharma, Sharma, Rajput, Mann, Singh, & Gandhi, 2021). There are several different kinds of PAGE, differentiated based on the type of gel and/or buffer used. Native and sodium dodecyl sulfate (SDS) PAGE are two of the most common types of gel electrophoresis for milk protein separation. Native PAGE, which relies on tris/glycine buffers (25 mM tris, 192 mM glycine and 20% (v/v) methanol), separates proteins in their native form in non-reducing conditions (Sharma et al., 2021). Because charge, shape and size of the

protein is taken into account (Estéves, Fuciños, Bargiela, Pastrana, Tovar, & Rúa, 2016), native PAGE is useful for qualifying κ-casein complexes, however, the remaining caseins do not separate well due to similar isoelectric points (Sharma et al., 2021). SDS-PAGE utilizes tris/glycine/SDS buffer (25 mM tris, 192 mM glycine and 0.1% (v/v) SDS) and accurately distributes all milk proteins across the gel based on molecular weight. Two different types of SDS-PAGE are commonly used, non-reducing and reducing. The reducing agent, β-mercaptoethanol, breaks disulfide bonds that form between proteins during denaturation and aggregation. Comparing non-reducing and reducing gels allows for visualization of κ-casein-denatured whey protein complex formation via thiol/disulfide interactions (Bogahawaththa, Buckow, Chandrapala, & Viasiljevic, 2018).

Another method to visualize the separation of milk proteins is microfluidic gel electrophoresis (MGE), known as lab-on-a-chip, which yields similar results to reducing and non-reducing SDS-PAGE, however, it is a faster and more highly reproducible method (Bütikofer, Meyer, & Rehberger, 2006). The variation in technique and equipment are the two main differences when comparing SDS-PAGE to MGE, performed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Both methods yield gel images that help qualify the amount and type of protein present in the sample, however, SDS-PAGE is known to be more time consuming, uses more materials, including samples, buffers, solvents and staining/destaining solutions whereas MGE uses less than 0.5 mL total material, all of which are loaded into one chip (Fig. 2) and combined during analysis (Anema, 2009).

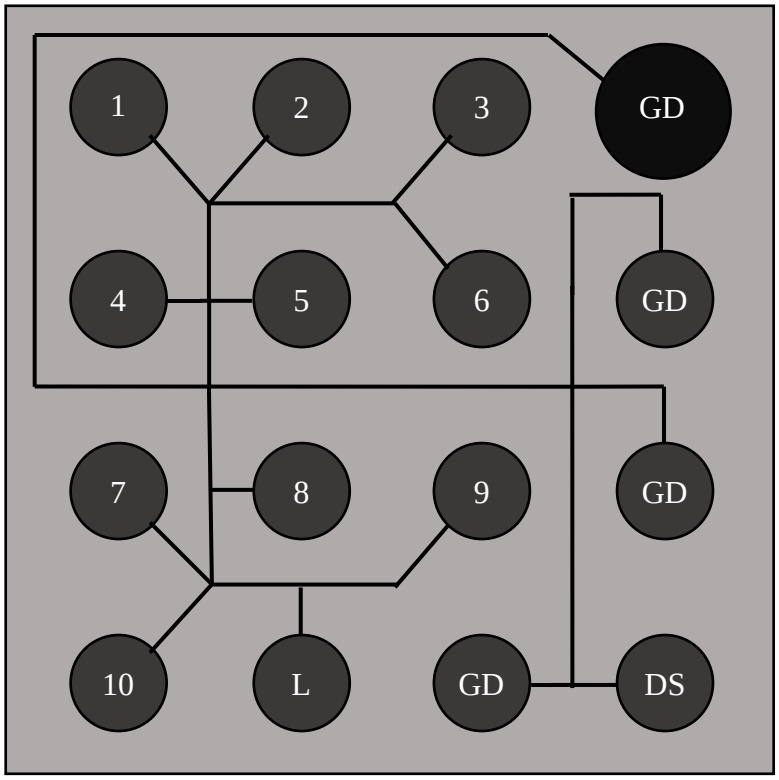


Figure 2: Lab-on-a-chip used in microfluidic gel electrophoresis. GD: Gel/dye matrix, Abbreviations are DS, Destaining solution; L, Ladder; 1-10, Samples 1-10.

Source: Anema, 2009; Agilent Technologies

While MGE, or lab-on-chip, is more costly, it typically takes < 1 hr to prepare and analyze 10 samples, while SDS-PAGE may take several days. Similar methodologies exist between the two analyses, while SDS-PAGE charges the gel field, MGE operates with individual electrodes placed in each sample well (Anema, 2009). Another difference between the two methods is the molecular weight the proteins migrate to, as the Agilent 2100 Bioanalyzer shifts the size of milk proteins (Table 7) to increase clarity and distinction between the five main proteins detected (Nitsche, 2009).

Table 7: Molecular weights of milk proteins from two gel electrophoretic methods.

Proteins	MW PAGE (kDa)	MW 2100 Bioanalyzer (kDa)
α-casein	23/25	37
β-casein	24	33
κ-casein	19	46
β-lactoglobulin	18	18
α-lactalbumin	14	12

Nitsche, 2011

MW: Molecular weight, PAGE: Polyacrylamide gel electrophoresis

2.8.4 – Fluorescence Spectroscopy

Fluorescence spectroscopy can be used to detect protein denaturation by measuring the fluorescence of tryptophan and Maillard products (Singh and Amamcharla, 2021). Because tryptophan fluoresces, it is useful as a measure of protein denaturation as it is exposed when β-Lg unfolds by thermal denaturation (Albani, 2015). Two tryptophan residues are found in β-Lg, one in a hydrophobic region (AA 19) and the other on the surface (AA 61). Albani (2015) found that the tryptophan in the hydrophobic region is responsible for fluorescence, thus, with heat treatment, hydrophobic bonds are broken, and fluorescence varies. Maillard reaction products, which are created during the thermal treatment of milk, include hydroxymethylfurfural, pyrrole and imidazole, increase with denaturation and fluorescence (Shaikh and O'Donnell, 2017).

There are two different types of fluorescence spectroscopy, right angle and front face, which are differentiated based on the angle of detection and the sample used. Right angle

fluorescence spectroscopy occurs on the filtrate of the fraction soluble at pH of 4.6 and is used to obtain the fluorescence of advanced Maillard products and soluble tryptophan (FAST) index. The FAST index is a measure of denaturation and is derived from the fluorescence of Maillard emission products (360/420 nm) divided by tryptophan emission products (290/340 nm) (Rathod and Amamcharla, 2021). Birlouez-Aragon, Sabat and Gouti (2002) reported FAST index values of 10.4 for raw milk, 18.6 for milk heated at 78 ^{o}C for 8 min and 75.2 for milk heated at 95 ^{o}C for 8 min and concluded that the higher the heat treatment, the higher the FAST index. Front face fluorescence spectroscopy, run on yogurt mixes, is also used to detect denaturation based on the intensity of the peaks from both tryptophan and Maillard emission and Maillard excitation spectra. While Maillard products generally increase with heat treatment, tryptophan products decrease with heat treatment in right angle but increase in front face fluorescence spectroscopy (Singh and Amamcharla, 2021).

2.9 – Previous Research

Previous research has found that heating yogurt mix prior to fermentation increases the gel firmness and G' values and improves stability of the gel (Vasbinder, Alting & de Kruif, 2003). Understanding the effect of heat treatment and MSNF on milk proteins, including the denaturation and aggregation that occurs, during heating of the yogurt mix is critical to yogurt production. Vasbinder et al. (2003) reported that the ideal heating regimen for yogurt mixes was between 70 and 90 ^{o}C for 5 to 30 min, which results in the favorable denaturation and aggregation of whey proteins. Others agree, including Jørgensen et al., 2017 and Lesme et al., 2019, that temperatures in this range are critical to the formation of gel structure in yogurt, and

produced yogurts from mixes heated at 75 or 95 ºC for 5 min and 90 ºC for 5 min, respectively. However, a lack of information exists between the relationship of yogurt firmness and how the effect of individual protein denaturation at a specific temperatures and denaturation rates of α-La and β-Lg affects yogurt structure based on temperature and total solids. Anema (2008) reported that as the concentration of denatured whey proteins and κ-casein complexes increase in 10 to 25% MSNF, the structure of proteins that creates the gel network becomes denser and resistance to shear increases by ~5x. However, it has been reported that the protein source mattered; i.e., NFDM, whey protein concentrates, sodium caseinates or microfiltration retentates, and the structure of the gel changes due to the ratio of casein to whey protein (Jørgensen et al., 2017). Jørgensen et al (2015) reported that in set yogurts at a casein:whey protein ratio of 45:55, firmness was maximized after heating yogurt mix at 95 ºC for 5 min, whereas a lower heat treatment of 75 ºC for 5 min favored the control, with no addition of whey protein concentrate, nearly doubling the firmness when compared to the 45:55 and 75 ºC treatment. Due to these variable factors, this research focused on analyzing the protein component during yogurt production while simultaneously monitoring texture and gel quality. To ensure this, protein denaturation and aggregation were analyzed with nitrogen fractionation, gel electrophoresis and fluorescence spectroscopy, which were key to filling in gaps from previous research in regard to yogurt texture and MSNF.

Chapter 3 – Research Objectives

The objective of this research was to create a yogurt with maximum firmness by altering the processing conditions or protein contents in the yogurt mix. Two phases were completed. In the first phase, a high milk solids not fat system was used as the model to investigate the impact of heat treatment of the mix on the yogurt quality. Whereas in the second phase, two lower milk solids not fat systems were used as models to determine the impact of protein denaturation of the mixes on yogurt texture.

Phase one study: Investigate the effect of yogurt mix heat treatment (70, 78, 86 and 95 ^{o}C for 30 min) on texture in yogurts with high MSNF concentration (16%).

Phase two study: Analyze the impact of MSNF concentrations (9 and 12%) and mix heat treatments (70, 75 and 85 ^{o}C for 30 min) on milk protein denaturation and its effect on yogurt firmness and texture.

A review of literature was conducted to analyze the potential of plant-based milks to produce nondairy food alternatives.

Chapter 4 – Materials and Methods

4.1 – Experimental Design

For the phase one study, set-style yogurts were produced from mixes with 16% MSNF that were subjected to one of four heating regimens of 70, 78, 86 and 95 ^{o}C for 30 min. Three replications were performed resulting in 12 yogurts and 15 yogurt mixes, which included a nonheated control mix (M16-C). Yogurts produced from heated mixes were designated as Y16-70, Y16-78, Y16-86 and Y16-95 while mixes were designated as M16-70, M16-78, M16-86, M16-95 and M16-C. Statistical analysis was performed using SAS University Edition and SAS Studio (SAS Institute Inc., Cary, NC) using a one-way analysis of variance (ANOVA) with the mixed procedure. Data was analyzed for significance at a $P \leq 0.05$ level using LSmeans with Tukey's adjustment.

For the phase two study, set-style yogurts were produced from mixes made with one of two MSNF contents (9 or 12%) and heated to one of three temperatures (70, 75, or 85 ^{o}C) for 30 min. Three replications were performed resulting in 18 yogurts and 24 mixes, designated as Y9-70, Y9-75, Y9-85, Y12-70, Y12-75 and Y12-85 and M9-70, M9-75, M9-85, M12-70, M12-75 and M12-85, respectively, and the non-heated control (M9-C and M12-C) mixes. Statistical analysis was performed using SAS® University Edition and SAS® Studio (SAS® Institute Inc., Cary, NC) using a two-way analysis of variance (ANOVA) with the mixed procedure. Significance was determined at a $P \leq 0.05$ level. Significant factors and interactions were differentiated using LSmeans with Tukey's adjustment at a $P \leq 0.05$ level (Zamberlin & Samaržija, 2017).

4.2 – Materials

For the phase one study, Dillon's brand, Vitamin A and D fortified skim milk was obtained at a local grocery store, Dillon's Food Store (Hutchinson, KS) in Manhattan, KS. The milk was blended with Grade A low heat NFDM (Fresno, CA) at a ratio of 12.2:1 (16% MSNF). For the phase two study, Grade A low heat NFDM obtained from Dairy America (Fresno, CA) was blended with DI water to obtain 9 or 12% MSNF. Due to availability, NFDM originated from the same lot for the phase one study, separate from the phase two study. Future work should be conducted with different lots of NFDM to ensure results seen in both studies are independent of NFDM treatment or date/time of year of production.

4.3 – Production of yogurts

All glassware and other equipment used, including 1000 mL beakers (Fisher Scientific, Waltham, MA), glass one-gallon jars (ULINE, Pleasant Prairie, WI), volumetric flasks (Fisher Scientific), stir bars (Fisher Scientific), were washed and held in ~2400 ppm chlorine bath for at least 20 min before air drying. In the phase one study, skim milk and NFDM were hydrated to 16% MSNF, whereas in the phase two study, NFDM was added to DI water to 9 or 12% MSNF 24 hrs prior to yogurt production. To hydrate, NFDM was added to either skim milk or DI water and mixtures were stirred on stir/heat plates (Cat. 50-949-783, Electron Microscopy Sciences Corning Digital PC-420 Hot Plate/Stirred, 120 V, 60-1150 RPM, Fisher Scientific) with magnetic stir bars (Cat. 22-261734, Bel-Art™ SP Scienceware™ Egg-Shaped Spinbar™ Magnetic Stir Bars, Fisher Scientific) using the stir function only in 1000 mL beakers (Fisher Scientific) at 22 $\pm$ 1 ^{o}C. The mixtures were stirred for 1 hour, then covered with two layers of

aluminum foil (Reynolds Wrap, Reynolds Kitchens, Richmond, VA) and stored at 4 ± 1 ºC until yogurt manufacture.

To prepare the yogurt, the yogurt mix (which consisted of NFDM and skim milk or DI water) was heated with constant stirring on stir/heat plates covered with aluminum foil (Reynold's) until the desired temperature (70, 78, 86, 95 or 70, 75, and 85 ºC, for the phase one and phase two study, respectively) was reached determined by temperature probes (Fisher Scientific). Stir bars were removed and two new layers of aluminum foil (Reynold's) were placed on top of the 1000 mL beakers. Beakers were placed into hot water baths (Model No. FSGPD20, Fisherbrand Isotemp General Purpose Deluxe Water Baths, Fisher Scientific) pre-set at the desired temperatures ± 2 ºC and held in place for 30 min. The heated mixes were then removed (phase one study mixes were heated in several beakers then combined in one-gallon glass jars (ULINE)) and placed into ice baths until the mixes reached 40 to 45 ºC, ~10 to 15 min.

To inoculate yogurt mixes, culture (DuPont, Danisco, YO-MIX™ 111 LYO 750 DCU or YO-MIX™ 495 LYO 100 DCU, Mississauga, ON, Canada, phase one and phase two study, respectively) was added at 0.02%. To culture cooled mixes, ~20 g of 42 ± 2 ºC mix was removed and placed into a 60 mL polypropylene (PP) cup (Great Value) with 0.02% culture. Cooled mix and culture were stirred for ~30 seconds then combined with the remaining mix and stirred for an additional ~60 seconds, then set aside for 5 min. The inoculated yogurt mix was then poured into various sterilized and/or sanitized containers, which consisted of a combination of 120 mL sterile translucent PP cups (Fisherbrand), 60 mL PP cups (Great Value), 50 mL PP centrifuge tubes, 250 mL glass volumetric flasks (Fisherbrand). These containers were placed into an incubator (Model No. 650D, Fisherbrand Isotemp Microbiological Incubator, Fisher Scientific) set at 42 ± 1 ºC until the pH reached 4.6, which took ~5 hours, however the exact

fermentation time was not recorded. Yogurts were placed into storage (Whirlpool 18.0 Cu. Ft. Top Freezer Refrigerator, Benton Harbor, MI) at 4 ± 1 ºC until testing.

4.4 –Analyses

4.4.1 – pH and Titratable Acidity

The pH and TA (percent lactic acid) of the mixes and yogurts were measured before, during and after incubation. The pH was measured using a pH/mV/Ion meter (Cat. 13-636-AP125, Fisherbrand™ Accumet™ AP110 Portable pH Meter, accumet AP110 Meter Kit, Fisher Scientific) after standardization with pH 4.0 and 7.0 buffer solution (S25849A/B, Fisher Science Education) at 22 ± 2 ºC.

The TA was measured using an acid-test buret (Cat. 11-301-40, DWK Life Sciences A620F1, Fisher Scientific) attached to a 2000 mL Erlenmeyer flask (Cat. 10-090E, Pyrex™ Wide Neck Heavy-Duty Erlenmeyer Flask, Fisher Scientific) with a 75 mL bulb (Cat. 15-000-501, Fisherbrand™ Pipet Filling Bulbs, Fisher Scientific). To measure the TA, the flask was filled with 0.1 N sodium hydroxide (Cat. AC124190010, ACROS Organics™, Fisher Scientific) and 1% phenolphthalein (Cat. 5620-16, Ricca Chemical Company, Fisher Scientific) was used as the indicator. In order to find the TA, 9 g of yogurt was weighed into a 100 mL glass beaker and then ~0.5 mL phenolphthalein indicator was added. The yogurt was titrated with base until the first permanent pink color change (~30 seconds). The acid-test buret was read directly for the percent acidity, or the percentage of lactic acid in the yogurt (Hooi, Barbano, Bradley, Budde, Bulthaus, Chettiar, Lynch and Reddy, 2004):

Equation 1

$$\%\ Acidity\ Expressed\ as\ Lactic\ Acid\ = \frac{mL\ NaOH\ \times\ Normality\ of\ NaOH\ \times\ 9}{Sample\ Weight}$$

4.4.2 – Moisture

Moisture contents were measured on fluid milk, NFDM, and prepared yogurt. Similar procedures were used to determine the moisture content of the fluid milk and yogurt (IDF-ISO-AOAC 925.23). The container of fluid milk was inverted 10 times gently to avoid foaming, whereas the yogurt was stirred for 30 seconds using a plastic spoon. Two to 3 g of sample were weighed and placed into individual pre-weighed, pre-heated 20 mL aluminum weighing dishes (Cat. No 08-732-100, Fisherbrand, Fisher Scientific). The third sample, NFDM, was weighed into aluminum weighing dishes as well in amounts between 1 to 1.5 g (AOAC 927.05). Each sample was placed into a forced-air convection oven (Model No. 750F, Fisherbrand Isotemp General Purpose Heating and Drying Oven, Fisher Scientific) set at 102 ± 1 ^{o}C for 4 hours. Samples were removed and cooled in a desiccator for a minimum of 30 min. The samples were then weighed, and the moisture was calculated with the following equations (Hooi et al., 2004):

Equation 2

$$\%\ Moisture\ = \frac{Wet\ Sample\ Weight - Dry\ Sample\ Weight}{Wet\ Sample\ Weight} \times 100$$

Equation 3

$$\%\ Solids\ =\ 100 - \%\ Moisture$$

4.4.3 – Texture

To measure yogurt firmness, 90 g of yogurt mix had been fermented in 120 mL sterile specimen containers (Fisherbrand 120 mL Specimen Containers, Cat. 16-320-730, Fisher

Scientific) using a method adapted from Amatayakul et al. (2006). On test day, the texture analyzer (Stable Micro Systems, Model TAXT2, Texture Technologies Corp, Hamilton, MA) with a back extrusion rig (TA-094BE, Texture Technologies Corp, Hamilton, MA) and a 35 mm aluminum probe was used to measure the consistency providing values for adhesiveness (g.mm), the energy needed to detach the sample from contact surfaces i.e. the tongue, teeth or palette, cohesiveness (g), ratio of force required to overcome stick, consistency (g*mm), resistance to force to masticate the sample, firmness (g), the effort required to masticate the sample in a single bite, and work of cohesion (g*mm), the energy to deform a sample, using a single compression test cycle with a 30-kg load cell (Breene, 1975; El-Zeini, Ali, Awad & El-Ghany, 2018). To obtain these values the texture analyzer was calibrated using a 300 g weight after attaching the probe. The following settings were selected on Exponent software (Stable Micro Systems): pre-test speed 1.0 mm/s, test speed 1.0 mm/s, post-test speed 10 mm/s, distance 30 mm with a trigger force of 10 g. The yogurt containers were placed directly underneath the probe and held in place during the test to prevent movement. The probe was cleaned of excess yogurt between each sample test.

4.4.4 – Water Holding Capacity (Phase One Study)

To determine the WHC of yogurts, a method was adapted (Hassan, Frank, Schmidt, & Shalabi, 1996). After the mix was cultured, 20 g were added to 50 mL PP centrifuge tubes, which were placed in an upright position during fermentation and storage (4 ± 1 ^{o}C). The yogurt samples were equilibrated to 24 ± 2 ^{o}C for 30 min before testing. Tubes were centrifuged (Marathon 21000R, Fisher Scientific) for 30 min at 13500 x *g* at 10 ^{o}C. The supernatant, or expelled whey, was removed and weighed. The following equation was used to calculate WHC:

Equation 4

$$WHC(\%) = \frac{Yogurt\ Weight - Whey\ Expelled\ Weight}{Yogurt\ Weight} \times 100$$

4.4.5 – Water Holding Capacity (Phase Two Study)

A different method was used for the phase two study due to the decrease in MSNF content (from 16% to 9% and 12%). High *g* force used in phase one study (13500 x *g*) was not suitable for the phase two study due to lack of variation in WHC when used on yogurts with 9 and 12% MSNF. The WHC was determined using the method of Meletharayil, Patel and Huppertz (2015). Yogurt samples, 20 g in 50 mL centrifuge tubes, were centrifuged (Marathon 21000R, Fisher Scientific) for 15 min at 3,000 x *g* at 4 ± 1 °C. The supernatant was removed and weighed. The WHC was calculated with Equation 4.

4.4.6 – Color

Yogurt color was measured using a Hunter Lab MiniScan EZ 4500L spectrophotometer (Hunter Associates Laboratory Inc., Reston, VA). The spectrophotometer was calibrated using a white tile and black glass standards (Delikanli & Ozcan, 2016). Yogurt was stirred and placed into round opaque polypropylene disposable containers with a diameter of 40 mm and depth of 11 mm (Cat. NC9050158, Decagon Devices Inc, Fisher Scientific) and leveled with a flat edge. Color was tested in triplicate by rotating the container 120° between each measurement and values were averaged. Values were obtained for L*, a*, and b* (McClements, 2002b). The whiteness index (WI) was also calculated using the following equation (Vargas et al., 2008):

Equation 5

$$WI = 100 - [(100 - L*)^2 + (a*)^2 + (b*)^2]^{1/2}$$

4.4.7 – Syneresis (Phase One Study)

Syneresis, or the separation of whey from yogurt, is measured by the calculation of the percent of whey removed based on the total weight of the yogurt. The following method was derived from Lee and Lucey (2004). First, 45 g cultured yogurt mix was added to a 50 mL glass volumetric flask and incubated at 42 ± 1 ºC until the pH reached 4.6. Following incubation, yogurt was maintained at ~4 ºC. After 24 hr of storage, syneresis was siphoned from the surface of the yogurt using a needle and syringe and then weighed. Syneresis was calculated using Equation 6 (Kücükcetin, 2008):

Equation 6

$$Syneresis(\%) = \frac{Weight\ of\ Whey\ Removed}{Yogurt\ and\ Whey\ Weight} \times 100$$

4.4.8 – Syneresis (Phase Two Study)

The method used in phase one yielded no syneresis however it was concluded that syneresis free yogurt was not made. Therefore, when producing yogurts with less solids in the phase two study, a different published method was selected (Sanchez Alan, Subbiah & Schmidt, 2019) that would better fit the structure of yogurts produced. The method chosen was known to detect differences in gel syneresis in systems with less total milk solids and protein (~3.5% protein). Syneresis was measured following a modified method described by Sanchez Alan, Subbiah and Schmidt (2019). Cultured yogurt mix, 90 g, was added to 120 mL sterile cup prior to incubation. Yogurts were removed from storage (4 $\pm$ 1 ºC), held at an ~30º angle, instead of

an 8^{o} angle, for 30 sec, instead of 2 hr, then the surface whey was removed using a syringe within 15 sec. Equation 6 was used to determine the amount of syneresis (Kücükcetin, 2008).

4.4.9 – Graininess

The method to analyze graininess was adapted from Remeuf et al. (2003) in which images were captured of the yogurt and analyzed. For each sample, 1 g yogurt was dispersed in 10 mL deionized ~22 ± 2 ^{o}C water by stirring manually with a plastic spoon (Target) for 15 seconds. The dispersion of yogurt in water was poured into a ~10 cm round glass petri dish (Fisherbrand) and placed on a piece of red cardstock (Astrobrights, Neenah,WI). The image was captured immediately after stirring using an iPhone 7plus (Cupertino, CA) elevated 15 cm above the sample. Image J software (National Institute of Health, Bethesda, MD) was used to analyze images. Grains were defined as those particles that possessed a diameter $\geq$ 1 mm and were counted. Images were captured in triplicate from each sample and the number of grains were averaged. Graininess images are found in Appendix E and F.

4.4.10 – Rheology- Flow Properties (Phase Two Study)

The rheological properties of the yogurt were measured using an Anton Paar rheometer (MCR-92 Rheometer, Anton Paar, Graz, Austria) equipped with a parallel plate – plate geometry (PP25) (25 mm diameter and a 2.0-mm gap setting, 20 ^{o}C). To measure the rheology, yogurts were equilibrated to ~20 ^{o}C, then stirred gently 20 times prior to placing samples (~1.5 mL) on the stage.

Yogurts were subjected to varying logarithmic shear rates of 0.1 to 150 s^{-1} to obtain flow curves (Lesme et al., 2019). Data was fit to the Herschel-Bulkley equation (Equation 7) in Excel (Microsoft) using the shear stress (σ) and shear rate (γ) data.

Equation 7

$$\sigma = \sigma_o + \mathrm{K}\gamma^n$$

Values for yield stress (σ_o), consistency coefficient (K) and flow behavior index (n) were extracted with Excel (Microsoft) using nonlinear regression (Lesme et al., 2019). Apparent viscosity (η) was determined at a shear rate of 50 s^{-1} from Anton Paar software.

4.4.11 – Rheology - Viscoelastic Properties (Phase Two Study)

The viscoelastic properties of yogurts were measured using an Anton Paar rheometer (MCR-92 Rheometer, Anton Paar, Graz, Austria) equipped with a parallel plate – plate geometry (PP25) (25 mm diameter and a 2.0-mm gap setting, 20 ^{o}C). Yogurt samples were equilibrated to ~20 ^{o}C, then stirred gently 20 times prior to placing samples (~1.5 mL) on the stage.

A dynamic oscillation test was conducted using the method of Marafon et al. (2011). Yogurt samples were subjected to a small amplitude oscillatory measurement. Shear stress ramped from 1.0 to 50.0 Pa at a constant frequency of 1 Hz (150 s). The storage modulus (G'), and loss modulus (G"), were derived using the equipment software.

4.4.12 – Confocal Scanning Laser Microscopy (Phase One Study)

The method for confocal scanning laser microscopy (CSLM) was similar to that of Ciron, Gee, Kelly and Auty (2010). Yogurts and yogurt mixes were stained with Fast Green FCF (0.1 %, w/v, in DI water) for protein (Sigma-Aldrich, St. Louis, MO, USA) and Nile Red (0.125%,

w/v in propane-1, 2-diol) for fat (Molecular Probes, Thermo Fisher Scientific, Waltham, MA) and analyzed using an LSM 5 PASCAL (Zeiss, Thornwood, NY, USA) with a Zeiss Axiocam HR digital camera (Zeiss). A drop of yogurt mix, and a mid-layer sample of yogurt was individually stained with 10 μL Nile Red-Fast Green mixture dye (3:1, v/v). Dyed samples were stored at 4 ^{o}C until analysis. Three images of each samples were taken at 63 × magnification at excitation wavelengths of 488 nm and 633 nm for fat and protein, respectively, from random positions on all samples for all replications. Captured images were analyzed with ImageJ Software (US National Institute of Health, Bethesda, MD, USA).

4.4.13 – Protein Analysis

The proteins in yogurts mixes were analyzed by two different methods. First, the protein contents in the mixes were separated and quantified using the method of Rowland (1938) in order to determine the impact of heating on the non-casein protein fraction in particular. Second, mixes were analyzed using electrophoresis to determine and compare the levels of native and denatured proteins in the four yogurt mixes. The combination of these two methods will allow for further understanding of the effect of heat treatment on the denaturation and aggregation of proteins (Anema, 2008; Dissanayake, Ramchandran, Donkor, & Vasiljevic, 2013).

4.4.13.1 – Protein Fractionation

Following Rowland's method (1938), the protein in mixes was chemically separated into one of three nitrogen-containing fractions, which were analyzed for nitrogen content using a modified Kjedahl method (AOAC International, 2010, Methods 991.20, 991.21 and 998.05). Nitrogen contents were multiplied by the conversion factor of 6.38 to obtain total protein, non-

casein protein (NCP) and non-protein contents in the three fractions (Rowland, 1938; Sodini, Remeuf, Haddad, & Corrieu, 2004). True protein, soluble protein (SP) at pH 4.6, and insoluble protein (IP) at pH 4.6 (Sodini et al., 2004; Outinen et al., 2010) were calculated to determine the relative degree of denaturation (DD) as follows:

Equation 8

$$TP = T - NP$$

Equation 9

$$SP = NCP - NP$$

Equation 10

$$IP = T - NCP$$

Equation 11

$$DD\ (\%) = \frac{SP_{MX-C} - SP_{MX-YY}}{SP_{MX-C}}\ x\ 100$$

Where $SP_{MXX\text{-}C}$ is the SP in the control mix and SP_{MXX} represents the SP in one of the heated mixes where: X equals 9 or 12 and YY equals 70, 75 or 85 (phase two study).

4.4.13.2 – Microfluidic Gel Electrophoresis (Phase One Study)

Electrophoresis was used to detect proteins in the mix samples using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) with the Protein 80 kit (Agilent Technologies). Samples, gels, destaining and denaturing solutions were prepared according to Agilent Protein 80 Kit Guide (2016). Yogurt mix samples were diluted 1:10 with deionized

water and then divided in half. Half of the samples were analyzed directly (non-reducing conditions), and half were combined with 3.5% β-mercaptoethanol (60-24-2, Fisher Scientific) (reducing conditions). Gel like images were formed using Agilent software by the mobility of the milk proteins (Nitsche, Agilent, 2011).

4.4.13.3 – Polyacrylamide Gel Electrophoresis (Phase Two Study)

Due to research planning issues during the COVID-19 pandemic, the Agilent 2100 Bioanalyzer (Agilent Technologies) was unavailable for the phase two study, therefore, SDS and native PAGE gels were produced from modified methods of Sanchez Alan et al. (2017), Rathod and Amamcharla (2021) and Singh and Amamcharla (2021). Proteins were analyzed using precast gels, 4 to 15% Tris-glycine and 12% Tris-glycine eXtended gels (Bio-Rad Laboratories, Hercules, CA, USA), respectively, with the Mini-Protean II dual slab cell system (Bio-Rad Laboratories). Yogurt mixes were combined with either reducing, non-reducing or native sample buffers (Bio-Rad Laboratories) to a 0.5% protein concentration and vortexed. Reducing and non-reducing samples were heated at 90 ^{o}C for 5 min, then cooled for 3 min at 0 ^{o}C. Then 15 μL of sample was loaded into the precast gels along with 15 μL of the protein ladder (Bio-Rad Laboratories) and submerged in either SDS, tris/glycine/SDS, or native, tris/glycine, PAGE buffers (Bio-Rad Laboratories). When the bands reached the bottom of the gels, after running at ~70 V for 15 min then ~100 V for 1 hr, gels were stained with Coomassie Blue G-250 (Bio-Rad Laboratories) for 1 hr. Finally, gels were destained with a 10% acetic acid, 10% methanol solution for ~12 hr.

4.4.13.4 – Fluorescence Spectroscopy

Tryptophan and Maillard products emission and excitation spectra were obtained using a Perkin-Elmer LS50B Luminescence spectrometer (Waltham, MA) using the method from Singh and Amamcharla (2021) with slight modifications. Five scans were recorded from each mix using FL Data Manager Software (Perkin-Elmer). The tryptophan emission spectra, captured as a measurement of the amount of exposed tryptophan on amino acid 19 of β-Lg, were observed from 305 to 450 nm at an excitation wavelength of 290 nm while the Maillard products emission spectra, 380 to 480 nm, had an excitation wavelength of 360 nm. The Maillard products excitation spectra was observed at 260 to 350 nm at an emission wavelength of 410 nm. All spectra were obtained from samples at 22 $\pm$ 2 ºC.

4.4.13.5 – Right-Angle Fluorescence Spectroscopy

Spectra were obtained from the soluble protein phase (Rowland, 1938) of control and heated yogurt mixes in a 10 mm fluorometer cell (Starna Cells, Inc., Atascadero, CA). Right-angle fluorescence spectroscopy was performed on a Perkin-Elmer LS50B Luminescence spectrometer (Waltham, MA) with five scans recorded on each emission spectra. Adaptations from Singh and Amamcharla (2021) included spectra slit widths of 10.0 and 7.0 nm. Tryptophan and Maillard products emission observed at 290/340 nm and 360/420 nm, respectively, were used to calculate the fluorescence of advanced Maillard products and soluble tryptophan (FAST) index (Singh and Amamcharla, 2021).

Equation 12

$$FAST\ Index\ =\ 100\ x\ \frac{F_{AMP}}{F_{TRP}}$$

Where F_{AMP} is the fluorescence of advanced Maillard products and F_{TRP} is the fluorescence of tryptophan products.

4.4.13.6 – Front Face Fluorescence Spectroscopy

Front-face fluorescence spectroscopy was performed on control and heated yogurt mixes in a 20 mm fluorometer cell (Starna Cells, Inc.) using a method adapted from Singh and Amamcharla (2021), but all spectra had slit widths of 10.0 and 7.0 nm. All spectra were captured with an 1% attenuation filter in the excitation slit.

Chapter 5 – Results (Phase One Study)

5.1 – Yogurt

Yogurts Y16-70, Y16-78, Y16-86 and Y16-95 had an average pH of 4.40, TA of 1.38% expressed as lactic acid, MSNF of 16.58% and TP of 6.43%. As no significant differences were present in pH, TA, MSNF or TP, it was concluded that the effects of heat treatment did not prohibit the production of yogurts, which had a fermentation time of ~5 hr.

5.2 – Gel Electrophoresis

Gel like images (Fig. 3) were captured using the Agilent Bioanalyzer 2100 with the Agilent Protein 80 kit. The gel like image from M16-C in reducing conditions (Fig. 3, lane a) displayed all expected bands for milk proteins and were similar when compared with M16-70, M16-78, M16-86 and M16-95 (Fig. 3, lanes b-e). When mixes were prepared without a reducing agent (Fig. 3, lanes f-j), M16-78, M16-86 and M16-95 (Fig. 3, lanes h-j) displayed an increased band intensity located between ~30 and 40 kDa (casein proteins) and a decreased band intensity and/or lack of bands located between 12, 18 and 46 kDa (α-La, β-Lg, and κ-casein, respectively) (Nitsche, 2011). Additionally, bands at ~63 kDa and ~71 kDa, which increased in size with heating regimen, are high molecular weight proteins and soluble protein complexes, respectively.

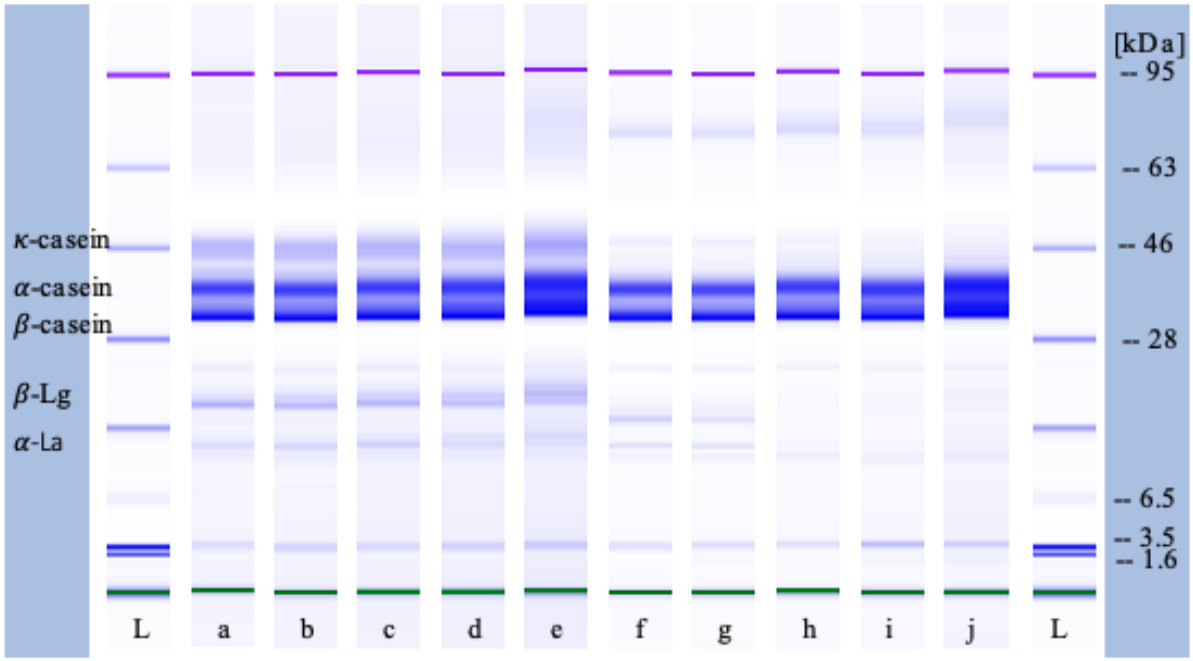


Figure 3: Microfluidic gel electrophoresis results formed during reducing (lanes a-e) and non-reducing (lanes f-j) conditions. Electrophoretic patterns are of yogurt mixes not heated (lanes a and f) and mixes heated at 70 (lanes b and g), 78 (lanes c and h), 86 (lanes d and i), and 95 ^{o}C (lanes e and j) for 30 min, while L is the ladder.

More drastic differences were observed amongst samples under non-reducing conditions (Fig. 3, lanes a-e), specifically at 12 and 18 kDa (α-La and β-Lg, respectively) and ~32-46 kDa (α-, β-, and κ-casein). The formation of complexes between denatured β-Lg, α-La and κ-casein can be interpreted as their bands dissipate at $\geq$ 78 ^{o}C (Fig. 3, lanes h-j) (Bogahawaththa et al., 2018; Jovanovic Barac, Macej, Vucic, & Lacnjevac, 2007).

When β-mercaptoethanol was added, disulfide bonds between casein and whey proteins are broken and thus, the bands for the individual proteins are present (Oldfield et al., 2005). Variation in band intensity (Fig. 3, lanes a-e) visualizes irreversible denaturation due to disulfide

interactions (Oldfield et al., 2005). While bands are in similar positions, the molecular weight appears greater suggesting protein unfolding (Anema & Li, 2003).

5.3 – Texture

The texture of yogurts, defined by firmness and adhesiveness, affects consumer perception of the yogurt gel (Delikanli & Ozcan, 2016). Significant differences ($P \leq 0.05$) for firmness and adhesiveness of yogurts are shown in Table 8. Firmness was greatest in Y16-78 and Y16-86 followed by Y16-95 while Y16-70 was the least firm, ~3-fold less. Adhesiveness, which is inversely related to firmness, was greatest (lowest value) in Y16-70 and least (highest value) in Y16-78, Y16-86 and Y16-95 (Table 8). The lack of variation in adhesiveness and firmness in Y16-78. Y16-86 and Y16-95 is a result of the high percentage of protein (6.43%) in the yogurt samples (Damin et al., 2009). At 16% MSNF, distinction in texture based on heat treatment and, thereby, thermal denaturation, was not exhibited when heating $\geq$ 78 ^{o}C when the casein/whey protein ratio was ~80/20 (Sodini et al., 2004). Mistry and Hassan (1992) reported that poor quality yogurts may result when protein content exceeds 5.6% and Damin et al. (2009) reported no variation in firmness in yogurts produced from rehydrated skim milk powder at 12, 13, 13.5, 14, or 14.5% MSNF (4.65 to 5.2% protein) that had been heated at 90 ^{o}C for 5 min.

Table 8: Texture and quality indices of yogurts made from mixes with 16% milk solids nonfat heated at four different temperatures (70, 78, 86 and 95 ^{o}C).

Yogurts	Texture		Quality Indices	
	Firmness (g)	Adhesiveness (g.sec)	Graininess (grains/ 1 g)	WHC (%)

Y16-70	237.53^{c}±15.02	-261.54^{a}±21.17	72.78^{c}±17.44	22.28^{b}±1.05
Y16-78	684.72^{a}±46.43	-696.41^{b}±45.15	148.70^{b}±11.96	28.33^{a}±0.82
Y16-86	607.64^{ab}±66.01	-620.48^{b}±53.36	207.55^{a}±10.08	28.51^{a}±1.27
Y16-95	527.24^{b}±37.78	-606.41^{b}±38.78	236.14^{a}±28.84	27.65^{a}±0.66

$^{a-c}$ Means (n=3) in the same column with different superscripts significantly differ ($P \leq 0.05$).

Yogurts, Y16-70, Y16-78, Y16-86, and Y16-95, were made from mixes heated at 70, 78, 86, and 95 ^{o}C for 30 min, respectively.

Abbreviations are WHC, Water holding capacity.

5.4 - Microstructure

In CSLM images (Fig. 4a), Y16-70 displayed a more evenly dispersed but less dense arrangement of the green area, or the protein network in the yogurts, while Y16-78, Y16-86 and Y16-95 appear to have more densely clustered proteins and a more discontinuous network (Jørgensen, Abrahamsen, Rukke, Johansen, Schüller, & Skeie, 2015). The gel network in Y16-70 appears to have more protein likely due to the lack of interactions between the β-Lg and κ-casein, and, therefore, lack of aggregation. When the mix is heated to 70 ^{o}C, few whey proteins are denatured and, therefore, are not bound to the casein micelles and, though smaller, are more dispersed and appear more numerous (Sodini et al., 2004). No red areas, which represent fat, were found in confocal images taken despite NFDM and skim milk containing <1.25 and 1.5% fat, respectively.

Microscopic images for yogurts also assist in explaining the differences reported in texture. When the mix is heated to 70 ^{o}C, native whey proteins are not bound to the casein

micelles (Fig. 4a) (Sodini et al., 2004). The gel network of Y16-70 in CLSM images (Fig. 4a) appears to have more evenly dispersed protein, which may be due to the lack of interactions between β-Lg and κ-casein, which do not occur at 70 ^{o}C. The structural differences observed in Fig. 4a-d support the quality tests done on Y16-70, in which this yogurt possessed a less firm and less adhesive texture. The gel network in Y16-95 appears to have larger protein aggregates, displayed in areas with larger, denser green aggregates which occurs as the proteins in the yogurt mix denature during heat treatment. The result of denaturation due to increased heat treatment translates to whey-casein complexes that increase in size and number while appearing less numerous due to aggregation (Hassan, Ipse, Janze, & Qvist, 2003).

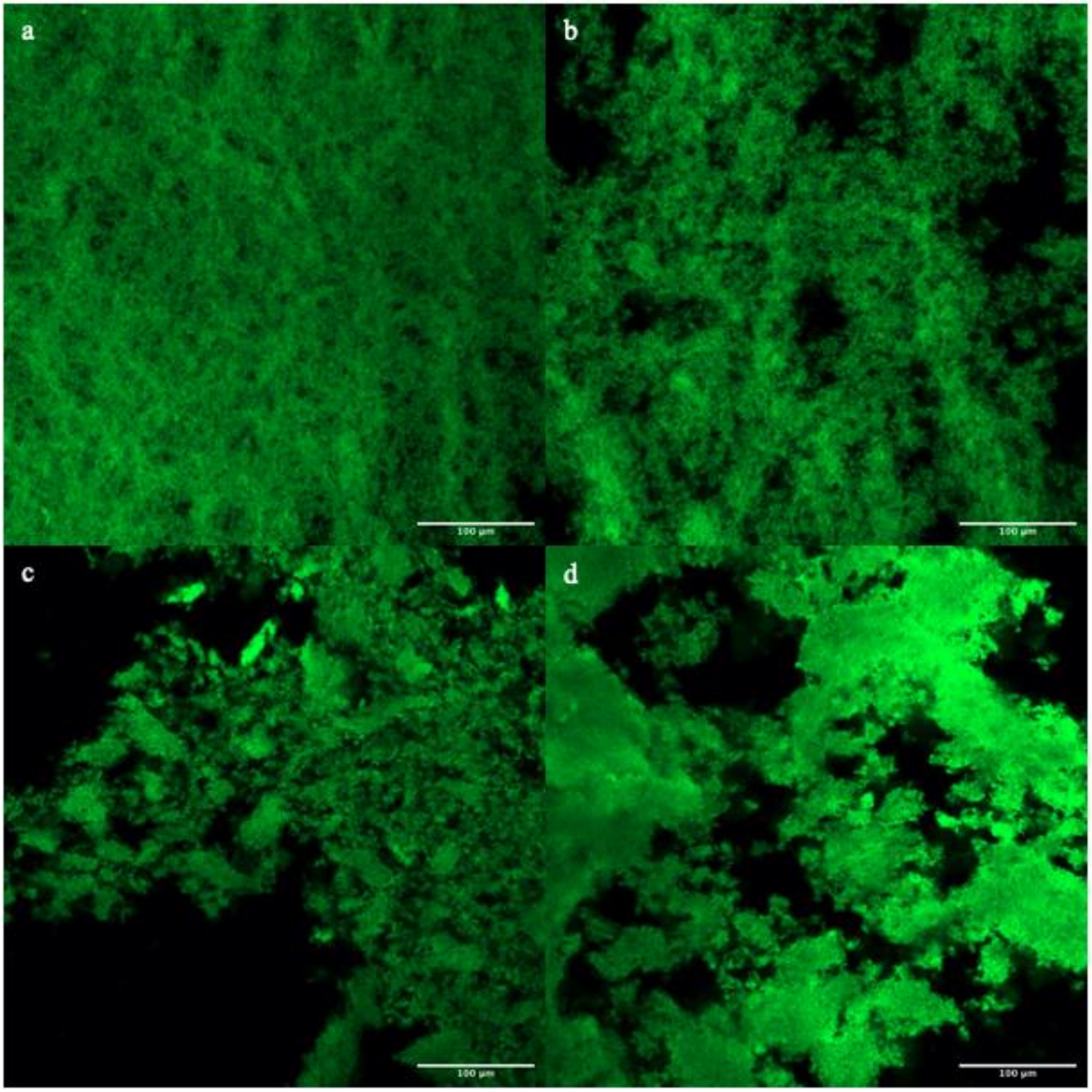


Figure 4: Confocal scanning laser microscopy images of yogurts made from mixes heated at 70 (a), 78 (b), 86 (c) and 95 ^{o}C (d) for 30 min.

Proteins and the protein network are stained green. Bars equal 100 μm.

5.5 - Quality Indices

Quality indicators, which include WHC and graininess, of yogurts are in Table 8. Statistically, Y16-70 had less WHC than Y16-78, Y16-86 and Y16-95. Syneresis was not observed in any yogurt sample. However, this study has probably not found a method to produce syneresis-free yogurt. Graininess, a negative textural attribute, was found in the least amounts in Y16-70 followed by Y16-78, whereas Y16-86 and Y16-95 had more than twice the number of grains (Table 8).

Not only do denatured whey protein/casein complexes affect firmness and rheological properties of gels, but they also affect gel quality indices (Table 8) (Körzendörfer & Hinrichs, 2019). In this case, the high MSNF, e.g., 16.58%, increased gel strength and stability, provided by the higher concentration of protein, may have discouraged structural rearrangements and gel contraction prohibiting syneresis (Lucey, 2002). Graininess, a count of the number of grains in 1 g yogurt, increased in Y16-86 and Y16-95, perhaps due to α-La/β-Lg and κ-casein aggregation, which resulted in larger protein complexes that remained intact upon stirring, yielding more numerous grains (Kücükcetin, 2008).

Overall, results show the interactions between the β-Lg/κ-casein aggregation occurs due to thermal denaturation. Evidence supported by the CSLM images, and proof that protein bonds result from disulfide interactions, further confirm that variation in firmness is due to protein denaturation. When the mix heat treatment $\geq$ 78 ^{o}C, differences in whey protein aggregation and whey protein involvement in disulfide bonding may be responsible for variation in firmness of Y16-70 and Y16-78, Y16-86 and Y16-95 (Meletharayil et al., 2015). The texture of the gels may also be predicted by looking at the micrographs of the yogurts (Jørgensen et al., 2015). Yogurts with increased aggregation (increasing from Y16-78, Y16-86 to Y16-95) display areas of denser

protein networks and a more consistent structure between protein complexes which, in turn, produce firmer gels (Vargas et al., 2008). The size of the micelles present in yogurts may be an indicator of the yogurt texture quality, predicting the graininess and WHC of the yogurt (Remeuf et al., 2003). For example, gels that have micelles that are more distinct (Y16-95) suggest that yogurts will have higher graininess, which was observed in this study (236.14 grains/1 g) (Jørgensen et al., 2015). Branching around the micelle may provide structure and resistance to compression, increasing firmness, while also physically trapping and retaining water under force (Ercili-Cura et al., 2013).

Chapter 6 – Results and Discussion (Phase Two Study)

6.1 – Compositional profile

Overall, yogurts did not differ significantly in pH, TA expressed as lactose acid or fat content, which overall averages of 4.51, 1.32% and 0.15%, respectively. As expected, yogurts made from the 12% MSNF mixes had greater true protein (average of 3.84%) and MSNF (average of 12.17%) contents compared with yogurts made from 9% MSNF (averages of 3.01 and 9.13%, respectively). Thus, neither heat treatment nor MSNF concentration deterred yogurt production (fermentation time ~5 hr).

6.2 – Protein denaturation

6.2.1 – Protein Fractionation

During protein fractionation, the supernatant removed after acidification to pH 4.6 represents the SP, comprised of native whey proteins while the IP proteins consists of casein and denatured whey proteins (Singh & Amamcharla, 2021). Mixes statistically differ in total protein (3.20 and 4.07%) and non-protein (0.20 and 0.24%) contents as a function of concentration (9 and 12%, respectively). On the other hand, SP and IP were significantly affected by concentration and heating regimen, but not the interaction (Table 9). As expected, more SP was in the mixes containing 12% MSNF compared with mixes containing 9% MSNF and as the mix heating temperature increased, SP decreased while IP increased. Mixes heated at 85 ^{o}C had ~1.5-fold and 6-fold greater denaturation than mixes heated at 75 ^{o}C and 70 ^{o}C, respectively. It is important to note that the DD does not include denaturation that may have occurred during NFDM processing and is relative in this case to non-heated mix, which may explain why values are lower than other studies.

Table 9: Soluble protein, insoluble protein and degree of denaturation of yogurt mixes as functions of milk solids nonfat (9 or 12%) concentration and heat treatment (70, 75, or 85 °C for 30 min).

Mixes		Protein Denaturation		
		Soluble protein (%)	Insoluble Protein (%)	Degree of Denaturation
MSNF Concentration				
	9%	$0.77^{b}\pm0.28$	$2.24^{b}\pm0.28$	35.86 ± 23.79
	12%	$1.03^{a}\pm0.32$	$2.81^{a}\pm0.29$	32.06 ± 19.14
Heat regimen				
	C	$1.21^{A}\pm0.20$	$2.26^{C}\pm0.33$	-
	70	$1.09^{B}\pm0.18$	$2.39^{BC}\pm0.36$	$9.91^{C}\pm1.45$
	75	$0.79^{C}\pm0.13$	$2.63^{AB}\pm0.35$	$33.47^{B}\pm5.32$
	85	$0.50^{D}\pm0.14$	$2.81^{A}\pm0.41$	$58.50^{A}\pm7.54$

[a-e] Means (n=12) $\pm$ standard deviations in the same column with different lowercase superscripts significantly differ ($P \leq 0.05$) by concentration.

[A-D] Means (n=6) $\pm$ standard deviations in the same column with different uppercase superscripts significantly differ ($P \leq 0.05$) by heating temperature.

C is for non-heated mixes, 9 and 12% represent the MSNF concentrations. The heating regimens of 70, 75, and 85 represent the mix heating regimens of 70, 75, and 85 °C for 30, respectively.

The SP and IP contents in the yogurt mixes are key to understanding texture variations in the resulting yogurts. A decrease in SP relates to an increase in denatured whey protein/casein

micelle complex formation (Anema, 2000). When comparing SP to IP in this study, there was a decrease in SP and an increase in IP when comparing contents in control mixes to those heated at 85 °C, from 1.21 to 0.50% (SP) and 2.26 to 2.81% (IP), which indicates that heating temperature drove aggregate formation (Table 9). Values for the DD (average for heat treatments) in 9 and 12% MSNF mixes, 35.86 and 32.06%, respectively, did not statistically differ.

Qian et al (2017) calculated the DD as the ratio of whey protein in the soluble protein phase in raw and heated milk from SDS-PAGE after the quantitative scanning of the supernatant phase of milks. The DD in milks heated at 75 °C for 30 min was ~41% while milks heated at 85 °C for 30 min resulted in a DD of ~90%. Meanwhile, Sodini, Montella and Tong (2005) reported a DD that ranged ~72.9-76.7% in skim milk (enriched with NFDM) and whey protein concentrates blends (4.5% protein) after heating at 90 °C for 55 min by calculating the percentage of difference in soluble protein content before and after heat treatment. In another study, Law and Leaver (1997) reported ~75% ($\pm$ 5%) total whey protein denaturation after 20 min of heating at 80 °C in skim milk and milk with 123 and 146% whey protein and 9.1 and 20% casein of the original skim milk samples using chromatography to determine denaturation. The DD values reported in this study are lower than the values reported above likely due to several conditions. The pasteurization of skim milk using a plate heat exchanger at 105 °C for 4 sec resulted in ~17.3% denaturation, calculated as the difference in soluble protein (Akkerman, Rauh, Christensen, Johansen, Hammershøj & Larsen, 2016). The thermal processing NFDM underwent prior to being used in the yogurt mixes has been found to denature ~80 to 91% β-Lg and ~33 to 45% α-La during the preheat treatment (110 or 120 °C for 2 or 3 min, respectively). The pretreatments of yogurt mixes prior to their use in this study may result in the decreased DD values (Table 9).

6.2.2– Fluorescence spectroscopy

Data calculated for FAST index (Table 10) on the soluble protein phase using right angle fluorescence spectroscopy quantifies protein denaturation. The greater the FAST index, the greater the protein denaturation (Sun et al., 2008), and in this case that is M12-85. Values reported by Singh and Amamcharla (2021) for unheated samples with ~5% protein was 10.3 at pH 6.8, while values reported in this article were 6.39 and 8.49 for 9 and 12% TS mixes, respectively. The lower FAST index values were likely a result of the decreased protein contents (3.01 and 3.84% protein). Variations in peak height of front face spectrofluorometric spectra exist primarily during advanced Maillard products emission (Fig. 5a) and excitation (Fig. 5b), while variation in the tryptophan emission spectra exists (Fig. 5c) but is less dramatic. Advanced Maillard products emission and excitation peaks vary based on heat treatment because of the formation of advanced Maillard products (Liu, Zamora, Castillo, & Saldo, 2018). It can be assumed that the greater amount of protein in the 12% MSNF mix allows for the production of more advanced Maillard reaction products when compared to 9% MSNF mixes. The increase in advanced Maillard products emission and excitation peaks due to heating is solely an indicator of the formation of advanced Maillard products and does not necessarily indicate greater denaturation (Albani, 2015).

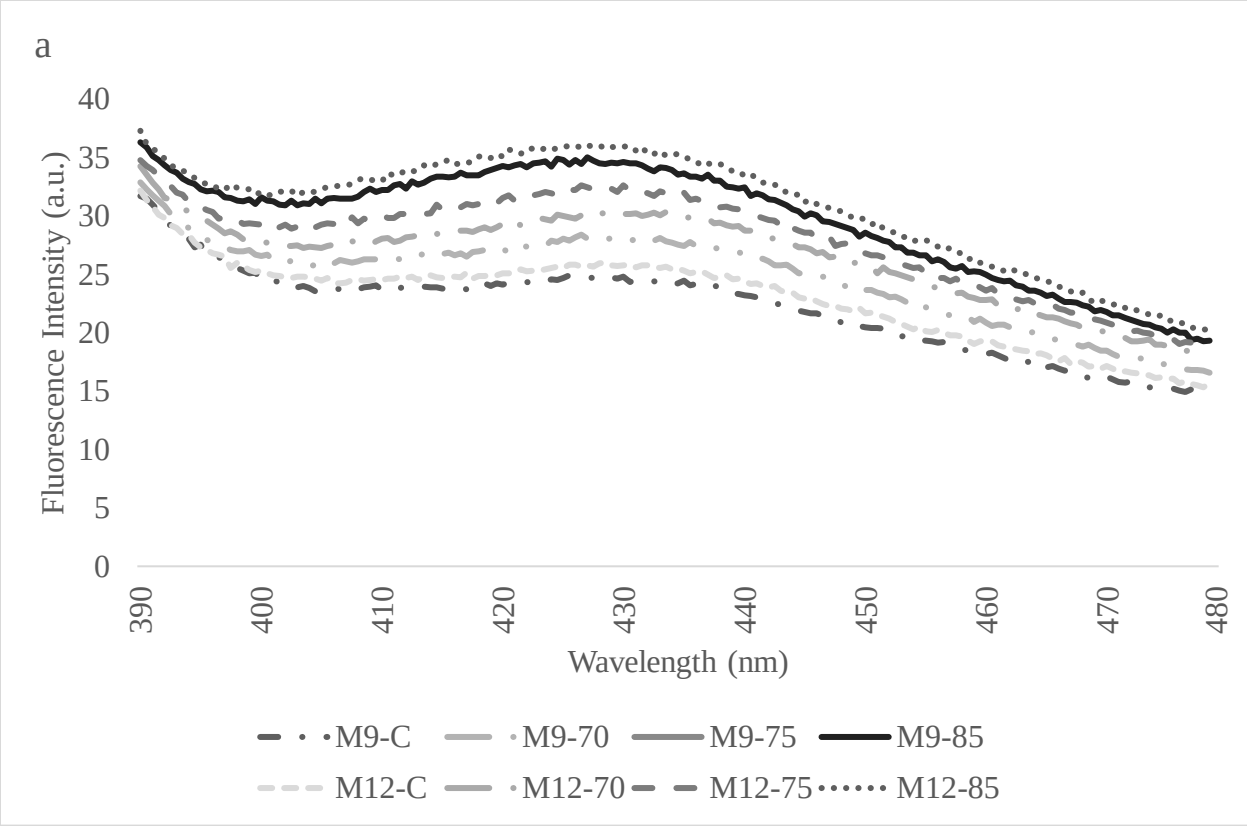
Table 10: FAST indices of yogurts as a function of milk solids nonfat (9 versus 12%) concentration and mix heat (70, 75, or 85 °C for 30 min).

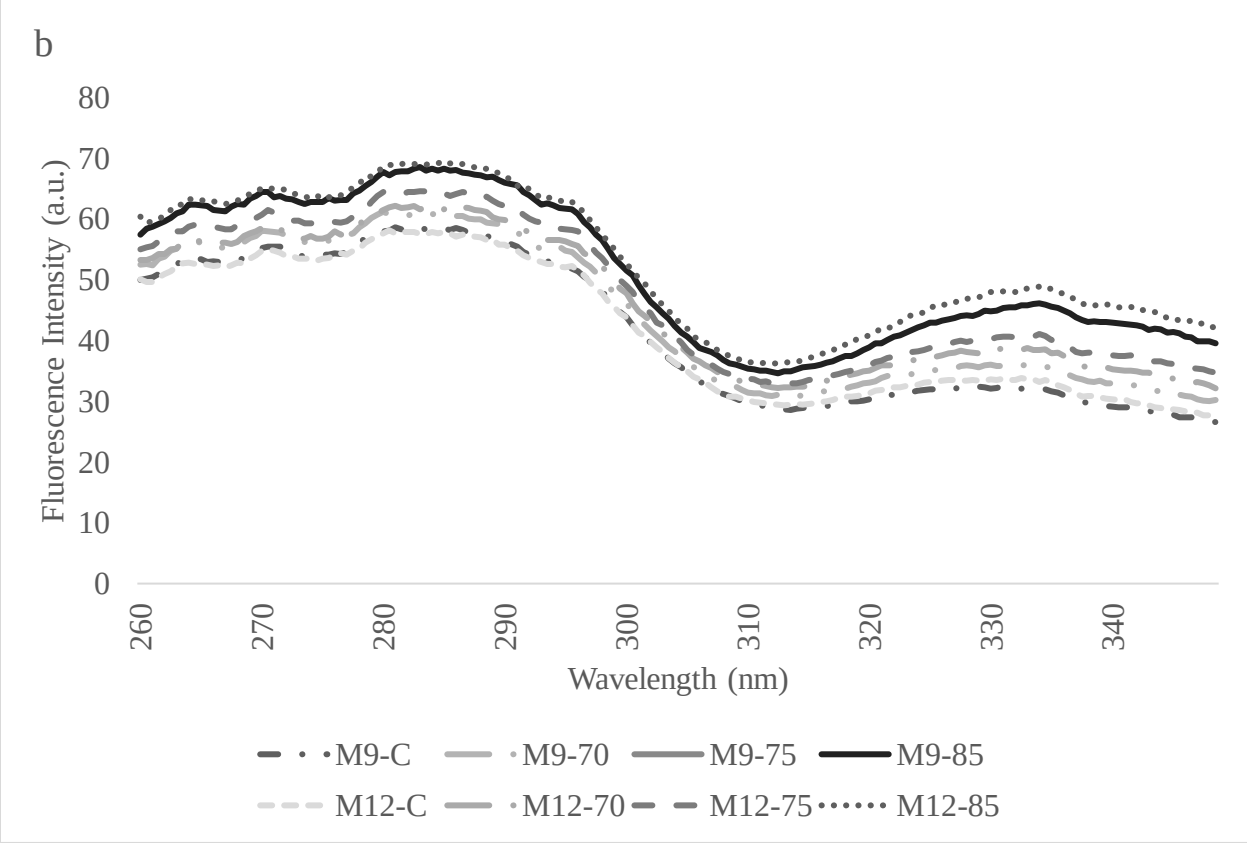
Mixes	Protein
	FAST Index
M9-C	$6.39^{f} \pm 0.16$
M9-70	$7.69^{ef} \pm 0.55$
M9-75	$12.55^{cd} \pm 0.75$
M9-85	$40.28^{b} \pm 2.45$
M12-C	$8.49^{ef} \pm 0.57$
M12-70	$10.56^{de} \pm 0.38$
M12-75	$15.21^{c} \pm 0.47$
M12-85	$54.49^{a} \pm 2.49$

[a-e] Means (n=3) ± standard deviations in the same column with different lowercase superscripts significantly differ based on interaction ($P \leq 0.05$).

Abbreviations are FAST ($100 \times F_{AMP}/F_{TRP}$): Fluorescence of advanced Maillard products and soluble tryptophan; F_{AMP}: Fluorescence of advanced Maillard products; F_{TRP}: Fluorescence of tryptophan products.

Mixes M9-C, M12-C, M9-70, M9-75, M9-85, M12-70, M12-75, and M12-85 represent 9 and 12% MSNF concentrations, not heated (C) or heated at 70, 75, and 85 °C for 30 min.


a
Fluorescence Intensity (a.u.)
0
5
10
15
20
25
30
35
40
390
400
410
420
430
440
450
460
470
480
Wavelength (nm)
M9-C
M9-70
M9-75
M9-85
M12-C
M12-70
M12-75
M12-85


b
Fluorescence Intensity (a.u.)
0
10
20
30
40
50
60
70
80
260
270
280
290
300
310
320
330
340
Wavelength (nm)
M9-C
M9-70
M9-75
M9-85
M12-C
M12-70
M12-75
M12-85

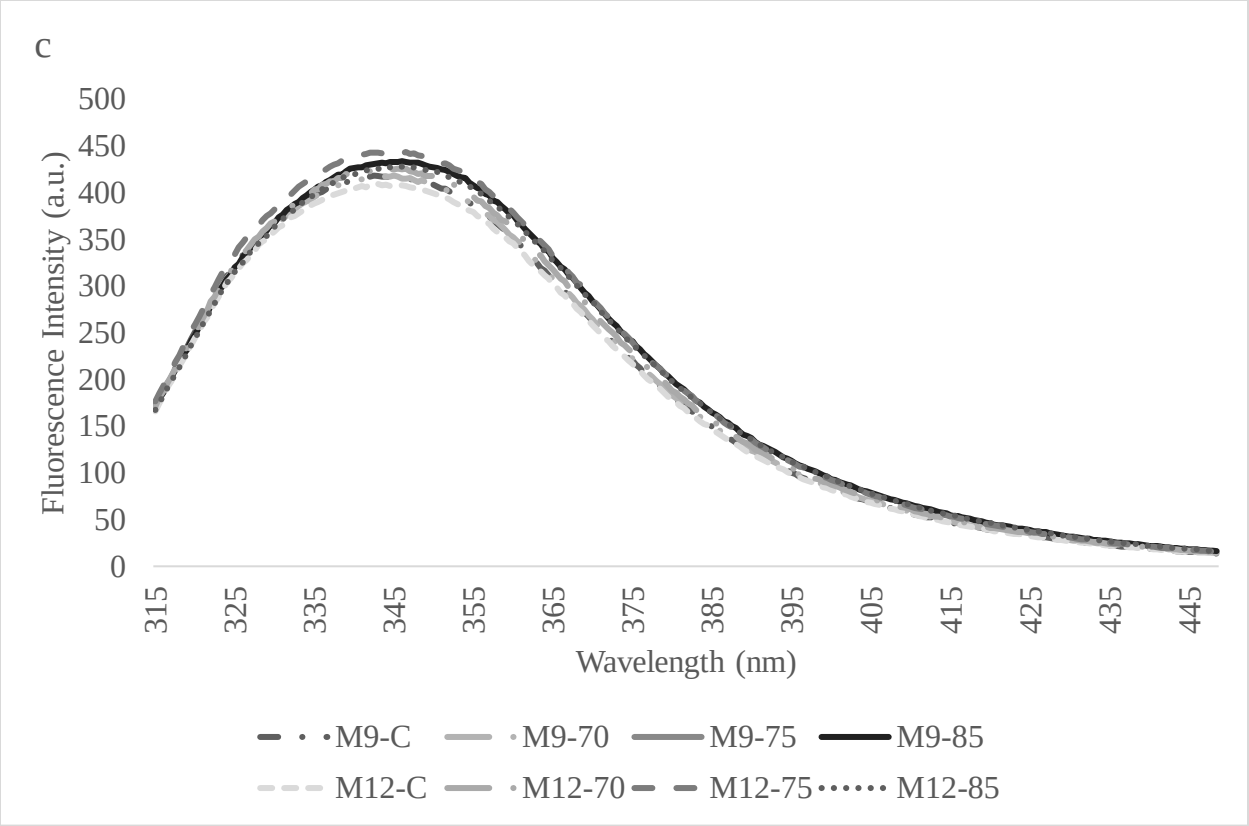


Figure 5: Averages (n=3) of front face fluorescence spectroscopy of mixes containing either 9% or 12% milk solids nonfat.

Mixes heated to 70, 75, 85 ^{o}C for 30 min, denoted as M9-70, M9-75, M9-85, M12-70, M12-75, and M12-85, respectively. M9-C and M12-C represent 9 and 12% mixes that were not heated. Front face spectra include advanced Maillard products emission (a), advanced Maillard productions excitation (b) and tryptophan emission (c).

Front face spectral images for tryptophan visualize disulfide interactions, particularly those involving β-Lg. The 19th amino acid of β-Lg, tryptophan, fluoresces if it is not involved in aggregation (Liu et al., 2018). Spectra for the non-heated mixes (M16-C) are similar to mixes heated to 70 ^{o}C, indicating few disulfide interactions occurred during this heating regimen. The

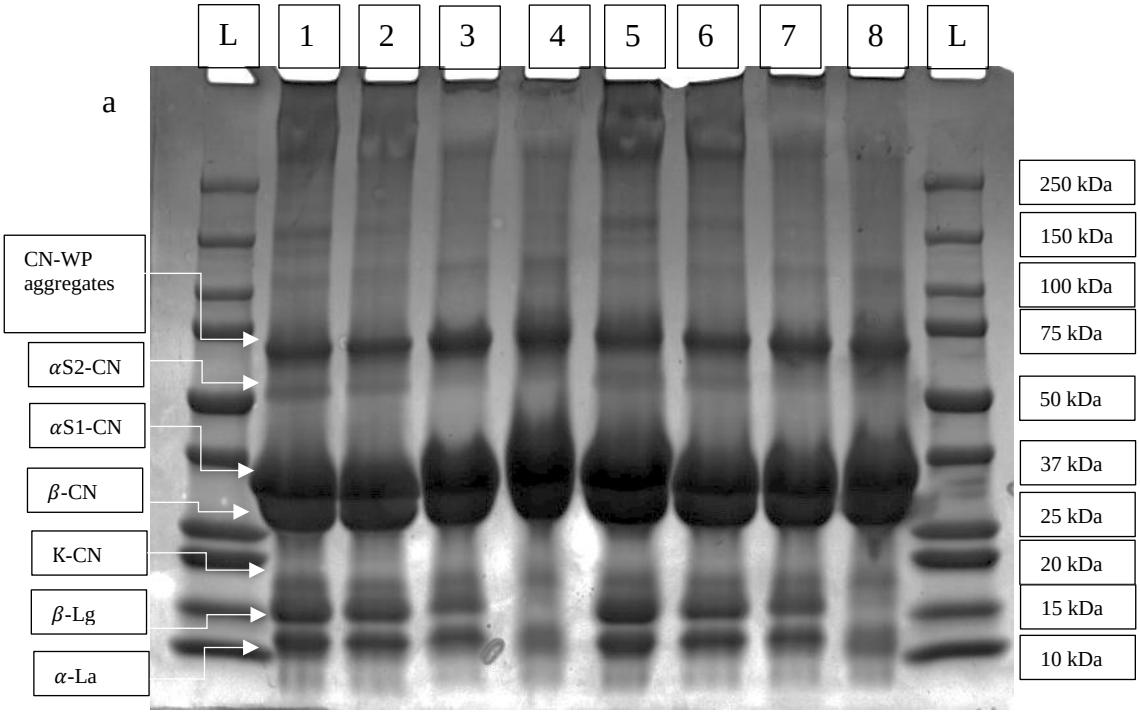
decreased peak intensity in M85 spectra compared with the M75 spectra indicate less tryptophan (amino acid 19 on β-Lg). The fluorescence of tryptophan occurs after denaturation, but prior to aggregation, when it is obscured by β-Lg/β-Lg or β-Lg/α-La aggregation (Modler & Kalab, 1983; Kronman & Holmes, 1965; Albani, 2015). A shift in the peak of tryptophan spectral images between 340 and 350 has been linked to protein-protein associations, however the minor shift in this study may be due to lower protein contents in this study (< 3.84% protein) than a study completed by Singh and Amamcharla, 2021 (5% protein). These researchers reported a decrease in tryptophan fluorescence intensity at increasing heating temperatures and concluded that when solutions contained 5% protein, prepared with ultrafiltration retentate and MPC with 80% whey protein, the degree of peak intensity decrease related to the length and temperature of the heat treatment (Singh & Amamcharla 2021).

6.2.3- Gel Electrophoresis

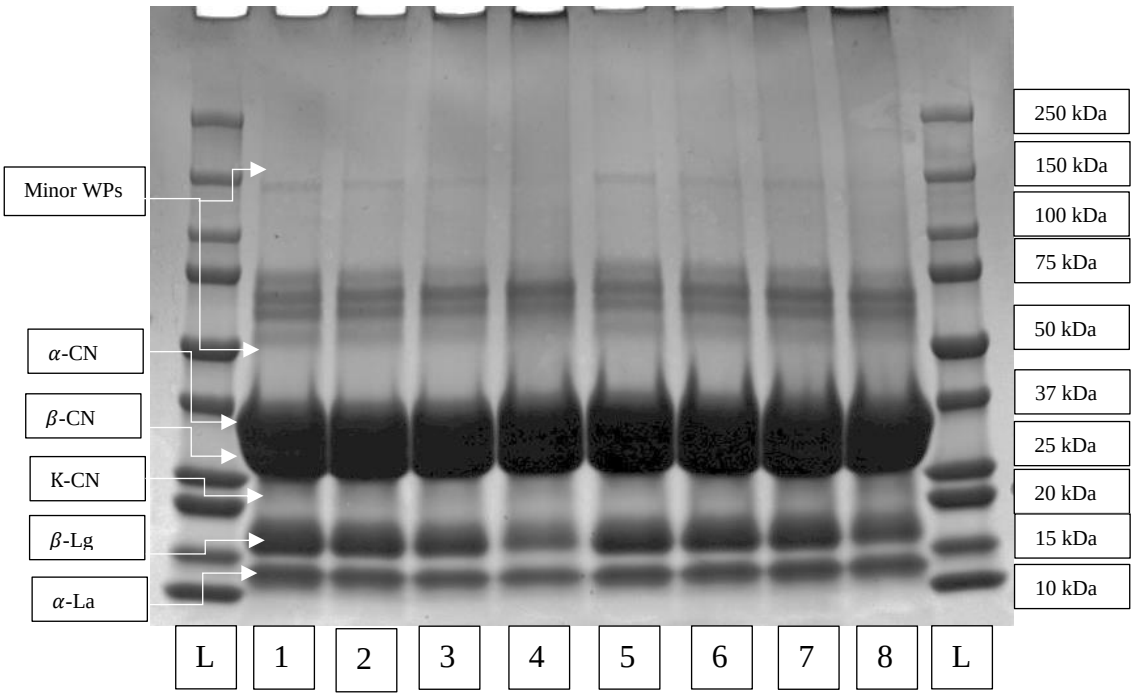
Gel electrophoresis was incorporated to visualize the movement and interactions of proteins at 9 and 12% MSNF concentrations upon heating with increasingly severe heat treatments. Gel images (Fig. 6) were captured in non-reducing (Fig. 6a), reducing (Fig. 6b) SDS, and native (Fig. 6c) PAGE conditions. Non-heated mixes, M9-C and M12-C, (Fig. 6a, lanes 1 and 5) and mixes heated at 70 ºC, M9-70 and M12-70 (Fig. 6a, lanes 2 and 6) in non-reducing conditions display all expected bands for milk proteins. Comparing M9-70, M9-75, and M9-85 (Fig. 6a, lanes 2-4) and M12-70, M12-75 and M12-85 (Fig. 6a, lanes 6-8) in non-reducing conditions allows for visualization of denaturation and aggregation resulting from disulfide interactions. At ~14, 18, and 19 kDa (α-La, β-Lg and κ-casein, respectively), bands decrease in size and intensity with increasing temperature while bands between 25 and 50 kDa remain

relatively unchanged, as β- and α-casein located between 25 and 37 kDa, respectively are more heat stable than whey proteins (Jovanovic et al., 2007). The absence of differentiation in the band at ~14 kDa in the non-heated control mix and mixes heated at 70 and 75 ^{o}C in non-reducing conditions provide further evidence that the native form α-La remained in the mixes until heating conditions reached 85 ^{o}C. Bands >70 kDa, are aggregates from the interaction of β-Lg, α-La and κ-casein, which increase in size and intensity with increased heat exposure (Jovanovic et al., 2007). Bogahawaththa and Vasiljevic (2020) reported that heating at $\geq$ 80 ^{o}C for 5 min resulted in the formation of protein aggregates between β-Lg, α-La and κ-casein increasing with denaturation, which were displayed as bands in high MW regions on non-reducing gels.

a



b



c

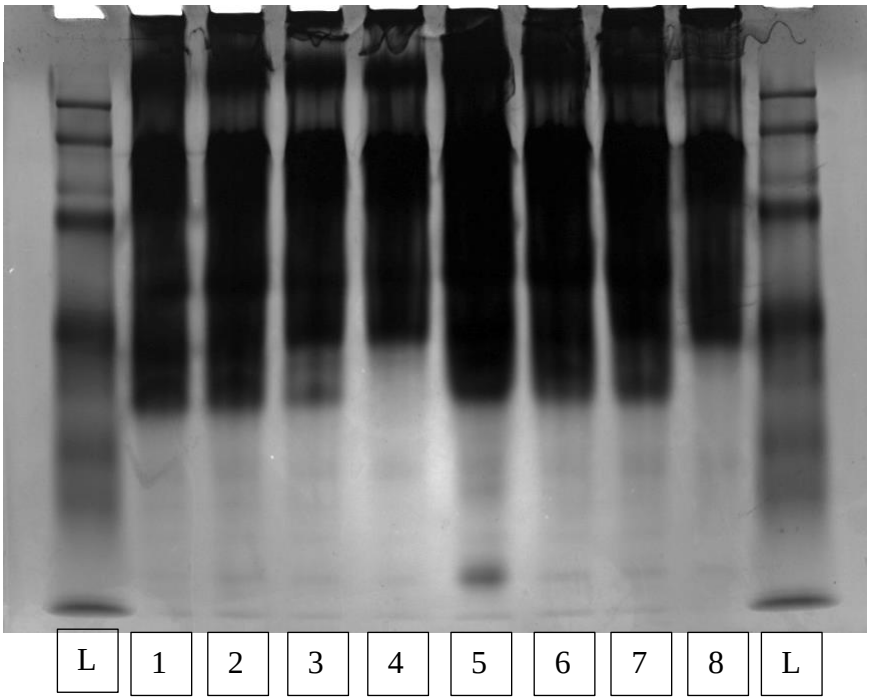


Figure 6: Effects of milk solids nonfat concentration (9 and 12%) and heating regimen (none, 70, 75, and 85 ^{o}C for 30 min) on non-reducing (a), reducing (b) and native (c) PAGE results of yogurt mixes.

Yogurt mixes contained 9 or 12% MSNF heated at 70, 75 or 85 ºC for 30 min and were denoted as M9-70 (Lane 2), M9-75 (Lane 3), M9-85 (Lane 4), M12-70 (Lane 6), M12-75 (Lane 7), and M12-85 (Lane 8), respectively. M9-C (Lane 1) and M12-C (Lane 5) represent 9 and 12% MSNF mixes that were not heated.

Abbreviations are: α-La, lactalbumin; β-Lg, lactoglobulin; CN, casein; WP, whey proteins.

The addition of β-mercaptoethanol (β-ME) (Fig. 6b) to the mixes breaks the disulfide bonds that form between β-Lg, α-La and κ-casein during aggregation; thus, the bands for individual milk proteins are present (Oldfield et al., 2005). When comparing the lanes in reducing conditions with the corresponding lanes in non-reducing conditions (Fig. 6a), bands are less distinct and exhibit greater spread between 50 and 75 kDa in reducing gels (Fig. 6b) than those bands between 10 and 15 kDa in non-reducing gels (Fig. 6a). These variations in band distinction and spread from reducing to non-reducing gels is interpreted as the dissociation of disulfide bonds, confirming that interactions of denatured whey-denatured whey proteins and/or denatured whey-casein micelle complexes were formed via disulfide interactions (Singh & Amamcharla, 2021).

In the native PAGE gels, proteins are separated based on net charge, size and shape, rather than size as in SDS PAGE (Sharma et al., 2021). Because α- and β- casein have similar charges, the bands show a continuous spread, not individual bands in the lane (Sharma et al., 2021). The “bands” in the lanes appear to be more condensed and to move to higher molecular weight regions, in particular M9-85 and M12-85 (Fig. 6c, lanes 4 and 8) reflecting protein aggregation caused by heat treatment (Pesic, Barac, Stanojevic, & Vrvic, 2014).

For this study, DD (Table 9), fluorescence spectroscopy (both FAST index (Table 10) and front face fluorescence spectroscopy (Fig. 5)) and electrophoresis (Fig. 6) individually provide partial evidence of denaturation, aggregation and bonding mechanisms between milk proteins. Gel images show the interactions between β-Lg and κ-casein more than the DD analysis and FAST index (Tables 9 and 10) and reinforce the β-Lg/κ-casein aggregation occurs as a result of disulfide interactions in M75 and M85, which were cleaved under reducing conditions (Sanchez Alan et al., 2017). Whereas the DD and FAST index indicate the heating conditions resulted in interactions between proteins as whey proteins thermally denatured. The tryptophan spectra (Fig. 5c) showed the behavior of the proteins in the mix after denaturation, but prior to aggregation, as aggregation obscured tryptophan fluorescence (Albani, 2015). From these multiple analyses it is concluded that when mix heat treatment exceeds 75 ^{o}C, different pathways occur during denatured whey protein aggregation (e.g., with or without disulfide bonding) which affects gel firmness. In combination with evidence of α-La denaturation and aggregation when heating temperatures $\geq$ 85 ^{o}C, confirmation of differentiation in the protein components of M9-85, M12-75 and M12-85, with association to increased protein concentration, may be responsible for the increase in texture from Y9-85, Y12-75 to Y12-85 (Meletharayil et al., 2015).

6.3 – Texture

Firmness, the force required to achieve deformation; adhesiveness, a negative area related to stick; and cohesiveness, the negative force achieved during probe retraction, all positively impact the perceived thickness of the yogurt gel (Delikanli & Ozcan, 2016). Mean

differentiations ($P \leq 0.05$) for the significant interactions of firmness, adhesiveness, cohesiveness, consistency, and work of cohesion are shown in Table 11.

The differences in gel structure may be attributed to denaturation and the interactions that occur between the β-Lg and α-La proteins and κ-casein when mixes are heated. No yogurts were made from control mixes as preliminary work (not disclosed) determined firmness of control yogurts was not desirable. However, it can be assumed that the texture of Y9-70 and Y12-70 resulted from minimal denaturation (reversible) to no protein interactions, as > 90% of whey proteins remain intact and do not interact with casein when heated at 70 °C, reported in a study by Bogahawaththa & Vasiljevic, 2020. When little to no protein denaturation occurs, yogurt firmness is low and syneresis is high, as no aggregation is present to support the yogurt structure (Ozcan, Horne, & Lucey). This supports the results that firmness, adhesiveness and cohesiveness of Y9-70 and Y12-70 were statistically equivalent.

Table 11: Textural characteristics of yogurts made from mixes containing either 9 or 12% milk solids nonfat and heated at 70, 75, or 85 °C for 30 min.

Yogurts	Texture				
	Adhesiveness (g.sec)	Cohesiveness (g)	Consistency (g.sec)	Firmness (g)	Work of Cohesion (g.sec)
Y9-70	$-138.61^{a} \pm 18.01$	$-86.16^{ab} \pm 3.03$	$3476.74^{b} \pm 80.84$	$128.02^{d} \pm 2.02$	$-140.09^{a} \pm 6.84$
Y9-75	$-190.96^{b} \pm 13.59$	$-90.42^{ab} \pm 4.48$	$3541.38^{b} \pm 70.19$	$152.24^{cd} \pm 2.52$	$-165.82^{b} \pm 2.67$
Y9-85	$-219.75^{b} \pm 18.92$	$-109.41^{b} \pm 2.73$	$4042.32^{b} \pm 197.72$	$173.57^{c} \pm 12.78$	$-208.89^{c} \pm 7.96$
Y12-70	$-177.82^{ab} \pm 38.8$	$-80.89^{a} \pm 18.35$	$2539.24^{c} \pm 367.2$	$152.42^{cd} \pm 5.08$	$-239.03^{d} \pm 10.30$
Y12-75	$-390.82^{c} \pm 15.12$	$-196.34^{c} \pm 15.1$	$5753.31^{a} \pm 236.29$	$242.22^{b} \pm 15.12$	$-364.26^{e} \pm 3.78$

Y12-85	-371.94^{c}±22.78	-244.77^{d}±10.05	6268.09^{a}±399.89	310.83^{a}±14.50	-474.79^{f}±13.24

$^{a-f}$ Means (n=3) ± standard deviations in the same column with different superscripts significantly differ ($P \leq 0.05$). Yogurts, Y9-70, Y9-75, and Y9-85, made from mixes with 9% MSNF and Y12-70, Y12-75, and Y12-85, made from mixes with 12% MSNF, heated at 70, 75, and 85 ^{o}C for 30 min, respectively.

During heating and subsequent fermentation of the mix, variations in the protein aggregation and attachment patterns influence the texture of the yogurt. Thus, the compression of the gel is a function of the strength and density of the protein network. Increased β-Lg, α-La, and κ-casein complexes affect the response of the yogurt gels to compression and equate increased resistance to penetration with increased consistency and work of cohesion (Delikanli & Ozcan, 2016). In this study, firmness was a function of MSNF if mixes were heated at 75 or 85 ^{o}C. However, yogurts made from 12% MSNF mixes had only 0.8% more protein than yogurts made from 9% MSNF mixes, but the firmness of the resultant heated yogurts nearly doubled when comparing Y9-85 with Y12-85, show casing the strength of the gel network as a function of protein chain density (Mistry & Hassan, 1992; Harwalkar & Kaláb, 1986). Theoretically, the increase in MSNF from 9 to 12% resulted in a ~0.64% and ~0.16% increase in casein and whey proteins, respectively (Table 9). Oldfield et al. (2005) reported that increasing the concentration of whey proteins from 0.54 to 1.16% did not increase the number of β-Lg aggregates formed via disulfide bonding; however, the increased concentration of whey proteins increased the amount of β-Lg associated to the casein micelle and the size of β-Lg/κ-casein aggregates (Haug, Høstmark & Harstad, 2007). It is important to note that while thermal denaturation of whey proteins is critical to the gel structure, the increase in available κ-casein is key to the formation of protein chains, which maximizes the firmness (Damin et al., 2009).

Comparing the individual effects of heating temperature and MSNF concentration on firmness helps explain the significance of each factor on texture. A ~2-fold increase in firmness was observed between Y12-70 and Y12-85 as opposed to the 1.2-fold increase in firmness between Y9-70 and Y9-85. This suggests that firmness would continue to increase with temperature and MSNF concentration; however, research indicates mixes heated > 90 ^{o}C have long range bridges between proteins (formed via irreversible denaturation), which disrupt the gel network, resulting in weaker gels (Needs et al., 2000). The irreversible denaturation and aggregation of β-Lg/α-La expected when mixes are heated to > 90 ^{o}C covers the exposed hydrophilic groups on the casein micelle surface, blocking any availability to form disulfide bonds between β -Lg/β-Lg and β-Lg/κ-casein (Wijayanti et al. 2014). Damin et al. (2009), Mistry and Hassan (1992) and Harwalkar and Kaláb (1986) report increased MSNF in mixes increase yogurt firmness, but firmness may plateau when MSNF exceeds 16% (Damin et al., 2009).

6.4 – Rheology

Rheological properties of yogurts analyze the breakdown of the microstructure with shear or oscillation (Paseephol et al., 2008). From the Herschel-Bulkley model, it was concluded that all yogurts produced were non-Newtonian (n <1) and exhibited shear thinning behavior (Tables 12 and 13) (Paseephol et al., 2008; Rathod & Amamcharla, 2021). A 3-fold decrease of n occurred based on concentration (9 to 12% MSNF) demonstrating that yogurts containing 12% MSNF behaved less like Newtonian fluids than the yogurts containing 9% MSNF. Concentration and temperature variations resulted in a significant difference of σ_o, which exhibited at least 2-fold increases from 9 to 12% MSNF and from 70 to 85 ^{o}C (Table 12). Paseephol et al. (2008)

stated that a greater protein concentration yielded a denser chain matrix, resulting in a gel structure with greater rigidity (σ_o values) when met with shear force which correlates with findings in this study.

Table 12: Flow behavior properties of yogurts made from mixes with either 9 or 12% milk solids nonfat concentrations heated at 70, 75, or 85 ºC for 30 min.

Treatment		Flow behavior property	
		n	σ_o (Pa)
Concentration			
	9%	$0.18^{a} \pm 0.06$	$16.66^{b} \pm 5.40$
	12%	$0.06^{b} \pm 0.01$	$33.19^{a} \pm 14.14$
Heat regimen			
	70	0.11 ± 0.06	$13.25^{B} \pm 4.53$
	75	0.13 ± 0.10	$28.87^{A} \pm 11.83$
	85	0.12 ± 0.08	$32.66^{A} \pm 14.06$

[a-b] Means (n=9) $\pm$ standard deviations in the same column with different lowercase superscripts significantly differ by concentration ($P \leq 0.05$).

[A-B] Means (n=6) $\pm$ standard deviations in the same column with different uppercase superscripts significantly differ by heat treatment ($P \leq 0.05$).

Abbreviations are: n, flow behavior index (dimensionless); σ_o, yield stress.

Concentrations are: 9 and 12% MSNF.

Heating regimens are: 70, 75 and 85 ºC for 30 min.

Table 13: Rheological properties of yogurts made from mixes with either 9 or 12% milk solids nonfat heated at 70, 75, or 85 ºC for 30 min.

Yogurts	Flow behavior properties		Viscoelastic properties	
	K (Pa.s^n)	η (mPa.s)	G' (Pa)	G" (Pa)
Y9-70	2.39^{c}±0.5	194.69^{e}±2.98	31.11^{d}±8.01	12.16^{e}±2.82
Y9-75	2.32^{c}±0.2	364.07^{cd}±31.00	90.13^{c}±2.71	36.66^{bc}±7.79
Y9-85	1.93^{c}±0.5	436.21^{c}±32.32	77.09^{c}±10.41	26.65^{cd}±2.75
Y12-70	3.80^{b}±0.4	260.66^{de}±26.78	42.24^{d}±4.76	16.72^{de}±1.60
Y12-75	4.58^{b}±0.8	790.58^{b}±49.83	121.83^{b}±1.49	41.45^{b}±0.5
Y12-85	6.53^{a}±0.2	1036.78^{a}±56.02	283.42^{a}±20.5	93.09^{a}±4.2

$^{a-e}$ Means (n=3) ± standard deviations in the same column with different superscripts significantly differ ($P \leq 0.05$).

Abbreviations are: K, consistency coefficient; η, viscosity at a shear rate of 50 s^{-1}; G', storage modulus; G", loss modulus.

Yogurts, Y9-70, Y9-75, and Y9-85, made from mixes with 9% MSNF, and Y12-70, Y12-75, and Y12-85, made from mixes with 12% MSNF, heated at 70, 75, and 85 ºC for 30 min, respectively.

Significant interactions ($P \leq 0.05$) were determined for factors of the consistency index, viscosity, storage modulus and loss modulus (K, η, G' and G", respectively) (Table 13). Similar to firmness, η exhibited a greater increase in 12% versus 9% MSNF yogurts when mixes where heated from 70 to 85 ºC (~3.98 and 2.25-fold, respectively). Torres et al. (2018) stated that when producing yogurts with NFDM and various increments of whey proteins, increasing the heat exposure resulted in greater denaturation and protein-protein interactions, which translated to

yogurts exhibiting increased viscosity and resistance to shear and oscillation. The greater the percentage of β-Lg/κ-casein complexes present, the stronger, more viscous the resulting gels (Torres et al., 2018), as demonstrated in this case by the ~2.4-fold increase in η from Y9-85 to Y12-85 due to MSNF concentration (Table 13). Lucey, Tamehana, Singh and Munro (1998) reported that denatured whey protein/casein micelle complexes, formed via disulfide bonds, increased viscosity, storage and loss moduli. When comparing Y12-70 to Y12-85 (Table 13) shows a ~6-fold increase in G' and G". The ability of proteins to interact with the surrounding proteins, forming bonds between β-Lg/β-Lg, β-Lg/ α-La and/or β-Lg/κ-casein, have also been associated to greater storage and loss moduli (Lucey, Tea, Munor & Singh, 1997). Results from rheological data corresponds to the results from texture profile analysis, highlighting that increased denatured whey protein-casein complexes, yields greater resistance to force, either via compression, shear or oscillation (Jøgensen et al., 2015; Torres et al., 2018).

6.5 - Quality Indices

Table 14 includes other quality indices, such as syneresis, TS, WHC, and WI of yogurts. The presence of syneresis in yogurts is a negative textural attribute and was the greatest in Y9-85 followed by the Y9-70 and Y9-75, which had > 3-fold less syneresis. Yogurts made with 12% MSNF had ~16-fold less syneresis than Y9-85 and did not differ based on the heating regimen. The WHC of yogurts made with 9% MSNF was ~15% less than those made with 12% MSNF. Values for WI show all treatments of Y9 were closer to white than Y12, regardless of mix heating temperature.

Table 14: Quality characteristics of yogurts made from mixes with either 9 or 12% milk solids nonfat concentrations heated at 70, 75, or 85 °C for 30 min.

Yogurts	Quality Index	Treatment		Quality Indices		
	Syneresis (%)			MSNF (%)	WHC (%)	WI
Y9-70	$2.09^{b}\pm0.5$	Concentration	9%	$9.13^{B}\pm0.07$	$46.80^{B}\pm2.55$	$87.36^{A}\pm0.14$
Y9-75	$2.31^{b}\pm0.2$		12%	$12.17^{A}\pm0.07$	$62.04^{A}\pm3.61$	$86.51^{B}\pm0.27$
Y9-85	$7.38^{a}\pm0.4$					
Y12-70	$0.25^{c}\pm0.05$					
Y12-75	$0.42^{c}\pm0.2$					
Y12-85	$0.73^{c}\pm0.02$					

[a-e] Means (n=3) ± standard deviations in the same column with different lowercase superscripts significantly differ ($P \leq 0.05$) by interaction.

[A-B] Means (n=9) ± standard deviations in the same column with different uppercase superscripts significantly differ ($P \leq 0.05$) by concentration.

Abbreviations are: MSNF, Milk solids nonfat; WHC, water holding capacity; WI, Whiteness index.

Yogurts, Y9-70, Y9-75, and Y9-85, made from mixes with 9% MSNF, and Y12-70, Y12-75, and Y12-85, made from mixes with 12% MSNF, heated at 70, 75, and 85 °C for 30 min, respectively.

Concentrations are: 9 and 12% MSNF.

Gel quality indices (Table 14) directly result from the type and amount of whey protein-casein complexes formed (Körzendörfer & Hunrichs, 2019). When yogurts contained 12% MSNF, increased gel strength and stability, provided by a higher concentration of protein, may have discouraged structural rearrangements and gel contraction thus decreasing syneresis

(Lucey, 2002). Viewed as a defect in yogurts, syneresis may be controlled by increasing the MSNF of yogurts, which been proven in this study and through literature (Akalin et al., 2012; Aryana & Olson, 2017; Das et al., 2019).

Increasing MSNF resulted in gels with greater protein chains, and a greater ability to retain water (Sodini et al., 2004). Literature reports that the greater the branching observed in the yogurt microstructure due to increased MSNF, the greater the stability of the gel structure under force, such as gravitational, resulting in greater WHC (Remeuf et al., 2003; Mahomud et al., 2016). Likewise, greater branching corresponds to a decreased porosity of the gel microstructure and is associated with greater WHC and firmness (Meletharayil et al., 2015).

Color changes in yogurts have been attributed to whey protein denaturation and the protein complexes formed in the yogurt mix (Vargas et al., 2008) (Table 14). As the gel network forms, the position or alignment of the proteins varies the color of the gel, which is a function of the amount of light reflected off the surface. Therefore, yogurts with lower WI are associated with increased protein aggregation (Needs et al., 2000).

Chapter 7 – Research Summary

In the phase one study, it was concluded that at 16% MSNF, the firmest yogurts were produced from mixes heated at 78, 86 and 95 ^{o}C. Additional heat did not benefit texture, as yogurts produced from mixes heated to 95 ^{o}C exhibited a decrease (not significant) in firmness. However, the decrease in quality, exhibited by the increased graininess of the yogurt produced from the mix heated at 95 ^{o}C may be a result of the irreversible aggregation of α-La onto the casein micelle surface, which decreased the hydrophobicity of the aggregates.

The lack of variations in yogurt texture at 16% MSNF due to heating regimen $\geq$ 78 ^{o}C was due to the high percentage of protein present in the mix. The temperatures used, specifically 78 and 86 ^{o}C, may have incorporated protein denaturation that was not intended, as β-Lg denaturation may have been nonreversible at 78 ^{o}C and α-La was involved in casein micelle complexes at 86 ^{o}C. Therefore, heating temperatures were adjusted for phase two study to 75 and 85 ^{o}C, in order to deter irreversible denaturation of β-Lg at 75 ^{o}C and maintain a low rate of aggregation with α-La at 85 ^{o}C, allowing for a more accurate assessment of the effects of denaturation and aggregation on yogurt firmness. Based on the phase one study, heating yogurt mix greater than 78 ^{o}C was not beneficial to yogurt quality.

In acknowledgment of the importance of protein concentration on gel firmness, it was hypothesized that decreasing the MSNF would allow for distinction in yogurt firmness based on heating temperature. Overall, for phase two study it may be concluded that increased heating regimens and higher levels of MSNF produce firmer, more viscous yogurts, or yogurts produced from mixes heated at 85 ^{o}C for 30 min with 12% MSNF. Further research to determine how β-Lg, α-La and κ-casein aggregate at different MSNF and the resulting gel structure formation is needed to understand the effect of denaturation and aggregation rate on yogurt firmness. The

potential for additional denaturation and aggregation may facilitate the production of firmer yogurts.

Chapter 8 – References

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Chapter 9 – Production and formulation of plant-based dairy food alternatives – A review

9.0 – Abstract

Plant-based dairy alternatives are becoming increasingly popular as consumer demand for products developed with plant-based ingredients grows. Plant-based milks, produced from either tree nuts, seeds, grains, or legumes, offer many nutritional benefits which increases their appeal to consumers. The production of dairy foods with plant-based ingredient substitutions poses several challenges to the physical, chemical and sensory properties of the product. Analysis techniques, such as melting behavior and overrun in ice cream or nondairy frozen dessert, or firmness and rheology in yogurt, may be monitored during nondairy food processing to assess similarities and differences. Sensory properties, such as taste, texture, aroma, and flavor, are critical to the consumer acceptability of dairy foods, and may rely on the functional properties of cow's milk. When dairy alternatives are utilized, the resulting product may have negatively perceived qualities. This review discusses the potential of plant-based milks to produce nondairy food products and the challenges that arise during production affecting the quality characteristics and consumer acceptance of the final product.

9.1 – Introduction

Plant-based milks provide many benefits, including high levels of protein, vitamins, and minerals, and may also offer a reduction in the risk of cancer or cardiovascular diseases, depending on the source of milk (Dervisoglu, Tazici, & Aydemir, 2005; Aboulfazli, Baba, & Misran, 2014). Plant-based milks do not contain milk proteins, lactose, or cholesterol, thereby

reducing the risk of allergy-related concerns, which pose a threat to ~80% of the population worldwide (Ahmadian-Kouchaksaraei, Varidi, Varidi, & Pourazarang, 2014; Huang et al., 2020). However, the production of dairy foods, such as yogurt or ice cream, with plant-based milks presents multiple challenges (Aboulfazli, Baba & Misran, 2015), not the least of which include consumers describing the texture as hard or gummy with beany off-flavors, especially with products are produced from soymilk (Aboulfazli et al., 2014; Ahmadian-Kouchaksaraei et al., 2014). Further, additional ingredients must be incorporated in order to mimic the texture, functional and sensory characteristics in the production of nondairy yogurts (Grasso et al., 2020).

Milk has been consumed since 7000 BC and is defined in the United States as the lacteal secretion collected from cows that is free of colostrum (Chalupa-Krebzdk, Long & Bohrer, 2018; 21CFR131.110, 2020). The consumption of milk is driven by its taste, odor, appearance and nutritive properties, contributing protein and fat and accounts for 52 to 67% of the recommended dietary intake of calcium in the U.S.; however, U.S. consumers purchase ~20 L less per capita per year in 2016 compared to 1997 (Chalupa-Krebzdak et al., 2018). The composition of milk is complex, existing as a nutrient delivery system, comprised of fat globules and proteins distributed as a colloidal dispersion. After some basic processing steps, such as homogenization and pasteurization, milk offers functional properties including emulsification, gelation, foaming, whipping and more (McClements, Newman & McClements, 2019). Milk is commonly incorporated as an ingredient in baked goods and used as a base in dairy foods, such as yogurt and ice cream. Milk solids nonfat (MSNF) includes all solids in milk except the milk fat (e.g., milk protein, carbohydrates (almost all lactose), vitamins and minerals) (Abdel-Haleem & Awad, 2015). Traditionally, nonfat dry milk (NFDM) may be added to boost MSNF in dairy foods, which benefits the structure and increases the protein content in ice cream and yogurt (Zhang &

Goff, 2003). The role of milk proteins, specifically whey and casein, in ice cream is to provide structure by stabilizing foam, while protein interactions in yogurts form the gel structure (Zhang & Goff, 2003; Aryana & Olson, 2017). In contrast, milkfat imparts a characteristic creamy mouthfeel and the unique properties of the milkfat globule provide partial stability to the food system (Mohan, Hopkinson & Harte, 204). Because milkfat is expensive, manufacturers are motivated to find alternative ingredients (Mostafavi, 2019); however, mouthfeel, flavor perception, and texture are negatively affected with a reduction or substitution in fat source (Chauhan, Lim, Powers, Ross, & Clark, 2010). Producing plant-based milk substitutes, colloquially referred to as milk despite not fitting the definition of the word, is motivated by consumers as they seek products that fit current diet fads and trends towards sustainability and environmentally conscious choices (Haas, Schnepps, Pichler & Meixner, 2019).

Ice cream is a popular dairy food primarily consumed as a dessert, consisting of air, water, fat, and protein (Akhtar, Blakemore, Clayton & Knapper, 2009) and is comprised of ice crystals, air bubbles, fat globules and an unfrozen serum phase (Mo et al., 2018). Base ingredients: milk, cream and sugar, are typically combined with hydrocolloid stabilizers, emulsifiers, flavors, colors, variegates and other inclusions, to produce the wide variety of flavors and styles available in the market (Abdel-Haleem & Awad, 2015; Muse & Hartel, 2004). While in the past, high fat ice creams dominated the market, today the number of frozen desserts that are low-calorie or adapted to specific diets are increasing rapidly (Bullock, Lahne, & Pope, 2020). Consumer trends towards a more health conscious diet that is low in carbohydrates and/or fat, and fads such as vegan or keto diets, have encouraged manufacturers to formulate ice creams made with non-dairy alternatives (Pereira, Resende, Abreu, Giarola, & Perrone, 2011). Plant-based milks, such as soy, coconut, oat or almond, are used in ice cream formulations, however,

in the U.S. the plant-based product is labeled as a nondairy frozen dessert (NDFD) (Warren, 2006). The market for NDFD is expected to reach $1.2 billion globally by 2025 (Bullock et al., 2020).

Yogurt is a fermented dairy food produced by the conversion of lactose into lactic acid by bacteria, specifically *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* cultures (Das, Choudhary & Thompson-Witrick, 2019). When produced with cow's milk, the yogurt gel structure relies on protein-protein interactions, which occurs during the heat treatment of the mix and subsequent protein gelation, which occurs during acidification (Aryana and Olson, 2017). Transforming yogurts into a plant-based product expands the opportunities of use for plant-based milks through fermentation, which may occur with either lactic acid bacteria, bacilli or yeast (Pandey, Ritz, & Perez-Cueto, 2021; Tangyu, Muller, Bolten, & Wittman, 2019). When producing plant-based yogurts (PBY) to have similar textural, nutritional and sensory characteristics as dairy-based yogurts, challenges arise, including off flavors and weak or unstable gels (Tangyu, Muller, Bolten & Wittmann, 2019). Interactions between plant proteins either through heating or fermenting, tend toward protein flocculation and phase separation, due to plant protein's low molecular weight and low hydrophobicity (Shi, Kraft & Guo, 2020).

In order to combat the unpleasant textural and sensory attributes, often stabilizers, hydrocolloids and/or flavoring agents are incorporated in the PBY formulation (Tangyu et al., 2019; Zhang, Hughes & Grafenauer, 2020). It is important to note that the ability of a PBY to meet the same nutritional standards as cow's milk yogurts almost always relies on fortification with vitamins, minerals or protein (Zhang et al., 2020). Nonetheless, PBY are gaining popularity, with a 20.2% growth in 2020 compared with 2019, which consisted of $343 million in sales in the United States (Plant Based Foods Association, 2020).

This review focuses on the formulation and production of plant-based dairy food alternatives, including milk, frozen desserts and yogurt. The effect of ingredient substitution for cow's milk with milk extracted from coconut, grains, legumes, nuts or combinations of both on texture, structure and sensory properties are presented. While in some instances, the plant-based ingredient substitution may function independently, in the majority of cases, additional ingredients must be added to mimic the qualities of the dairy counterpart. Each of the many different milk sources that have been used in the production of nondairy food alternatives yield products with unique characteristics.

9.2 – Production and sensory qualities of plant-based milks

Cow's milk is a complex colloidal system, consisting of protein, fat, carbohydrates and minerals (Table 15). Both the casein micelles and milk fat globules have unique structural properties, which are often exploited during the production of a dairy food. The components of cow's milk yield functional properties important to the production of dairy foods (McClements et al., 2019). The goal during the production of plant-based milks is to mimic the mouthfeel and appearance of cow's milk. Producing nondairy food substitutions that have similar physicochemical, textural and sensory characteristics to cow's milk ice creams may be challenging due to the vital role milk components have in the mouthfeel of fluid milk, the stable colloidal system in ice cream and the gel structure in yogurt (Aboulfazli et al., 2014, Loreto, Alonso-Miravalles & O'Mahony, 2020; Oyeyinka, Odukoya & Adebayo, 2019). Variations between cow's and plant-based milks affect the nutritional value, taste and consumer preference of food products. Nondairy milk has a different structure than bovine milk, as proteins in plants do not behave like milk proteins, rather than casein's flexible chains, plant proteins are compact

and do not form structures similar to dairy proteins upon association (McClements et al., 2019). To produce plant-based milks, plant matter, beans, nuts or seeds, are processed according to Fig. 7.

Table 15: Average composition of plant-based milks and cow's milks (skim and whole) presented for 100 mL of product.

Component	Soy	Coconut	Oat	Almond	Rice	Skim Milk	Whole Milk
Fat (g)	1.47	2.08	0.62	0.96	0.97	0.08	3.20
Proteins (g)	2.60	0.21	1.67	0.40	0.28	3.43	3.28
Carbohydrates (g)	4.92	2.92	5.83	1.31	9.17	4.89	4.67
Fiber	0.20	0.00	0.80	0.20	0.30	0.00	0.00
Calories (kcal)	43	31	33	15	47	91	60

Source: USDA, 2020

9.2.1 – Sources of plant-based milks

Soy milk is an emulsion high in iron, unsaturated fatty acids and niacin and is available either unflavored, sweetened or flavored (Jinapong, Suphantharika, & Jamnong, 2008; Ikya, Gernah, Ojobo & Oni, 2013). Briefly, soy milk is produced by grinding and straining soaked soybeans (Fig. 7) (Kolapo & Oladimeji, 1997). The resulting near white beverage has a similar appearance to cow's milk, contains no cholesterol or lactose and is safe for consumers with dairy or lactose sensitivities (Ikya et al., 2013). However, despite the stated benefits, consumer acceptability of soy milk limits its consumption (Oyeyinka, Odukoya & Adebayo, 2019), due to previously prominent "beany" flavors and odors, which resulted from the oxidation of

unsaturated fatty acids that occurred during production (Katayama & Wilson, 2008). Methods to combat the undesirable flavors or odors may include vacuum treatments, grinding at high temperatures or boiling beans are now incorporated as processing steps to remove volatile compounds (Silva, Silva & Ribeiro, 2020).

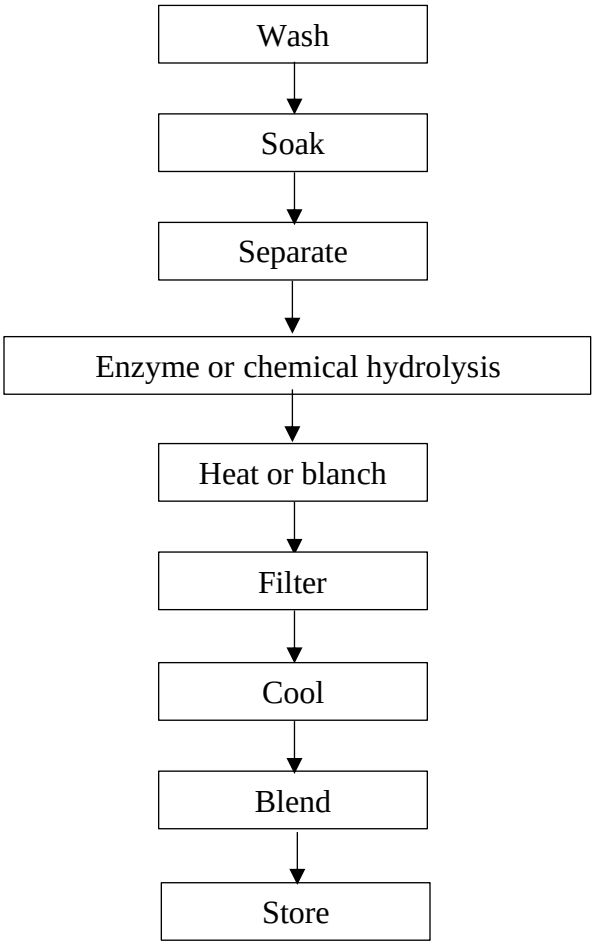

Figure 7: General process flow for plant-based milks.

Adapted from: Ashan, Zahoor, Hussain, Khalid, Khaliq, & Umar, 2015; McClements, Newman & McClements, 2019.

Figure 8: General process flow for plant-based milks.

Adapted from: Ashan, Zahoor, Hussain, Khalid, Khaliq, & Umar, 2015; McClements, Newman & McClements, 2019.

Coconut milk is the extract of grated coconut flesh primarily consumed in Asia and South America (Vanga & Raghavan, 2018). Coconut milk is digested easily and contains high levels of

calcium, phosphorus, potassium, vitamins B, C, E and antioxidants (~1.2% total minerals) (Aboulfazli et al., 2014; Olukoya & Dania, 2018). To produce coconut milk, the white flesh of coconuts is blended with water, then filtered through a sieve (Aboulfazli et al., 2014). Coconut milk contains lauric acid, which, when consumed, has been reported to lower cholesterol levels in humans (Olukoya & Dania, 2018) (Table 15).

On the other hand, oat milk is produced by filtering blended, soaked rolled oats or through enzymatic hydrolysis (Demir, Simsek, & Yildirim, 2021). Consumption of oat milk has been proven to decrease cholesterol and may reduce the risk of cardiovascular diseases in humans (Deswal, Deora, & Mishra, 2014). Oat milk is high in fiber when compared to dairy milk and is considered a relatively new product which is becoming increasingly popular (Syed, Gadhe, & Shaikh, 2020) (Table 15).

Almond milk is produced by soaking nuts in water, then grinding and straining the almond/water slurry (Topcuoglu & Yilmaz-Ersan, 2020). The strained milky liquid is high in monounsaturated fatty acids, protein, fiber, vitamin E and manganese (Vanga & Raghavan, 2018).

Rice milk is produced by mixing milled brown rice with water, however, during production, carbohydrates are degraded into sugars generating a naturally sweet taste in rice milk (Vanga & Raghavan, 2018). Although rice milk and cow's milk contain similar caloric values; rice milk has a ~50% more carbohydrates and ~90% less protein when compared to cow's milk (Vanga & Raghavan, 2018).

9.3 – Formulation and processing of nondairy frozen desserts

Some processing steps for the production of NDFD vary depending on the source of the milk, but several general steps are common. The processing steps for ice cream and NDFD are shown in Fig. 8. After the milk (plant-based or dairy) is blended with other ingredients, which may include sweeteners, flavors, emulsifiers, additional solids or fat, the ice cream or NDFD mix is pasteurized, cooled and aged (Ahsan et al., 2015). Mixes are flavored, frozen, packaged and hardened prior to storage and consumption. Variation in ice cream and NDFD dessert processing differ based on manufacturer, ingredients, flavor and available equipment. Góral et al. (2018) compared NDFD and ice creams and concluded that NDFD production was feasible with methods applied in ice creams production. Typically, ice creams and frozen desserts are evaluated on melt behavior, overrun, texture and/or sensory properties, each of which are affected by the "milk source" used in the formulation (Nistor, Pohrib, Mocanu, Constantin, & Ceclu, 2020).

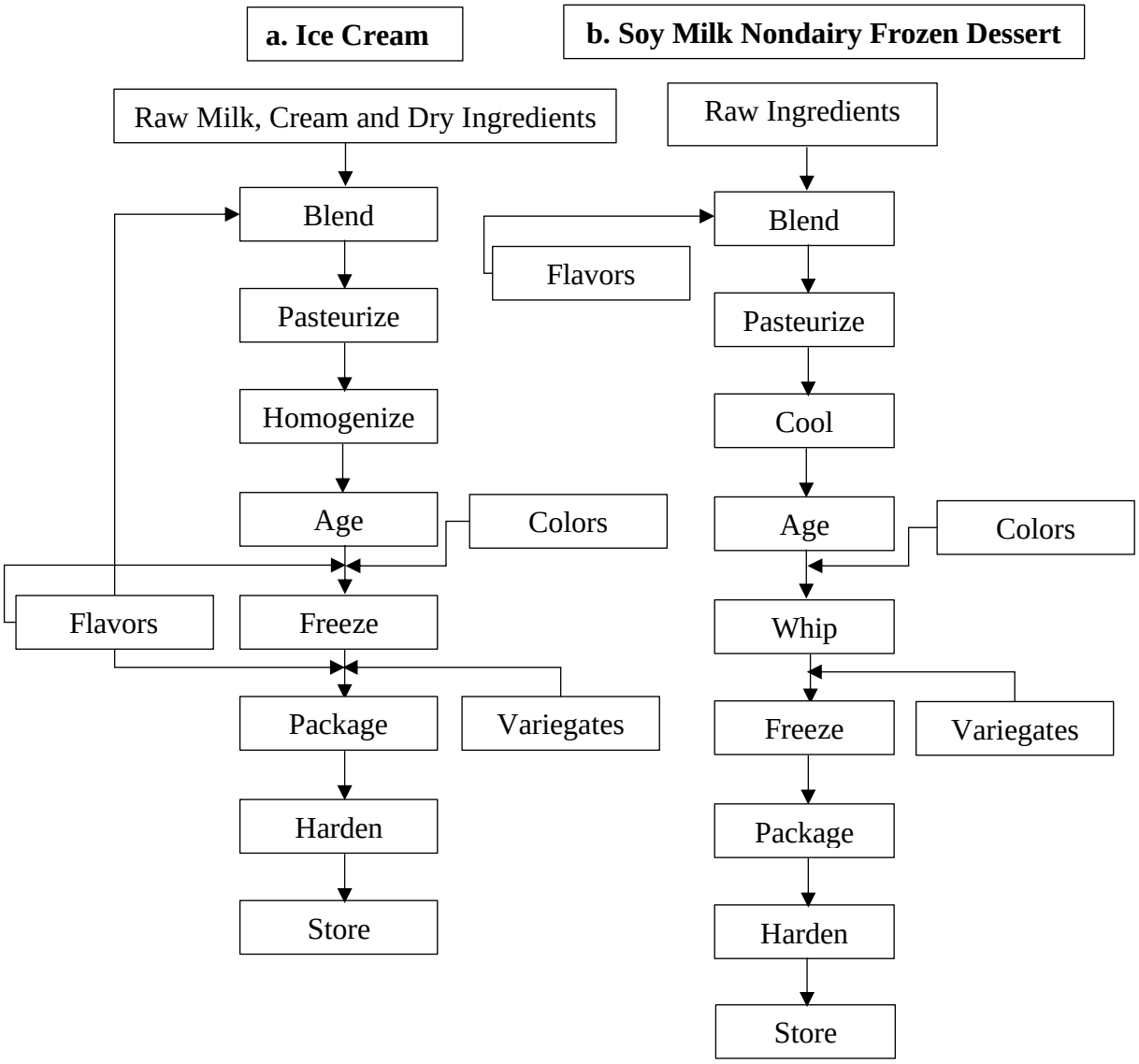

Figure 10: General process flow for the production of ice cream (a) versus a non-dairy frozen dessert (b) – depicting soy milk as the example.

Adapted from: Mohan, Hopkinson, & Harte, 2014; Hartel, Rankin, & Bradley, 2017; Ashan, Zahoor, Hussain, Khalid, Khaliq, & Umar, 2015.

Figure 11: General process flow for the production of ice cream (a) versus a non-dairy frozen dessert (b) – depicting soy milk as the example.

Adapted from: Mohan, Hopkinson, & Harte, 2014; Hartel, Rankin, & Bradley, 2017; Ashan, Zahoor, Hussain, Khalid, Khaliq, & Umar, 2015.

9.3.1 – Ingredients

Typical ingredients in ice cream and frozen desserts include milk, sweeteners, stabilizers, emulsifiers, fat, and flavors, all of which contribute a specific function to the final product (Syed et al., 2018). Sweeteners provide sweetness, flavor and increase the total solids (TS) content, depending on addition (typically ~8%) while depressing the freezing point. Stabilizers and/or emulsifiers (0.2-4% addition) are often incorporated into the mix to increase viscosity or provide melt resistance, and function by binding water molecules to stabilize the texture and structure (Góral et al., 2018). Ice cream typically contains ~3.5% protein (Table 16), which directly affects the freezing behavior (Daw & Hartel, 2015). Traditional protein sources include NFDM, whey protein isolates, whey protein or milk protein concentrates, which contribute high quality protein (Patel, Baer, & Acharya, 2006). While plant-based substitutions may include soy protein isolates (Aboulfazli et al., 2015), fat replacers, such as soy protein or vegetable fats, mimic the physical and sensory properties of milkfat in NDFD (Danesh, Goudarzi, & Jooyandeh, 2017; Puangmanee, Hayakawa, Sun, & Ogawa, 2004). Soy protein isolates are used as fat replacers in ice creams(~10-30% of TS) and are popular with consumers due to the health benefits linked to soy-fortified foods (Guo et al., 2018). Plant oils, such as coconut oil, are considered to be heart healthy, contain high levels of medium chain fatty acids, resist oxidation, contain no cholesterol and may function as milkfat replacers in NDFD (Choo et al., 2009). Flavors may be added to create variety, consumer interest or to obscure the off flavors of some plant-based milks (Syed et al., 2018; Mederiros, Filho & Bolini, 2019).

Table 16: Composition of ice creams and nondairy frozen desserts per 100 g of product.

Milk source	Total Solids (g/ 100 g)	Fat (g/100 g)	Protein (g/100 g)	Carbohydrates (g/100)	Calories (kcal)	Reference
Almond	-	7.61	3.26	28.26	185	USDA, 2020
Coconut	-	11.29	1.61	24.19	194	USDA, 2020
Cow	-	11.00	3.50	23.60	207	USDA, 2020
Oat	-	13.00	1.00	24.00	220	USDA, 2020
Rice	-	6.67	0	28.89	178	USDA, 2020
Soy	-	7.95	2.27	35.23	205	USDA, 2020
Cow	43.91	10.50	-	-	-	Aboulfazli et al., 2014
Coconut	43.16	10.40	-	-	-	Aboulfazli et al., 2014
Soy	43.94	10.50	-	-	-	Aboulfazli et al., 2014
Soy	32.17	2.52	4.06	-	-	Ahsan et al., 2015
Coconut	42.60	20.78	2.20	18.90	-	Góral et al., 2018
Jackfruit seed	-	0.19	3.04	0.24	-	Lumbantobing, Tanardi & Putra, 2019

9.3.2 – Melting behavior

Melting time is a measure of the length of time a pre-weighed amount of ice cream takes to liquify. Samples are typically placed into a mesh funnel and then drip time is recorded (Yangilar, 2016). Melting resistance, or meltdown, indicates how ice cream melts and often provides insight into the sensory characteristics of the ice cream. Factors affecting meltdown include the overrun (amount of air incorporated) and the type or network of ice crystals and fat globules. Generally, as viscosity increases, meltdown increases, which is calculated as the percentage of ice cream melted during a specific time (Patel, Dharaiya, & Pinto, 2015). Melting rate is determined by weighing the amount of melt from a pre-weighed ice cream sample over time, then plotting the ratio of drained ice cream to total sample weight, or weight of drip versus time (Akalin et al., 2018; Crizel, Araujo, Rios, Rech, & Flôres, 2014; Rawendra & Dwi, 2020).

Ashan et al. (2015) produced soymilk NDFD with varying levels (0 to 0.6%) of guar gum, which functioned as a stabilizer. Meltdown of the NDFD increased from 10.56 to 14.30% based on the addition of guar gum (0.0 to 0.6%). Similar to meltdown, the first drip time relates to quality. Dervisoglu et al. (2005) analyzed the effects of soy protein concentrate (SPC) concentration (0 to 4.5% SPC) substituted for NFDM in formulations of strawberry ice cream. At 5 ^{o}C, the viscosity of 4.5% SPC NDFD was ~3 fold greater than NDFD produced without SPC. The first drip occurred after ~10 min in NDFD with 0% SPC and ~45 min in NDFD with 4.5% SPC. The extended time for the SPC-containing NDFD was reported to be a result of the increased protein content, which resulted in a more viscous, full- bodied ice cream.

Aboulfazli et al. (2014) produced ice creams/NDFD with cow, coconut and soy milk. Cow's milk ice creams were reported to have greater melting rates, 35.88% (percent melted after 15 min), compared with coconut milk or soy NDFD, 27.00 and 16.27%, respectively. The

variation in melting rates was attributed to be a function of the difference in proteins. Whey proteins (cow's milk) have high amounts of surface activity, coconut and soy proteins do not, which reduces their solubility, decreasing the melting rate. Góral et al. (2018) produced NDFD with coconut milk and various stabilizers. The control NDFD, which did not contain stabilizers, had a first drop melt time of 25 min and a melt resistance of 21.0 mL in 45 min, whereas NDFD containing 4.0% inulin had a first drop melt time of 16 min and a melt resistance of 22.5 mL, respectively. The increase in TS, from ~42.6 to 46.9% and variations in size and number of ice crystals and fat particles were reported to be responsible for these results.

9.3.3 – Overrun

Overrun is defined as the amount of air incorporated into the ice cream during freezing (Aboulfazli et al., 2015). The whipping action and ice crystal formation combines milk proteins, partially coalesced fat and air bubbles into the structural basis of the frozen dessert. Overrun is calculated by the percent difference between the mass of a set volume of mix from the mass of the same volume of ice cream, divided by the volume of ice cream. Overrun is critical to the mouthfeel of ice cream and NDFD, contributing characteristics such as fluffiness, heaviness, and hardness, depending on the air percentage (Patel et al., 2015). In the U.S., different frozen desserts have different legal requirements for maximum overrun: 0% for frozen snacks, 20% for soft serve and super premium ice cream, 100% for ice cream, and 150% for frozen desserts, defined by the FDA (FDA, 2017).

Stabilizers have a crucial role in overrun as well and typically enhance the air incorporation process. Góral et al. (2018) reported that a control (0%) versus 4% inulin coconut milk NDFD had 8.76 to 15.31% overrun, respectively. Although, the overrun in the NDFD

increased with inulin addition, the overall low overrun was due to the lack of dairy products in the formulation (Table 17).

Ashan et al. (2015) produced soy NDFD with lab prepared and commercial soymilks. Varying amounts of guar gum (0, 0.3, 0.4, 0.5 or 0.6%) were included to analyze the effect of stabilizer on NDFD. It was reported that overrun increased from 39.42 to 60.85% when guar gum inclusion increased from 0 to 0.6%, respectively. Similar to the study completed by Góral et al. (2018), stabilizer addition increased overrun. In contrast, Aboulfazli et al. (2015) reported that overrun was constant despite the milk source: cow's milk ice cream and soy and coconut NDFD, ~29%. Additionally, NDFD made with rice or soy protein also generated similar overrun, ~42% (Medeiros, Filho and Bolini, 2019). The replacement of skim milk with soy extract, which is a spray dried protein product derived from defatted soy flour, completed by Pereira et al. (2011), at 0, 10, 20 and 30% was reported to have a significant effect on the overrun ($P < 0.05$). Overrun was greatest in the frozen dessert containing 10% soy extract addition (89.06%) compared to the frozen dessert containing 30% addition (64.60%). It was originally hypothesized by Pereira et al. (2011) that the greater the soy extract, the greater the overrun, however, it was concluded that the soy extract increased viscosity to the extent that it deterred overrun.

Table 17 – Comparison of overrun and hardness results of ice cream and nondairy frozen desserts reported in the literature.

Milk Source	Overrun (%)	Hardness (N)	Reference
Soy	39.42	19.0	Ashan et al., 2015
Whole milk powder	49.00	11.76	Medeiros et al., 2019
Rice protein	42.00	35.32	Medeiros et al., 2019
Soy protein	42.00	27.21	Medeiros et al., 2019
Coconut	8.76	2.41	Góral et al., 2018

9.3.4 – Texture

The texture attribute, hardness, is measured as the resistance to penetration when force is applied. Overrun, ice crystal size and amount, fat destabilization and the volume of the ice phase affect texture (Góral et al., 2018). An increase in hardness, measured in this case by a cone penetrometer (119.0 to 139.5 $1/10^{th}$ mm ($P < 0.05$)), is directly correlated with ice crystal formation, fat destabilization and ice phase volume (Patel et al., 2015). The larger the ice crystals, detectable in this instance through sensory analysis, the more resistance to compression, whereas "creamy" ice creams or NDFD are characterized by small ice crystals (Góral et al., 2018)

Ashan et al. (2015) analyzed the hardness of soy milk NDFD with and without guar gum addition. At 0% and 0.6% guar gum addition, the hardness was 19.0 N and 20.7 N, respectively; therefore, with 0.6% greater stabilizer addition, greater resistance to deformation is achieved. Firmness and adhesiveness of traditional cow's milk ice cream and rice and soy protein NDFD were analyzed by Medeiros et al. (2019). Values reported indicated that the greatest firmness and

adhesiveness was achieved in the rice protein NDFD (35.32 and 24.67 N, respectively) ($P < 0.05$). Firmness and adhesiveness decreased in soy protein NDFD, 27.21 and 20.17 N, and continued to decrease further in traditional ice creams, 11.56 and 5.57 N, respectively. The variation in texture was attributed to addition of cassava starch (8.5%) in rice and soy NDFD, which was incorporated as a source of fiber but functioned as a stabilizer (Table 17).

Góral et al. (2018) produced coconut milk NDFD with varying amounts of inulin (0.8 to 4%) and locust bean gum (0.2 to 0.8%) to determine their impacts on hardness and adhesiveness. The control and inulin NDFD had the lowest hardness (~2.6 N) statistically, which were ~1.7-fold less than locust bean gum NDFD (~4.4 N). The greatest adhesiveness was in NDFD containing 0.6% locust bean gum, followed by those containing 0.8 and 4.0% inulin. Overall, hardness was maximized by the type of stabilizer added (locust bean gum) rather than the amount added.

9.3.5 – Sensory properties

Sensory profiles of foods influence consumer acceptability and preference, which drives product sales. Typical sensory evaluations include hedonic tests, which are used to determine liking/acceptability. Scales for hedonic tests vary, from 5 to 100 points, where 0 may represent "extremely dislike" and the greatest point value may represent "extremely like" (Ashan et al., 2015). Flavor, odor, texture, aftertaste, melt quality, color, appearance and overall acceptability are characteristics that can be analyzed by sensory panelists (Crizel et al., 2014).

Olukoya and Dania (2018) conducted a study on unflavored, vanilla and strawberry coconut milk NDFD using a 10-point scale to evaluate liking. It was reported that strawberry NDFD scored the highest – 8.6, 8.7 and 8.3, for texture, flavor and taste, respectively, compared

with the unflavored (8.2, 8.2, and 7.5) and vanilla NDFD (7.9, 7.5, and 7.8). From this study, it was concluded that strawberry coconut NDFD were preferred.

Aboulfazli et al. (2014) compared vanilla flavored dairy ice cream to vanilla flavored soy and coconut NDFD using a 10-point scale for taste/flavor and 5-point scale for consistency or texture and appearance. The cow's milk ice cream scored highest for all attributes, 7.92, 4.04, and 4.08, followed by coconut milk, 6.50, 3.21, and 3.25, and, then soy milk NDFD, 5.08, 3.00, and 3.12, respectively. While the soymilk NDFD had improved ($P < 0.05$) viscosity and melting rate, 1120 mPa s and 16.27% respectively, compared to dairy ice cream, 363 mPa s and 27.00%, the sensory acceptability was low ($P < 0.05$). Based on these results, it was suggested that partial replacement of cow's milk with soy milk produced the best NDFD. In a follow-up study from Aboulfazli et al. (2015) further concluded that soy milk NDFD had low sensory acceptability, even though these NDFD did not have a sandy texture, crumbly body or sour taste. Atwaa et al. (2020) replaced camel milk with oat milk in NDFD at 10% increments to 40%. Flavor, body/texture, appearance and total evaluation scores (calculated out of 50, 40, 10 and 100, respectively) were highest at 40% oat milk addition, 45.3, 35.0, 8.7, and 89.0 when compared to 100% camel milk, 41.8, 24.0, 7.4 and 73.04, respectively, after 10 days of storage.

9.4 – Formulation and processing of nondairy yogurts

Plant-based yogurts (PBY) are produced similarly to dairy yogurts, through the fermentation of plant-based milk with lactic acid bacteria, however, while milk proteins stabilize during acid production, plant proteins are destabilized and weak gels result (Grasso, Alonso-Miravalles & O'Mahony, 2020). In order to combat the separation of the plant protein solution, stabilizers or gelling agents are added to mimic the gel-like texture expected from dairy yogurts.

Yogurt quality is typically described by evaluating texture, rheology, water holding capacity, syneresis, and sensory properties and these strategies are extended to PBY as well (Levy, Okun, Davidovich-Pinhas & Shpigelman, 2021).

9.4.1 – Ingredients

Ingredients in dairy yogurt include milk, additional MSNF, and culture, while sweeteners, flavors, and additional gelling agents are optional but occasionally included (Table 18) (Aryana & Olson, 2017). Plant-based yogurts also contain a milk source, such as soy, coconut, almond and others, and bacterial cultures. Other frequently incorporated ingredients include stabilizers, acidity regulators or thickeners (Grasso et al., 2020). Plant-based milks most commonly used in PBY include soy, coconut, almond or oat milk (Pandey, Ritz & Perez-Cueto, 2021).

Table 18 – Average composition of plant-based yogurts per 100 mL of product.

Component	Soy	Coconut	Almond	Skim Milk (Bovine)	Whole Milk (Bovine)
Fat (g)	1.80	3.50	3.47	0.18	3.25
Proteins (g)	3.50	0.31	2.79	5.73	3.47
Carbohydrates (g)	15.96	7.95	10.18	7.68	4.66
Fiber	0.20	0	-	0	0
Calories (kcal)	94	64	-	56	61

Source: USDA, 2020; Shi, Kraft & Guo, 2020.

9.4.2 – Texture

As previously stated, the texture of dairy foods is measured as a reaction to compression. When measuring the yogurt texture, texture profile analysis (TPA) yields results for firmness, adhesiveness, cohesiveness and consistency (Levy et al., 2021). Firmness (g) is a measure of the maximum force achieved during compression or the effort required to masticate the sample, while cohesiveness (g) is the maximum force achieved during probe retraction ratio or the force required to overcome stick (El-Zeini et al., 2018). Consistency (g*mm) is the sum of the area under the positive curve, formed during compression, or the resistance to force required to masticate the sample (Breene, 1975). Adhesiveness (g*mm) is the area under the negative curve, formed during probe retraction and the energy needed to detach the sample from contact surfaces i.e., the tongue, teeth, or palette, (Grasso et al., 2020).

Cruz et al. (2009) evaluated the effects of heat and/or pressure treatments on soy-PBY. Soymilks were exposed to ultra-high-pressure homogenization (UHPH) (200 or 300 MPa at 40 or 50 ^{o}C), ultra-high temperature pasteurization (UHT) (142 ^{o}C for 2 s) and autoclave sterilization (121 ^{o}C for 15 min) then stored. To produce PBY, soymilks were reheated to 45 ^{o}C, inoculated and fermented with lactic acid bacteria (*Streptococcus thermophilus* and *Lactobacillus delbruekii* subsp. *bulgaricus*) for 5 hr (pH 4.6). Firmness of soy PCY was the least in UHT treatment, roughly half of autoclave sterilization, ~1 N, while UHPH-treatment resulted in the firmest texture, ~3 to 5.5-fold greater than the PBY made from the autoclaved sterilization treatment ($P < 0.05$). As far as the UHPH-treatment, the firmest PBY was produced from conditions of 300 MPa at 40 ^{o}C, ~5.5 N. The poor firmness of the UHT and autoclave sterilization treatments was due to large water pockets and large fat droplets, respectively. UHPH

conditions of 300 MPa and 50 ºC, produced PBY with decreased firmness, which was attributed to greater protein denaturation resulting in protein clusters that weakened the gel ($P < 0.05$).

The type of heat treatment on oat milk PBY was studied by Demïr, Simsek and Yildirim (2021). Thermal (63 ºC for 30 min) and UV-C assisted thermal (77.67 J/mL at 60 ºC) treated oat milks and non-treated oat milk were acidified and evaluated for firmness and adhesiveness. After 7 days, PBY firmness and adhesiveness produced from raw and thermally treated oat milk were equivalent ($P > 0.05$), ~25 and 8.5 g, respectively, while yogurts produced from UV-C assisted thermally treated oat milk were ~1.8 and 1.6-fold greater, respectively ($P < 0.05$). In this study, the UV treated oat milks produced PBY, which exhibited the greatest gel stability, indicated by ~6-fold decrease in syneresis. Demïr et al. (2021) concluded that additional studies were needed to fully understand the impact of thermally assisted UV-C treatment on the microstructure of the resulting yogurts due to lack of conclusive results in this study.

Grasso et al. (2020) purchased six commercial PBY with soy, coconut, cashew, almond, and hemp milk from a local grocery store and compared textures, including firmness, consistency, and cohesiveness, to cow's milk yogurt (Table 19). Hemp PBY exhibited the greatest texture; however, the increased firmness, 1.78 N, consistency, 31.7 N*s, and cohesiveness, 1.06 N, were deemed the result of the agar and rice starch additions in the PBY formulation (ingredient contents in commercial samples were not disclosed) ($P < 0.05$). All other PBY and yogurts did not exhibit significant differences in texture (averaging a firmness of 0.54 N, consistency of 10.78 N*s and cohesiveness of 0.32 N) and it was concluded that no correlation between protein and texture existed, rather that stabilizers were successful at mimicking the texture of dairy yogurt, which was produced with only skim milk and culture (Grasso et al., 2020).

9.4.3 – Rheology

Measuring the rheological properties of yogurt describes the flow behavior and the effect of oscillation on yogurt gel structure (Grasso et al., 2020). Flow behavior analyses produces results for yield stress, apparent viscosity (measured at 200 s^{-1}), consistency coefficient (K) and the flow behavior index (*n*). Values for K and *n* are derived from the Herschel-Bulkley model, which is used to describe the flow behavior. In order for yogurts to fit the Herschel-Bulkley model, *n* must be <1, which indicates shear thinning behavior (Grasso et al., 2020). The effect of oscillation, or the viscoelastic behavior, produces values for storage and loss moduli (G' and G", respectively).

Brückner-Gühmann, Banovic and Drusch (2019) created a fermented oat PBY with 10 and 12% oat protein concentrate (OPC), suspended in oat milk and water, respectively. Oat PBY created with 10% OPC contained 17.8% MSNF while 12% OPC had 11.2% TS. Shear stress and storage moduli were greater in yogurts with 10% OPC than those with 12% OPC, ~10 and 2.3-fold, respectively ($P < 0.05$). Gels produced were described as soft fluid gels, as G' > G"; however, the 10% OPC gel had greater elasticity and the greater shear stress value indicated that more shear was required to collapse the gel structure ($P < 0.05$). The variation in the gels was attributed to the ~6% increase in MSNF in the lower protein gel, which consisted of carbohydrates, fat, and fiber. As previously stated, aggregated plant protein does not provide gel stability, whereas starches provide substance as "filler material" and decrease the amount of free water in the gel. Soy PBY produced by Donkor, Henriksson, Vasiljevic and Shah (2007) also exhibited gel like behavior (G' > G"), with $n < 1$ and K values of ~7.1 indicating PBY with expected shear-thinning behavior.

The flow behavior measured on soy, coconut, cashew, almond, and hemp PBY and dairy yogurts, produced by Grasso et al. (2020) indicated these systems exhibited shear-thinning behavior ($n < 1$) while K values (~2.9 Pa.s^n) of dairy yogurt were similar to all PBY ($P < 0.05$). The yield stress of cow's milk yogurts (11.7 Pa) was significantly less than PBY (28.5 Pa) while apparent viscosity of soy and almond PBY and cow's milk yogurts (~0.27 Pa s) was less than the coconut, cashew, and hemp PBY (~0.57 Pa s). The results were accredited to the addition of hydrocolloid stabilizers (stabilizer content in commercial samples was not disclosed) which included: maize starch and pectin (coconut PBY), tapioca starch (cashew PBY), and rice starch and agar (hemp PBY).

9.4.4 – Water holding capacity and syneresis

Water holding capacity (WHC) and syneresis in yogurt describes the behavior of whey under force or during spontaneous separation, respectively. In yogurt, WHC is a measure of the amount of water the gel retains under centrifugal force. The calculation of WHC is the difference of the weight of the removed whey, or supernatant, from the total volume of yogurt, expressed as a percent (Grasso et al., 2020). In dairy yogurts, increased WHC may result from increased MSNF or protein interactions, which are results of denaturation and aggregation caused by heating and acidification (Das, Choudhary, and Thompson-Witrick, 2019). Syneresis is the formation of whey on the surface of yogurt because of gel shrinkage (Aryana & Olson, 2017). Measured by collecting surface whey via drainage, the greater the syneresis, calculated as a percent of whey from the total amount of yogurt, the poorer the quality of the gel (Das et al., 2019). Methods to decrease syneresis, which poses a higher risk to PBY, include adding stabilizers or hydrocolloid polysaccharides, such as gums, starches, or carrageenan to function by

maintaining gel structure and prohibiting gel shrinkage over time (Das et al., 2019; Atwaa, Hassan & Ramadan, 2020).

As previously mentioned, soy-PBY manufactured by Cruz et al. (2009) were differentiated based on soymilk heat treatment. The resulting fermented gels were analyzed for WHC, which indicated that soymilks treated with UHT pasteurization (142 ^{o}C for 2 sec) produced poor quality (defined by low WHC and high whey expulsion) PBY. Unlike dairy yogurts, which benefit from denaturation, soy-PBY exhibited coarse gel networks with large gaps between proteins, decreasing the ability of the gel to retain water with a WHC ~85%. Autoclave sterilization (121 ^{o}C for 15 min) and UHPH-treated soymilks (combination of 200 and 300 MPa at 40 and 50 ^{o}C) generated PBY with a greater WHC, ~95%, due to the reduced size of the proteins and fat in the resulting soy PBY, which had a more evenly dispersed microstructure. Syneresis also improved, with similar syneresis values, ~2-7% ($P > 0.05$), in the autoclave sterilization and UHPH soy PBY, while UHT treatment generated PBY with greater syneresis, ~13 to 19% ($P < 0.05$). Ferragut, Cruz, Trujillo, Guamis and Capellas (2009) reported similar WHC and syneresis results for PBY made with UHPH-treated soymilk, ~95 and 2%, respectively. These researchers concluded that the increase in stability in the PBY was because the UHPH-treatment generated an abundance of hydrogen bonds between proteins as opposed to the hydrophobic interactions between proteins that predominated from the heat treatment. In other words, to manufacture PBY with the greatest gel stability, the hydrogen bonds must surpass the hydrophobic interactions.

Grasso et al. (2020) measured the WHC of PBY produced from soy (with and without stabilizer), coconut, cashew, almond, and hemp and cow's milk yogurt (Table 19). The dairy and soy PBY that were formulated without stabilizer addition had the lowest WHC, 75.7 and 82.8%,

respectively while the soy, coconut, cashew, almond, and hemp PBY, which were formulated with a stabilizing agent (percentage of stabilizer incorporated was not disclosed), had greater WHC, averaging ~95.9% ($P < 0.05$). The similarities of the syneresis and WHC of the soy PBY and dairy yogurts were attributed to the protein content, 4.60 and 5.10%, respectively, as the remaining yogurts contained less than 2.30% protein, while the enhanced WHC (~17%) of the remaining PBY was attributed to the pectin stabilizer (Table 19).

Table 19: Texture, water holding capacity, syneresis and rheological characteristics of yogurts made from plant-based and cow's milk.

Milk Source	Firmness	Cohesiveness	Consistency	Adhesiveness	WHC (%)	Syneresis (%)	Reference
Soy	0.46 N[a, b, c]	0.28 N[b, c]	9.98 N[a, b, c]	-	96.3[b, c, d]	-	Grasso et al., 2020
Coconut	0.44 N[a, b, c]	0.31 N[a, b, c]	10.00 N[a, b, c]	-	99.3[d]	-	Grasso et al., 2020
Cashew	0.51 N[a, b]	0.27 N[b, c]	8.71 N[a, b]	-	97.2c, d	-	Grasso et al., 2020
Almond	0.72 N[b, c, d]	0.44 N[a, b]	15.10 N[c, d]	-	91.0[a, b, c]	-	Grasso et al., 2020
Hemp	1.78 N[d]	1.06 N[a]	31.70 N[d]	-	95.9[b, c, d]	-	Grasso et al., 2020
Soy	1.33 N	-	-	-	~94.5	1.42	Ferragut et al., 2009
Soy	~1.6 N	-	-	-	~95	-	Cruz et al., 2009
Oat	~26 g	-	-	~8.8 g	-	~23	
Skim milk	0.36 N[a]	0.23 N[c]	6.81 N[a]	-	75.7[a]	-	Grasso et al., 2020

[a-d]Yogurts analyzed by Grasso et al (2020) with different superscripts within the same column significantly differed ($P < 0.05$).

0

9.4.5 – Sensory properties

Sensory characteristics of importance in yogurts include appearance, color, flavor, taste, texture, odor and/or and overall acceptability (Grasso et al., 2020; Kosterina, Yakovleva, Koniaeva & Iakovchenko, 2020). Tangyu et al. (2019) stated that overall, plant-based products are viewed by consumers as unpleasant in taste, with beany odors, green or grayish in color and chalky, sandy, or thin mouthfeel. Overcoming preconceived consumer expectations may be instrumental to increasing the willingness of consumers to purchase PBY (Tangyu et al., 2019).

Grasso et al. (2020) used hedonic testing (scale of 0 to 10, where 0 is "extremely dislike" and 10 is "extremely like") and reported that acceptability of commercial cow's milk yogurt and plain soy PBY, purchased from a local retail market, that contained pectin and acidifying agents in the formulations were similar (5.95), while plain soy PBY scored lower for acceptability (2.80) and flavor (2.54). Coconut PBY was the third most desirable, followed by cashew PBY and almond PBY, which both had flavor scores of 2.60 and 2.88, respectively. Plain soy, cashew and almond PBY scored significantly less (4.58) than flavored soy PBY, coconut PBY and cow's milk yogurts for odor (6.35).

Kosterina et al. (2020) presented results from sensory analysis on PBY produced with soy milk and with the addition of 20, 30, 40 or 50% coconut milk on a 9-point hedonic scale. The taste, texture and overall acceptability of yogurts was significantly greater ($P < 0.05$) with 30% coconut milk PBY (~7.9, 7.4 and 7.8, respectively) while 20% coconut milk addition PBY performed poorly in all categories, flavor, color, taste, texture, and overall acceptance (~4.8, 6.7, 4.6, 5.8 and 5.7, respectively). Flavor and color of the PBY did not vary with 30, 40 or 50% coconut milk inclusion (~7.3 and 7.7, respectively), however, PBY with 40 and 50% coconut milk addition decreased ($P < 0.05$) scores for taste, texture, and overall acceptance. Kosterina et

al. (2020) concluded that while the inclusion of coconut milk deters off flavors and odors found in primarily soymilk PBY, increasing the concentration of coconut milk beyond 30% was not beneficial.

9.5 – Conclusion

Overall, the production of plant-based dairy alternatives from sources such as soy, coconut, oat, or almond milk may result in products with similar characteristics as dairy milk, ice cream and/or yogurt. Some plant-based milk substitutions give rise to products comparable to their dairy counterparts although additional ingredients, such as gums or flavors, may be required to mitigate the differences in physical, chemical and/or sensory properties. It may be concluded that nondairy plant milks have the potential to be a viable substitute for cow's milk, meeting increasing consumer demand for such foods. Further research on the acceptability and preference for plant-based foods will expand the market for nondairy foods with the evolution of products that are indistinguishable to consumers based on milk source.

Chapter 10 – References (Review)

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Appendix A – SAS Codes (Phase One Study)

SAS code for yogurts produced from mixes heated at 70, 78, 86, or 95 ºC for 30 min.

```
data yogurt;
input Rep Temp pH TA MSNF WHC Syneresis Graininess Adhesiveness
G1 G2 A Ar t ap k n;
datalines;

proc print data = yogurt;
run;

proc means data =yogurt;
     class temp;
run;

ods graphics on;

proc glm data=yogurt;
     class Rep temp;
     model pH TA MSNF WHC Syneresis Graininess Adhesiveness G1
G2 A Ar t ap k n = Rep Temp;
     lsmeans temp / adjust = tukey;
     random rep;
run;
```

```
data mixes;
input Rep $ Temp $ Fast SP IP WPD TP NCP NP;
datalines;
```

SAS code for control mixes and mixes heated at 70, 78, 86, or 95 ^{o}C for 30 min.

```
proc print data = mixes;
run;

proc means data = mixes;
     class temp;
run;

ods graphics on;

proc glm data = mixes;
     class Rep temp;
     model Fast SP IP WPD TP NCP NP = Rep Temp;
     lsmeans temp / adjust = tukey;
     random rep;
run;
```

Appendix B – SAS Codes (Phase Two Study)

SAS code for yogurts produced from mixes heated at 70, 75, or 85 ºC for 30 min.

```
data yogurt;
input Rep Conc Temp Firmness Adhesiveness Consistency
Cohesiveness WCohesion Syneresis WHC FAST L a b WI Storage Loss
MSNF WPD Graininess pH TA yldstrss viscosity k n;
datalines;


proc print data = yogurt;
run;


proc means data = yogurt;
     class conc temp;
run;


ods graphics on;


proc glm data = yogurt;
     class Rep conc temp;
     model Firmness Adhesiveness Consistency Cohesiveness
WCohesion Syneresis WHC FAST L a b WI Storage Loss MSNF WPD
Graininess pH TA yldstrss viscosity k n = rep conc temp
conc*temp;
```

```
     lsmeans temp conc conc*temp / adjust = tukey lines;
run;
```

SAS code for non-heated control mixes and mixes heated at 70, 75, or 85 ^{o}C for 30 min.

```
data mixes;
input Rep Conc Temp Fat T NCP NP TP SP IP;
datalines;

proc means data = mixes;
run;

proc means data = mixes;
     class conc;
run;

ods graphics on;

proc glm data = mixes;
     class Rep conc temp;
     model Fat T NCP NP TP SP IP = rep conc temp conc*temp;
     lsmeans rep temp conc conc*temp / adjust = tukey lines;
run;
```

Appendix C – Raw data and ANOVA (Phase One Study)

Table C 1: Averages (n=3) for analyses performed yogurts produced from heated mixes, M16-70, M16-78, M16-86 or M16-95 (70, 78, 86, or 95 ^{o}C for 30 min, respectively) for three replications.

Rep	Temp	pH	TA (%)	MSNF (%)	WHC (%)	Syneresis (%)	Graininess	Adhesiveness (g.sec)	Firmness (g)
1	M16-70	4.38	1.40	16.58	21.50	0.00	89	-278.49	252.67
1	M16-78	4.40	1.36	16.66	27.97	0.00	149	-673.29	630.66
1	M16-86	4.38	1.37	16.61	27.05	0.16	199	-585.55	581.02
1	M16-95	4.39	1.40	16.44	27.58	0.14	226	-616.55	578.20
2	M16-70	4.42	1.41	16.49	23.47	0.00	75	-297.02	249.37
2	M16-78	4.40	1.42	16.59	29.27	0.00	161	-707.79	690.16
2	M16-86	4.37	1.37	16.61	29.40	0.00	205	-663.12	660.95
2	M16-95	4.42	1.38	16.54	27.03	0.47	269	-548.94	539.65
3	M16-70	4.40	1.40	16.58	21.88	0.00	54	-209.10	210.55
3	M16-78	4.42	1.39	16.60	27.75	0.00	137	-708.16	670.84

3	M16-86	4.43	1.33	16.59	29.07	0.00	219	-612.76	608.07
3	M16-95	4.42	1.34	16.60	28.35	0.00	214	-653.75	592.29

Abbreviations are MSNF, Milk solids nonfat; Rep, Replication; Temp, Temperature; TA, Titratable acidity; WHC, Water holding capacity.

Table C 2: Averages (n=3) for analyses performed on yogurts produced from heated mixes, M16-70, M16-78, M16-86 or M16-95 (70, 78, 86, or 95 ^{o}C for 30 min, respectively) for three replications.

Rep	Temp	η_{app} (Pa.s)	n (-)	K (Pa.s^{n})	A (Pa.s^{-1})	ΔA (Pa.s^{-1})	τ0 (Pa)	G' (Pa)	G" (Pa)
1	M16-70	80.97	0.99	1.42	979.81	195.96	4.86	176	71.97
1	M16-78	188.51	1.43	1.26	1503.85	300.765	18.34	1042.6	389.4
1	M16-86	182.34	1.31	1.31	1432.37	286.48	16.09	753.64	278.44
1	M16-95	144.87	1.34	1.31	1196.65	239.33	13.73	425.82	191.02
2	M16-70	89.57	1.03	1.39	1026.50	205.30	5.56	148.73	58.51
2	M16-78	195.04	1.42	1.26	1659.47	331.90	18.82	722.03	276.5
2	M16-86	189.19	1.39	1.27	1498.93	299.78	17.27	819.41	313.24
2	M16-95	175.27	1.32	1.33	1229.70	245.95	14.46	685.15	272.27

3	M16-70	78.90	0.94	1.47	726.23	145.32	5.04	131.52	52.96
3	M16-78	185.73	1.34	1.28	1353.27	270.76	15.78	1289.67	458.13
3	M16-86	184.80	1.37	1.28	1372.27	274.45	16.79	734.52	281.11
3	M16-95	178.24	1.32	1.32	1126.93	225.38	15.62	420.71	188.66

Abbreviations are Rep, Replication; Temp, Temperature; η_{app}, Apparent viscosity; n, Flow behavior index; K, Consistency index; A, Area under upward hysteresis curve; ΔA, Hysteresis loop area; $\tau 0$; G', Storage modulus; G", Loss modulus.

Table C 3: Averages (n=3) for analyses performed on control and heated yogurt mixes, M16-C, M16-70, M16-78, M16-86 or M16-95, (70, 78, 86, or 95 ^{o}C for 30 min, respectively) for three replications.

Rep	Temp	TP (%)	NCP (%)	NP (%)	SP (%)	IP (%)	DD	FAST Index
1	M16-C	5.69	1.24	0.28	0.96	4.71	-	10.70
1	M16-70	5.92	0.98	0.28	0.70	5.23	27.57	13.06
1	M16-78	5.72	0.51	0.29	0.22	5.50	82.92	45.58
1	M16-86	6.42	0.52	0.30	0.21	6.21	83.27	77.43
1	M16-95	6.27	0.54	0.29	0.25	6.02	79.27	83.49

2	M16-C	6.21	1.22	0.28	0.94	5.27	-	9.90
2	M16-70	6.35	1.00	0.29	0.71	5.63	24.32	13.00
2	M16-78	6.29	0.54	0.29	0.24	6.05	74.32	54.03
2	M16-86	6.45	0.50	0.29	0.22	6.23	77.03	74.35
2	M16-95	6.38	0.54	0.30	0.24	6.12	75.00	74.33
3	M16-C	6.36	1.19	0.29	0.90	4.99	-	9.69
3	M16-70	5.89	1.00	0.30	0.70	5.31	22.06	11.82
3	M16-78	6.01	0.54	0.28	0.25	5.50	71.89	41.20
3	M16-86	5.75	0.50	0.30	0.19	6.32	78.29	69.61
3	M16-95	6.51	0.51	0.29	0.22	6.25	75.80	72.96

Abbreviations are Rep, Replication; Temp, Temperature; TP, True protein, NCP, Non casein protein; NP, Non protein; SP, Soluble protein; IP, Insoluble protein; FAST ($100 \times F_{AMP}/F_{TRP}$), Fluorescence of advanced Maillard products and soluble tryptophan; F_{AMP}, fluorescence of advanced Maillard products; F_{TRP}, fluorescence of tryptophan products.

Table C 4: *P* values for yogurts and mixes made from heating at 70, 78, 86, or 95 ºC for 30 min. Significance was defined when $P \leq 0.05$.

	P (Rep)	P (Temp)
Adhesiveness (g.sec)	0.8962	0.0001
Firmness (g)	0.6013	<.0001
Syneresis (%)	0.4892	0.2894
WHC (%)	0.1914	0.0003
FAST index	0.1427	<.0001
L	0.1247	0.0002
A	0.0790	<.0001
B	0.1760	<.0001
WI	0.5562	0.0058
G’ (Pa)	0.9169	0.0061
G” (Pa)	0.9315	0.0036
MSNF (%)	0.6950	0.2609

DD	0.0680	<.0001
Graininess	0.2962	0.0001
pH	0.1389	0.6907
TA (%)	0.2401	0.1589
η_{app} (Pa.s)	0.1902	<.0001
n	0.2664	<.0001
A ($Pa.s^{-1}$)	0.0860	<.0001
ΔA ($Pa.s^{-1}$)	0.0850	<.0001
$\tau 0$ (Pa)	0.5424	<.0001
K ($Pa.s^{n}$)	0.4785	0.0009
NCP (%)	0.1136	<.0001
NP (%)	0.3734	0.5254
TP (%)	0.0678	0.0213
SP (%)	0.2935	<.0001
IP (%)	0.0535	<.0001

Abbreviations are MSNF, Milk solids nonfat; Rep, Replication; Temp, Temperature; WHC, Water holding capacity; FAST, Fluorescence of advanced Maillard products and soluble tryptophan; WI, Whiteness index; DD, Degree of whey protein denaturation;

TA, Titratable acidity; NCP, Non casein protein; NP, Non protein; TP, True protein; SP, Soluble protein; IP, Insoluble protein; η_{app}, Apparent viscosity; n, Flow behavior index; K, Consistency index; ΔA, Hysteresis area; $\tau 0$, Yield stress; G', Storage modulus; G", Loss modulus.

Appendix D - Raw data and ANOVA (Phase Two Study)

Table D 1: Averages (n=3) used in analyses performed on yogurts produced from heated mixes (70, 75, or 85 ºC for 30 min) from two different milk solids nonfat concentrations (9 and 12%) in three replications.

Rep	Conc	Temp	Firmness (g)	Adhesiveness (g.sec)	Consistency (g.sec)	Cohesiveness (g)	WCohesion (g.sec)	DD
1	9	70	126.80	-133.251	3567.42	-86.79	-147.91	8.33
1	9	75	154.43	-188.069	3548.12	-89.91	-168.70	37.50
1	9	85	170.22	-218.808	3936.35	-106.84	-205.52	63.46
1	12	70	150.08	-181.680	2951.00	-95.97	-228.35	11.78
1	12	75	227.92	-395.112	5481.98	-198.81	-360.85	34.62
1	12	85	318.19	-350.590	6728.97	-247.40	-470.51	49.52
2	9	70	126.91	-123.884	3450.56	-88.83	-137.11	9.16
2	9	75	149.49	-179.049	3468.06	-86.22	-165.31	38.27
2	9	85	187.70	-239.122	4270.44	-112.28	-217.98	69.27
2	12	70	148.93	-175.096	2245.74	-60.46	-239.84	8.45
2	12	75	258.05	-394.173	5913.83	-197.71	-363.60	25.96
2	12	85	294.12	-395.899	6013.08	-233.66	-464.23	50.72

3	9	70	130.35	-158.697	3412.23	-82.86	-135.25	11.07
3	9	75	152.81	-205.771	3607.95	-95.14	-163.44	27.68
3	9	85	162.80	-201.313	3920.18	-109.10	-203.18	57.98
3	12	70	158.25	-176.673	2420.98	-86.25	-248.90	10.65
3	12	75	240.70	-383.165	5864.11	-192.49	-368.33	36.80
3	12	85	320.17	-369.321	6062.23	-253.24	-489.64	60.05

Abbreviations are Rep, Replication; Conc, Concentration; Temp, Temperature; WCohesion, Work of cohesion; DD, Degree of whey protein denaturation.

Table D 2: Averages (n=2 or n=3) used in analyses performed on yogurts produced from heated mixes (70, 75, or 85 ^{o}C for 30 min) from two different milk solids nonfat concentrations (9 and 12%) in three replications.

Rep	Conc	Temp	MSNF (%)	Syneresis (%)	WHC (%)	Graininess	pH	TA (%)	L	a	b	WI
1	9	70	9.07	2.29	49.44	2	4.51	1.3	93.63	-2.76	10.65	87.29
1	9	75	9.08	2.09	46.15	8	4.5	1.33	93.56	-2.93	10.56	87.29
1	9	85	9.14	7.17	50.72	5	4.52	1.34	94.58	-2.41	10.98	87.52
1	12	70	12.06	0.19	65.26	4	4.49	1.29	92.41	-2.34	10.36	86.95

1	12	75	12.14	0.46	65.01	10	4.52	1.35	93.93	-2.48	11.78	86.52
1	12	85	12.28	0.75	56.34	12	4.53	1.36	94.59	-2.18	12.18	86.50
2	9	70	9.08	1.50	49.05	3	4.48	1.26	93.40	-2.78	10.22	87.52
2	9	75	9.22	2.43	43.34	9	4.52	1.35	94.21	-2.81	10.81	87.42
2	9	85	9.16	7.08	44.41	15	4.50	1.31	93.52	-2.62	10.68	87.24
2	12	70	12.19	0.28	64.05	5	4.52	1.33	92.57	-2.37	11.38	86.20
2	12	75	12.28	0.59	63.26	6	4.55	1.36	93.96	-2.62	12.03	86.29
2	12	85	12.16	0.72	62.18	7	4.53	1.30	94.86	-2.22	11.99	86.77
3	9	70	9.04	2.47	46.86	3	4.51	1.29	92.61	-2.75	10.11	87.18
3	9	75	9.16	2.42	47.00	3	4.50	1.32	93.96	-2.76	10.55	87.53
3	9	85	9.22	7.88	44.23	2	4.54	1.36	94.21	-2.67	11.04	87.25
3	12	70	12.17	0.27	59.94	13	4.47	1.26	92.61	-2.43	11.47	86.14
3	12	75	12.09	0.21	56.61	7	4.51	1.29	94.25	-2.58	11.90	86.53
3	12	85	12.19	0.71	65.69	6	4.49	1.28	95.00	-2.24	12.16	86.66

Abbreviation are MSNF, Milk solids nonfat; Rep, Replication; Conc, Concentration; Temp, Temperature; WHC, Water holding capacity, TA, Titratable acidity; WI, Whiteness index.

Table D 3: Averages (n=2) used in analyses performed on yogurts produced from heated mixes (70, 75, or 85 ºC for 30 min) from two different milk solids nonfat concentrations (9 and 12%) in three replications.

Rep	Conc	Temp	σ_o (Pa)	η (mPa.s)	K (Pa.s^n)	n	G' (Pa)	G" (Pa)
1	9	70	10.6548	197.89	2.96	0.13	30.592	10.380
1	9	75	18.7405	374.81	2.18	0.27	87.387	45.649
1	9	85	20.1090	402.18	1.96	0.22	83.013	27.388
1	12	70	18.5025	258.99	3.51	0.05	42.178	16.585
1	12	75	38.9343	778.68	4.35	0.07	122.740	41.953
1	12	85	54.8580	1097.15	6.38	0.05	306.945	97.321
2	9	70	8.6673	194.22	2.34	0.14	23.371	10.695
2	9	75	16.4560	329.12	1.81	0.25	90.185	32.501
2	9	85	23.3255	466.50	2.93	0.22	83.179	28.961
2	12	70	11.6530	234.75	3.75	0.05	37.517	15.193
2	12	75	42.2640	845.29	5.51	0.06	122.635	40.991
2	12	85	44.3245	986.49	6.50	0.05	273.945	89.001

3	9	70	10.6158	191.97	1.92	0.22	39.369	15.416
3	9	75	19.4135	388.27	1.80	0.14	92.806	31.837
3	9	85	21.9980	439.96	2.12	0.15	85.073	23.610
3	12	70	19.4115	288.24	4.21	0.06	47.029	18.389
3	12	75	37.3890	747.78	3.95	0.06	120.110	41.404
3	12	85	31.3350	1026.70	6.72	0.05	269.363	92.935

Abbreviations are Rep, Replication; Conc, Concentration; Temp, Temperature; σ_o, yield stress; η, viscosity; K, Consistency index; n, Flow behavior index; G', storage modulus; G", Loss modulus.

Table D 4: Averages (n=3) used in analyses performed on heated yogurts mixes (70, 75, or 85 ^oC for 30 min) from two different milk solids nonfat concentrations (9 and 12%) in three replications.

Rep	Conc	Temp	Fat (%)	T (%)	NCP (%)	NP (%)	TP (%)	SP (%)	IP (%)	FAST index
1	9	C	0.16	3.4324	1.1867	0.1914	3.2410	0.9953	2.2458	6.20
1	9	70	0.15	3.2538	1.1356	0.2233	3.0305	0.9123	2.1182	8.18

1	9	75	0.13	3.2793	0.8294	0.2074	3.0720	0.6221	2.4499	11.69
1	9	85	0.13	3.3048	0.5742	0.2105	3.0943	0.3637	2.7306	43.11
1	12	C	0.15	4.2108	1.5695	0.2424	3.9684	1.3270	2.6413	9.14
1	12	70	0.18	4.2746	1.4036	0.2329	4.0417	1.1707	2.8710	10.57
1	12	75	0.18	4.0704	1.1229	0.2552	3.8152	0.8677	2.9476	15.75
1	12	85	0.18	3.8790	0.9442	0.2743	3.6047	0.6699	2.9348	54.56
2	9	C	0.14	3.2538	1.3908	0.2074	3.0465	1.1835	1.8630	6.51
2	9	70	0.08	3.3686	1.2760	0.2010	3.1677	1.0750	2.0926	7.81
2	9	75	0.16	3.2538	0.9315	0.2010	3.0528	0.7305	2.3223	12.85
2	9	85	0.15	3.2666	0.5870	0.2233	3.0433	0.3637	2.6796	38.73
2	12	C	0.19	4.0577	1.7226	0.2520	3.8057	1.4706	2.3351	8.10
2	12	70	0.13	4.1087	1.5567	0.2105	3.8982	1.3462	2.5520	10.17
2	12	75	0.12	4.0577	1.2250	0.2424	3.8152	0.9825	2.8327	14.95
2	12	85	0.13	3.7642	0.9187	0.2648	3.4994	0.6540	2.8455	51.97
3	9	C	0.16	3.0369	1.1484	0.1691	2.8678	0.9793	1.8885	6.45
3	9	70	0.18	3.0496	1.0208	0.1499	2.8997	0.8709	2.0288	7.09

3	9	75	0.14	3.1007	0.8804	0.1723	2.9284	0.7082	2.2202	13.10
3	9	85	0.13	2.8072	0.5997	0.1882	2.6190	0.4115	2.2075	39.00
3	12	C	0.19	4.1215	1.5312	0.2137	3.9078	1.3175	2.5903	8.23
3	12	70	0.13	4.0577	1.3781	0.2010	3.8567	1.1771	2.6796	10.93
3	12	75	0.19	4.0832	1.0463	0.2137	3.8695	0.8326	3.0369	14.92
3	12	85	0.18	4.2108	0.7528	0.2265	3.9843	0.5264	3.4580	56.95

Abbreviations are Rep, Replication; Conc, Concentration; Temp, Temperature; T, Total protein; NCP, Non casein protein; NP, Non protein fraction; TP, True protein; SP, Soluble protein; IP, Insoluble protein; FAST, Fluorescence of advanced Maillard products and soluble tryptophan.

Table D 5: *P* values for yogurts and mixes made from heating at 70, 75, or 85 ºC for 30 min with either 9 or 12% milk solids nonfat in three replications. Significance was determined when $P \leq 0.05$.

	P (Rep)	*P* (Conc)	*P* (Temp)	*P* (Conc*Temp)
Adhesiveness (g.sec)	0.7828	<.0001	<.0001	<.0001
Cohesiveness (g)	0.2933	<.0001	<.0001	<.0001

Consistency (g.sec)	0.5582	<.0001	<.0001	<.0001
Firmness (g)	0.8737	<.0001	<.0001	<.0001
WC (g.sec)	0.6588	<.0001	<.0001	<.0001
Syneresis (%)	0.4305	<.0001	<.0001	<.0001
WHC (%)	0.6043	<.0001	0.5326	0.9284
FAST index	0.3659	<.0001	<.0001	<.0001
L	0.9909	0.7697	0.0001	0.0343
a	0.4007	<.0001	0.0001	0.4645
b	0.7929	<.0001	0.0043	0.3479
WI	0.6172	<.0001	0.7313	0.5985
G’ (Pa)	0.4638	<.0001	<.0001	<.0001
G” (Pa)	0.2870	<.0001	<.0001	<.0001
MSNF (%)	0.3834	<.0001	0.0965	0.7256
DD	0.9818	0.1552	<.0001	0.2219
Graininess	0.7584	0.2946	0.5082	0.6998
pH	0.5526	0.7408	0.1836	0.5047

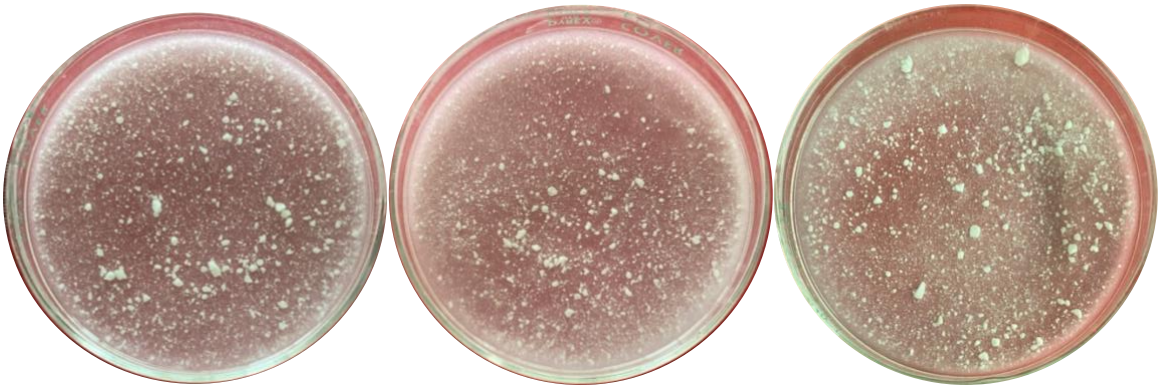
TA (%)	0.2930	0.7588	0.0574	0.6255
σ_o (%)	0.5241	<.0001	0.0002	0.0604
η (mPa.s)	0.9310	<.0001	<.0001	<.0001
K (Pa.s^n)	0.4912	<.0001	0.0018	0.0023
n	0.4758	0.0001	0.5347	0.6720
Fat (%)	0.1974	0.0986	0.5500	0.9578
T (%)	0.1854	<.0001	0.3663	0.9564
NCP (%)	0.0003	<.0001	<.0001	0.4097
NP (%)	<.0001	<.0001	0.0033	0.1961
TP (%)	0.3727	<.0001	0.2598	0.9262
SP (%)	0.0010	<.0001	<.0001	0.3657
IP (%)	0.2103	<.0001	0.0009	0.9598

Abbreviations are MSNF, Milk solids nonfat; WC, Work of cohesion; WHC, Water holding capacity; FAST, Fluorescence of advanced Maillard products and soluble tryptophan; WI, Whiteness index; Whey protein denaturation; TA, Titratable acidity; σ_o, Yield stress; K,

Consistency index; *n*, flow behavior coefficient; T, Total protein; NCP, Non casein protein; NP, Non protein fraction; TP, True protein; SP, Soluble protein; IP, Insoluble protein.

Appendix E – Non Significant Data Set (Phase One Study)

a



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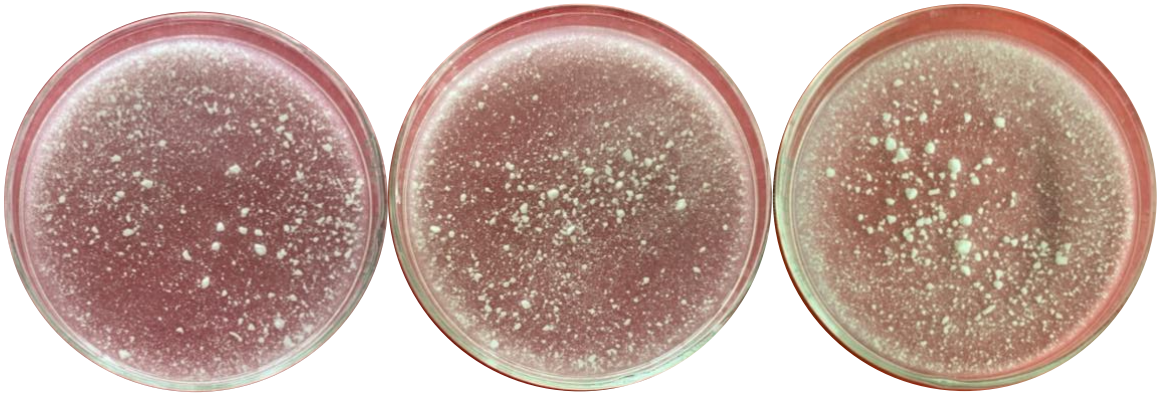

c

d

Figure E 2: Images displaying grains in diluted samples (1:10) of yogurts produced from mixes heated at 70 (a), 78 (b), 86 (c) and 95 ºC (d) for 30 min. One image was randomly selected for each replication. Grains with a diameter ≥ 1 mm were enumerated with ImageJ Software.

Figure E 3: Images displaying grains in diluted samples (1:10) of yogurts produced from mixes heated at 70 (a), 78 (b), 86 (c) and 95 ºC (d) for 30 min. One image was randomly selected for each replication. Grains with a diameter ≥ 1 mm were enumerated with ImageJ Software.

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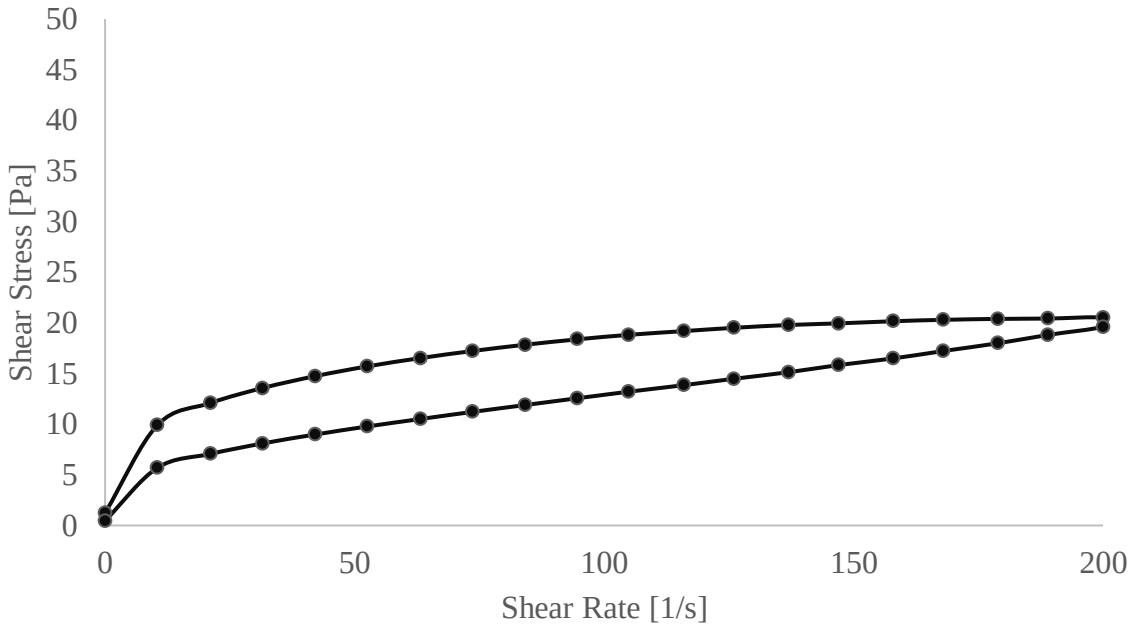
a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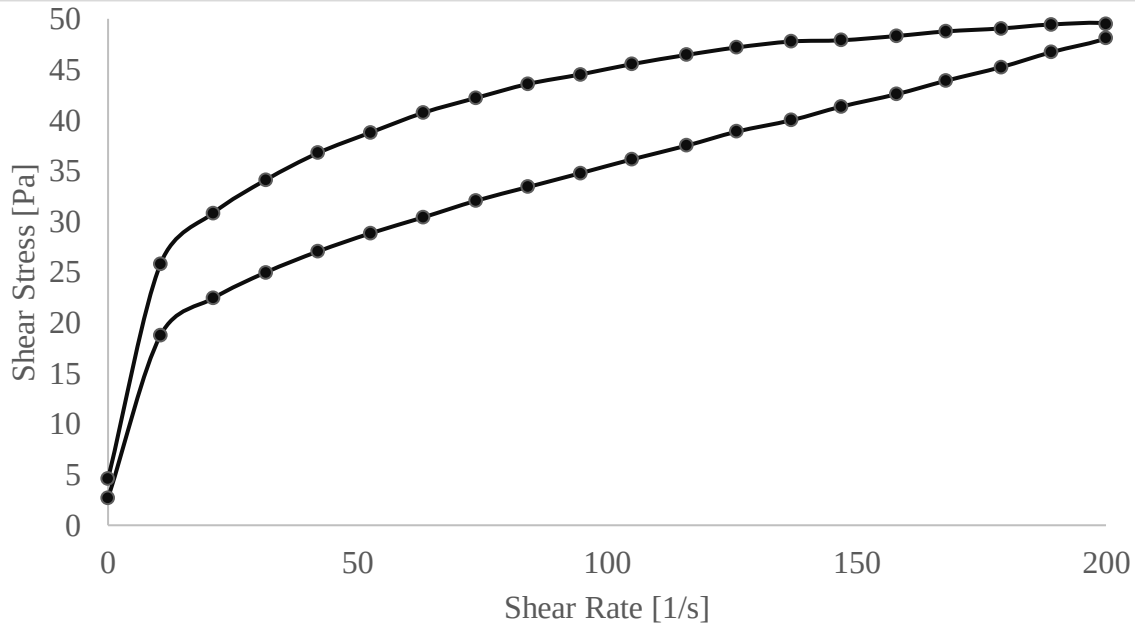
15
20
25
30
35
40
45
50
0
50
100
150
200
Shear Rate [1/s]

b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15
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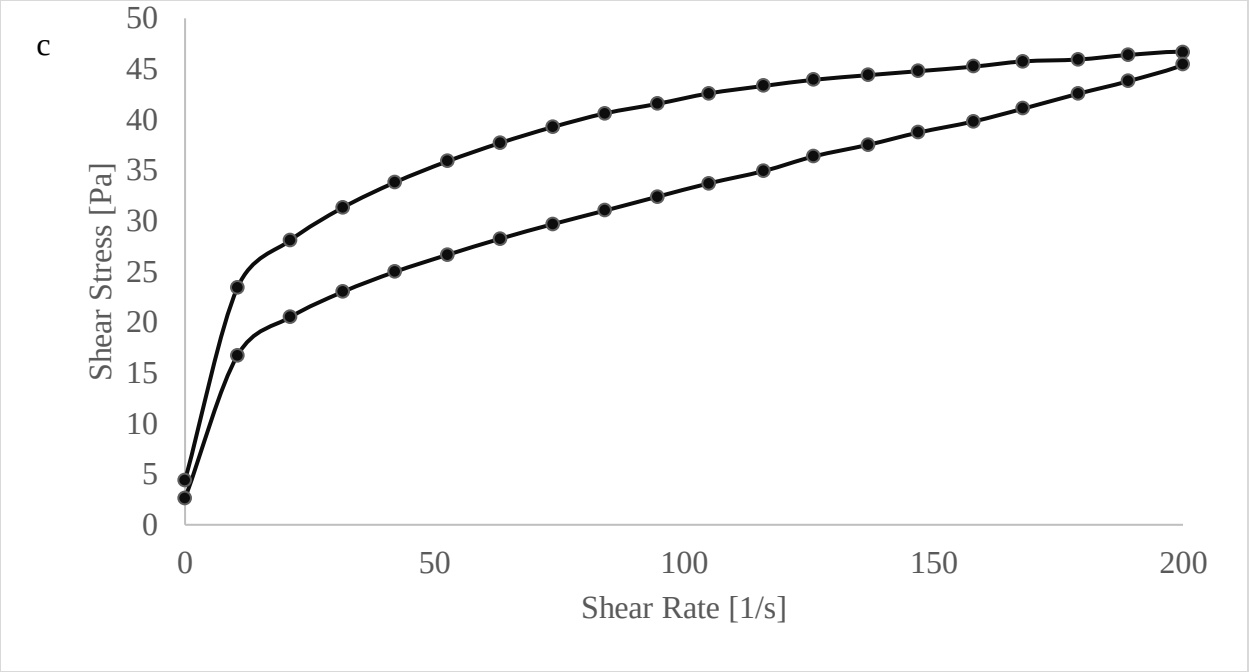
Shear Rate [1/s]



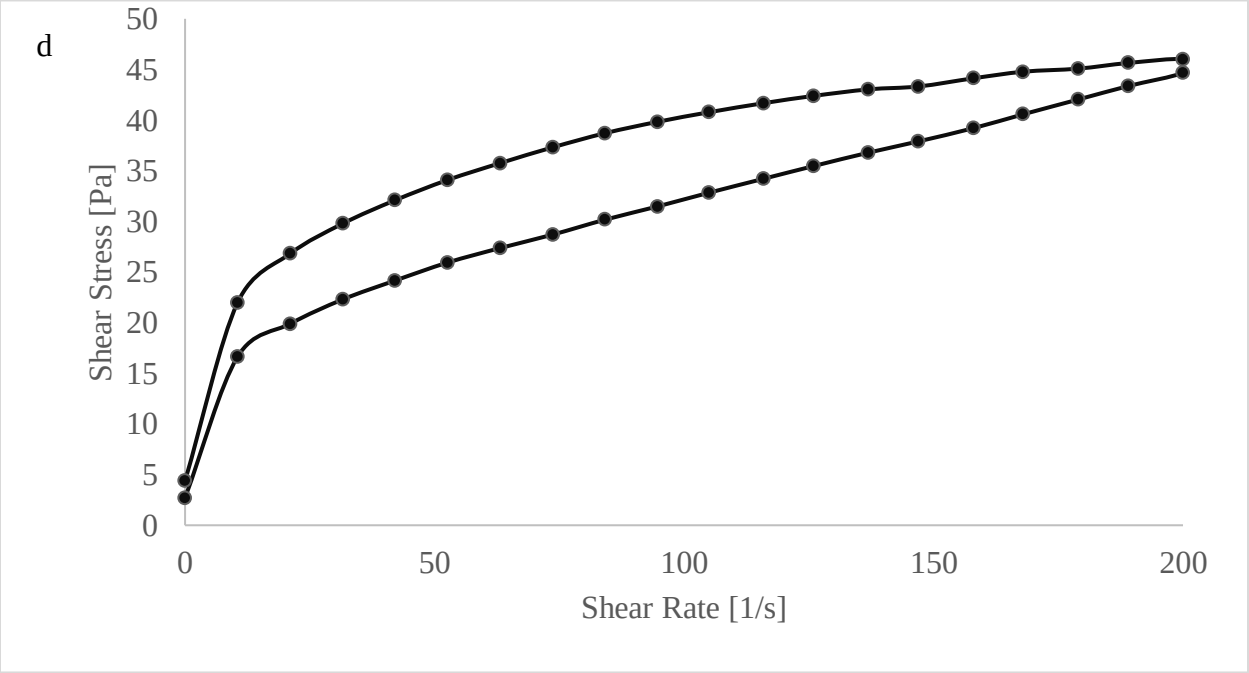


Figure E 4: Averages of flow curves for yogurts produced from mixes heated at 70 (a), 78 (b), 86 (c) and 95 ºC (d) for 30 min evaluated at 20 ºC.

Table E 1: Flow behavior properties of yogurts made from mixes heated at four different temperatures (70, 78, 86, and 95 °C for 30 min).

Yogurts	Flow Behavior				
	η_{app} (Pa.s)	n	K (Pa.s^n)	ΔA (Pa.s^{-1})	σ_o (Pa)
Y16-70	83.15^b±5.66	0.99^b±0.05	1.43^a±0.04	182.20^c±32.28	5.15^c±0.36
Y16-78	189.76^a±4.78	1.40^a±0.05	1.27^b±0.01	301.14^a±30.57	17.65^a±1.63
Y16-86	185.45^a±3.47	1.36^a±0.02	1.29^b±0.02	286.91^a±12.67	16.72^{ab}±0.60
Y16-95	166.13^a±18.47	1.33^a±0.01	1.32^b±0.01	236.86^b±10.50	14.60^b±0.95

Means (n=3) in the same column with different superscripts significantly differ ($P \leq 0.05$).

Yogurts, Y16-70, Y16-78, Y16-86, and Y16-95, were made from mixes heated at 70, 78, 86, and 95 °C for 30 min, respectively.

Abbreviations are η_{app}, Apparent viscosity; n, Flow behavior index; K, Consistency index; ΔA, Hysteresis area; σ_o, yield stress.

Table E 2: Viscoelastic properties of yogurts made from mixes heated at four different temperatures (70, 78, 86, and 95 °C for 30 min).

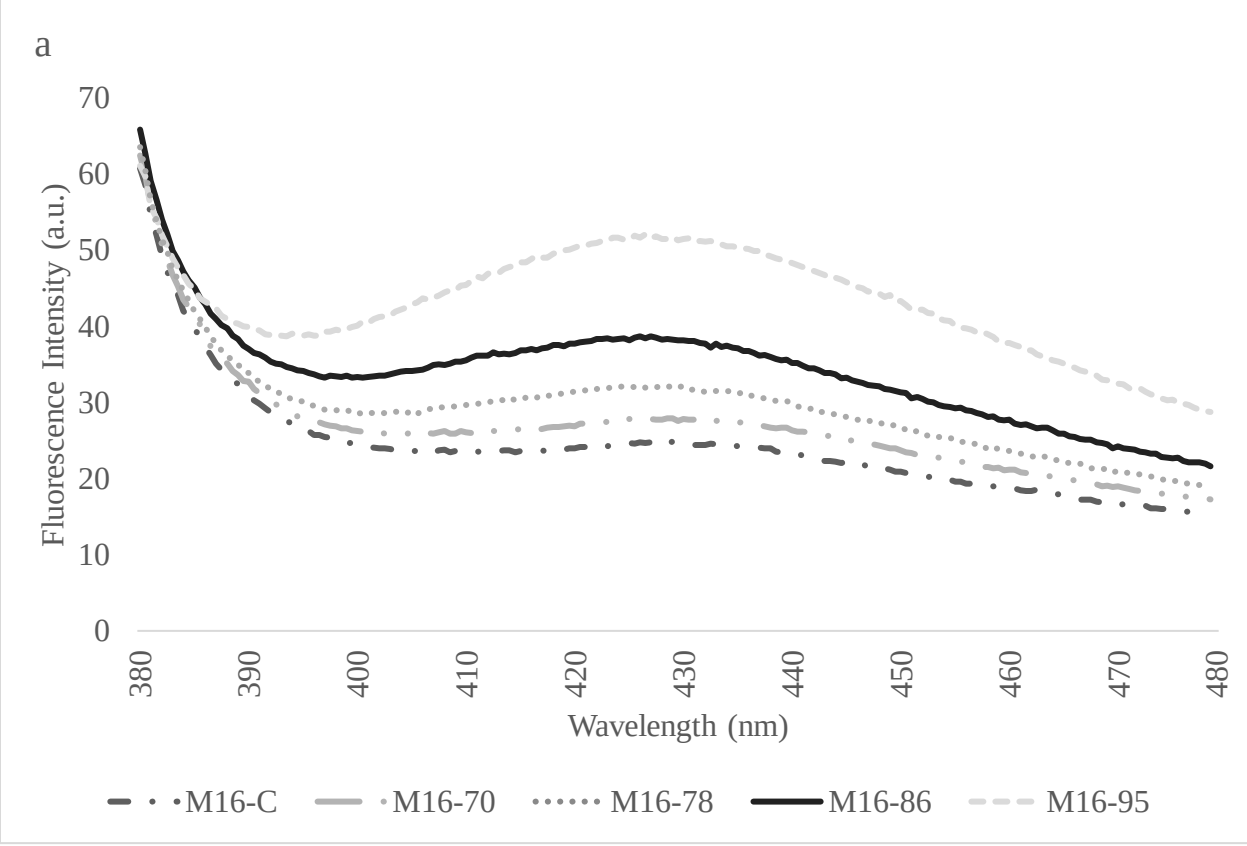
Yogurts	Viscoelastic Properties		Color
	G' (Pa)	G" (Pa)	WI
Y16-70	152.08^b±22.43	61.15^b±9.8	86.88^b±0.07
Y16-78	1018.10^a±284.61	374.68^a±91.7	87.41^a±0.13

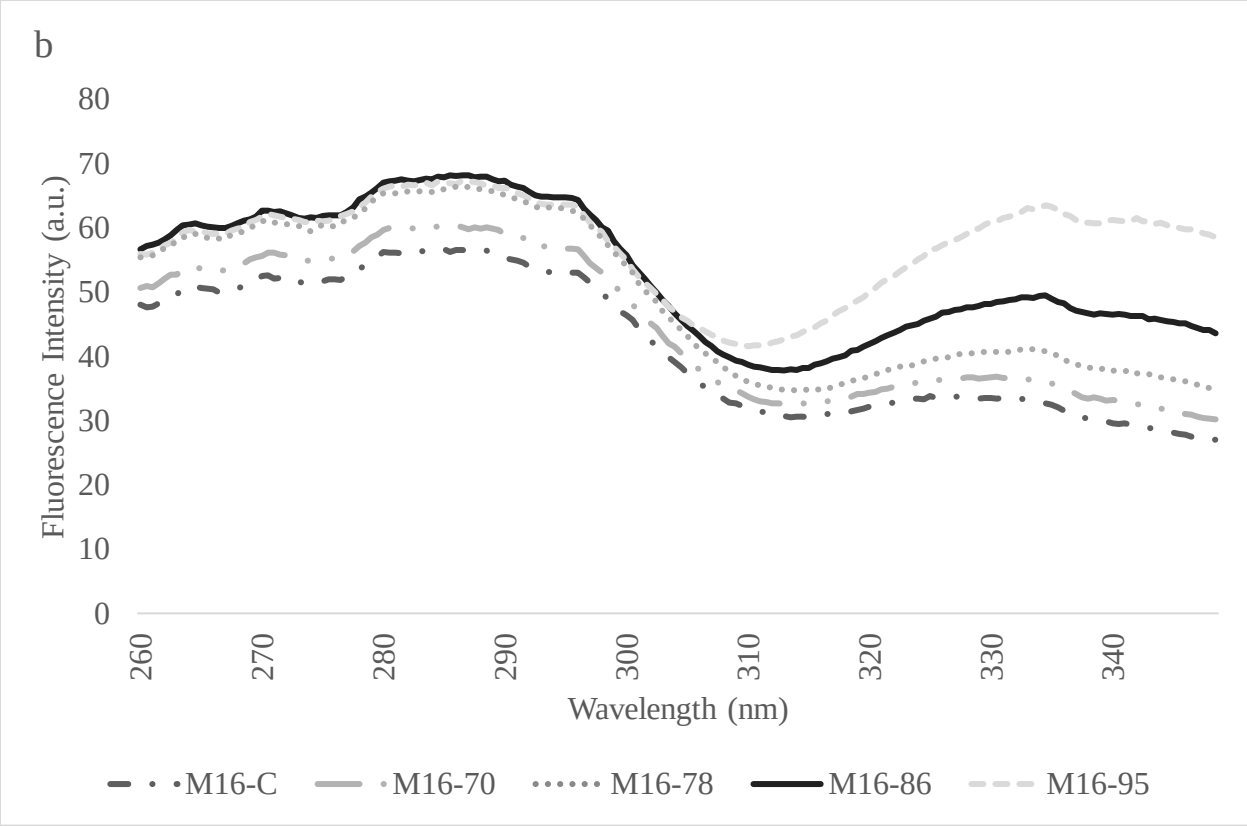
Y16-86	769.19[a]±44.53	290.93[a]±19.4	86.86[b]±0.16
Y16-95	510.56[ab]±151.22	217.32[ab]±47.6	85.20[c]±0.19

Means (n=3) in the same column with different superscripts significantly differ ($P \leq 0.05$).

Yogurts, Y16-70, Y16-78, Y16-86, and Y16-95, were made from mixes heated at 70, 78, 86, and 95 ºC for 30 min, respectively.

Abbreviations are G', Storage modulus; G", Loss modulus; WI, Whiteness index.




b
Fluorescence Intensity (a.u.)
0
10
20
30
40
50
60
70
80
260
270
280
290
300
310
320
330
340
Wavelength (nm)
M16-C
M16-70
M16-78
M16-86
M16-95

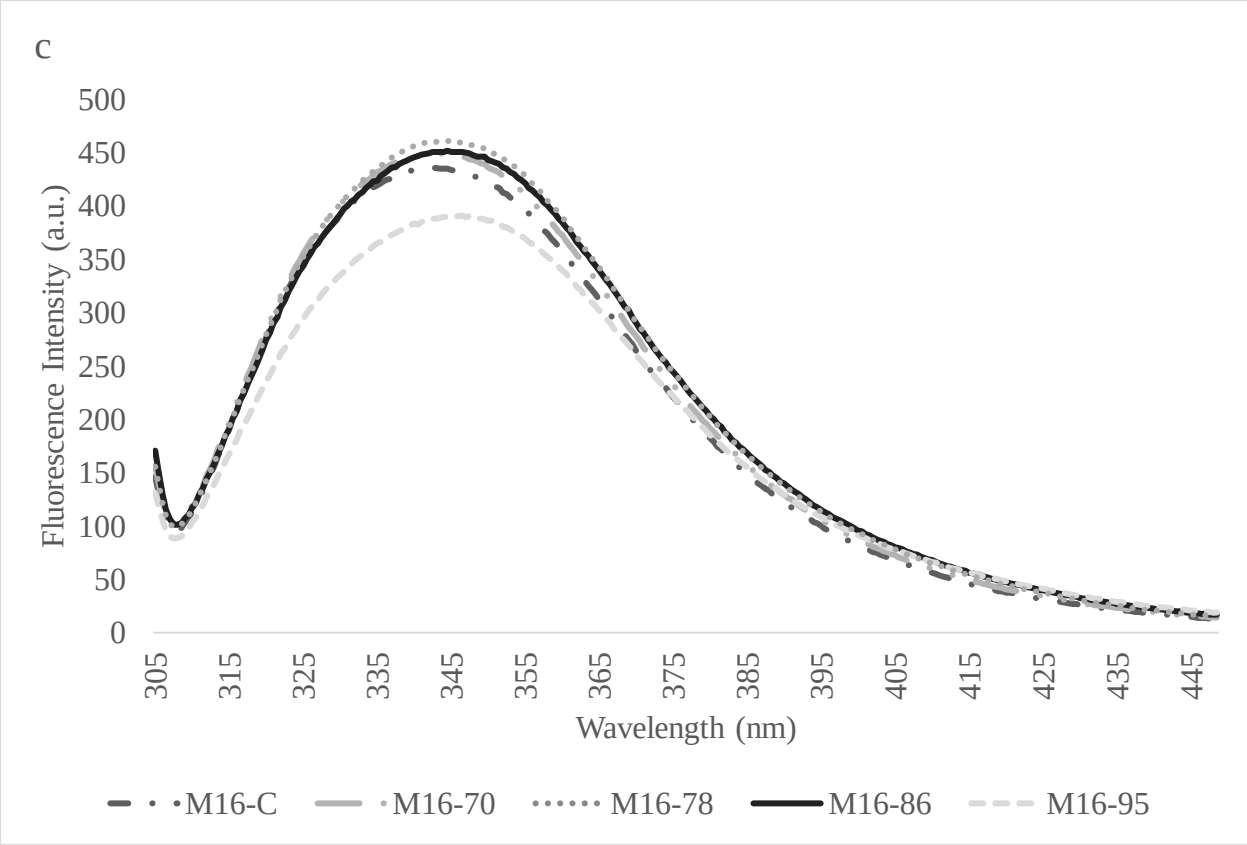

c
Fluorescence Intensity (a.u.)
0
50
100
150
200
250
300
350
400
450
500
305
315
325
335
345
355
365
375
385
395
405
415
425
435
445
Wavelength (nm)
M16-C
M16-70
M16-78
M16-86
M16-95

Figure E 5: Averages of front face fluorescence spectroscopy for control yogurt mixes and mixes heated to 70, 78, 86 or 95 ^{o}C for 30 min, denoted as M16-C, M16-70, M16-78 and M16-95, respectively. Front face spectra include Maillard emission (a), Maillard excitation (b) and tryptophan emission (c).

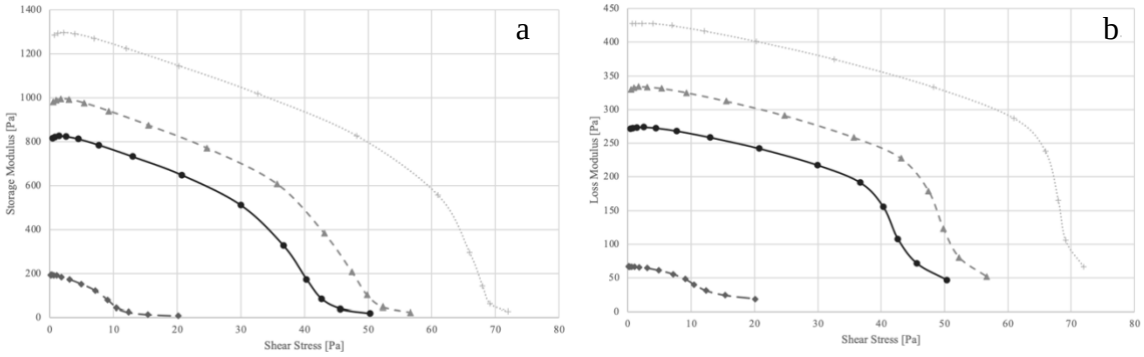


Figure E 7: Storage modulus (G') (a) and loss modulus (G") (b) measured on day 2 at 20^{o}C. Analyses was performed on yogurts produced from mixes heat at 70 (♦), 78 (+), 86 (△) and 95^{o}C (●) for 30 minutes.

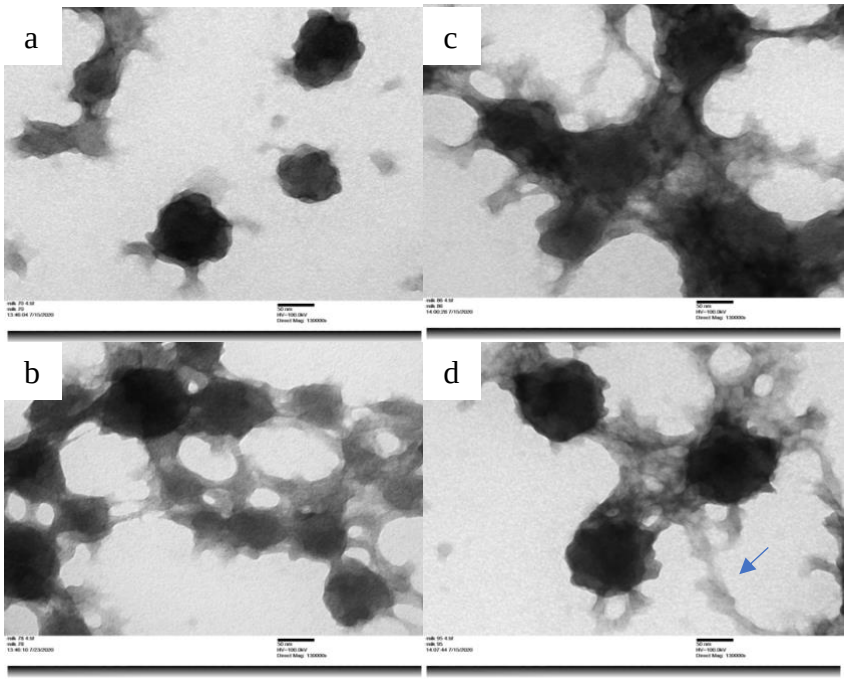


Figure E 8: Transmission electron microscope images at 130,000x of yogurt mixes prepared from mixes heated to 70, 78, 86 and 95°C for 30 minutes, M16-70 (a), M16-78 (b), M16-86 (c) and M16-95 (d), respectively on day 1. Black areas represent proteins and the protein network. Arrow indicates long range β-Lg appendages.

Figure E 9: Transmission electron microscope images at 130,000x of yogurt mixes prepared from mixes heated to 70, 78, 86 and 95°C for 30 minutes, M16-70 (a), M16-78 (b), M16-86 (c) and M16-95 (d), respectively on day 1. Black areas represent proteins and the protein network. Arrow indicates long range β-Lg appendages.

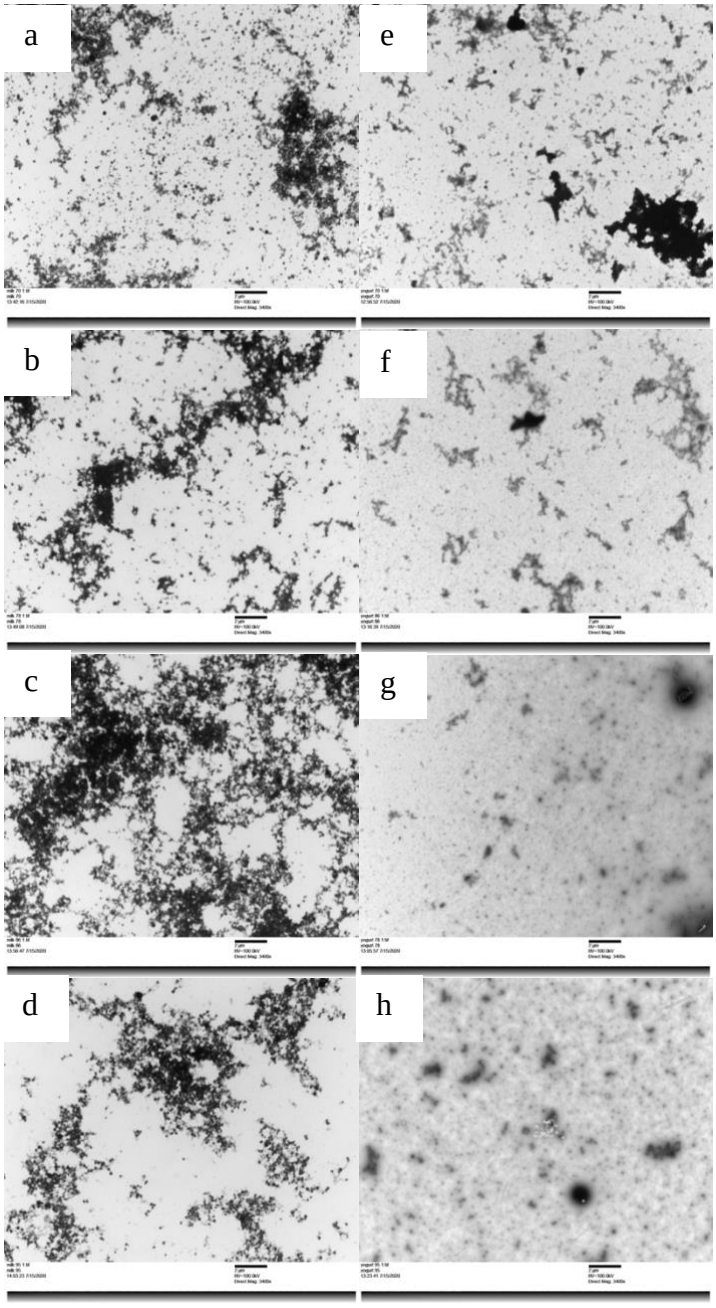


Figure E 11: Transmission electron micrographs at 3400x of yogurt mixes heated to 70, 78, 86 and 95^{o}C for 30 minutes, M16-70 (a), M16-78 (b), M16-86 (c) and M16-95 (d), and yogurts prepared from mixes heated to 70, 78, 86 and 95^{o}C for 30 minutes, Y16-70 (e), Y16-78 (f), Y16-86 (g) and Y16-95 (h). Black areas represent proteins and the protein network.

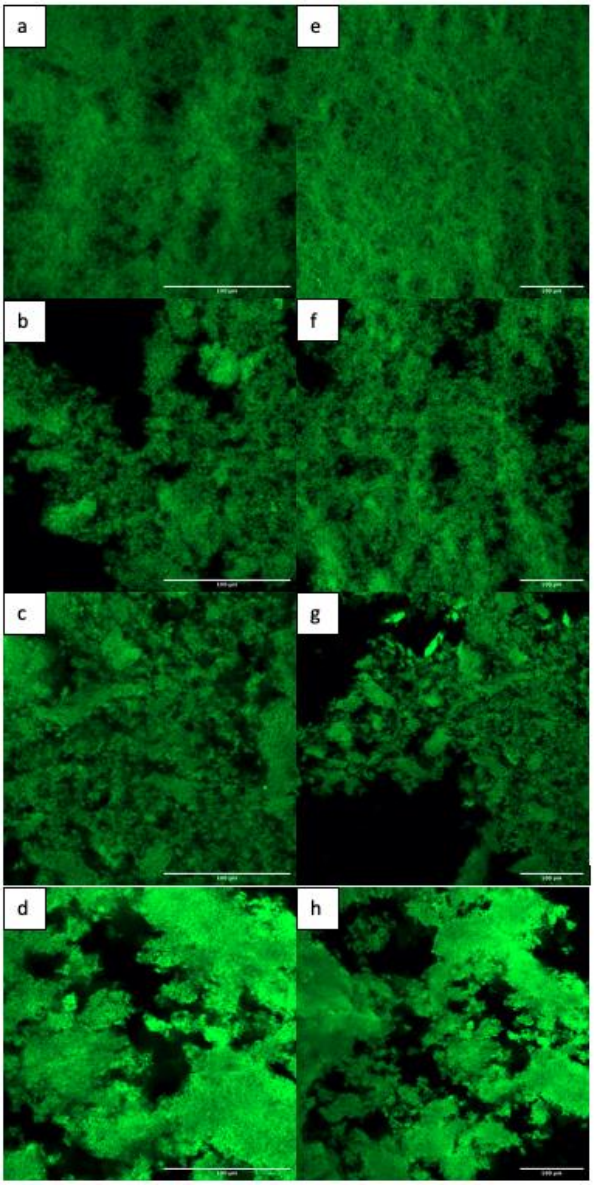


Figure E 14: Confocal scanning laser microscopy images at 20x of yogurts made from mixes heated at 70, 78, 86 and 95ºC for 30 minutes, Y16-70 (a and e), Y16-78 (b and f), Y16-86 (c and g) and Y16-95 (d and h) for 30 minutes, respectively. Proteins and the protein network are stained green.

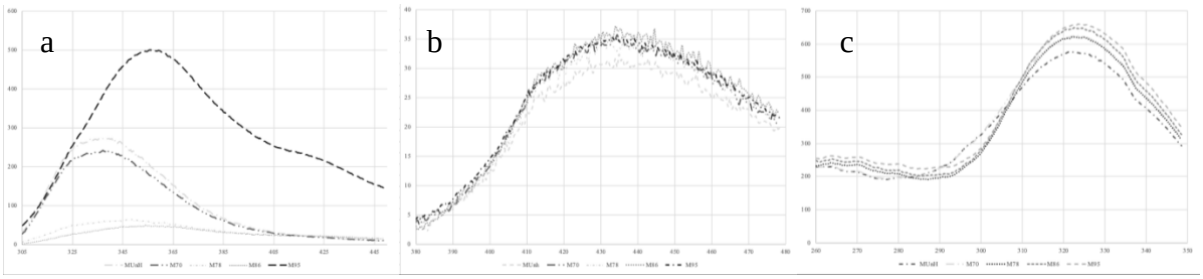


Figure E 16: Right angle fluorescence spectroscopy for soluble protein of unheated yogurt mix and mixes heated to 70, 78, 86, and 95ºC for 30 minutes, denoted as M16-C (- · -), M16-70 (- · ·), M16-78 (· · ·), M16-86 (- - -) and M16-95 (- - -), respectively. Right angle spectra include Tryptophan emission (a), Maillard products emission (b) and Maillard products excitation (c).

Appendix F – Non Significant Data Set (Phase Two Study)

Table F 1: Graininess of yogurts as functions of milk solids nonfat (9 versus 12%) concentration and mix heat (70, 75, or 85 ^{o}C for 30 min).

Yogurts	Graininess
Y9-70	2.67±0.57
Y9-75	6.67±3.21
Y9-85	7.33±6.81
Y12-70	7.33±4.93
Y12-75	7.67±2.08
Y12-85	8.33±3.21

Means (n=6) ± standard deviations did not significantly differ ($P \geq 0.05$).

Yogurts Y9-70, Y9-75, Y9-85, Y12-70, Y12-75, and Y12-85 represent 9 and 12% milk solids nonfat concentrations heated at 70, 75, and 85 ^{o}C for 30 min, respectively.

a

b

c

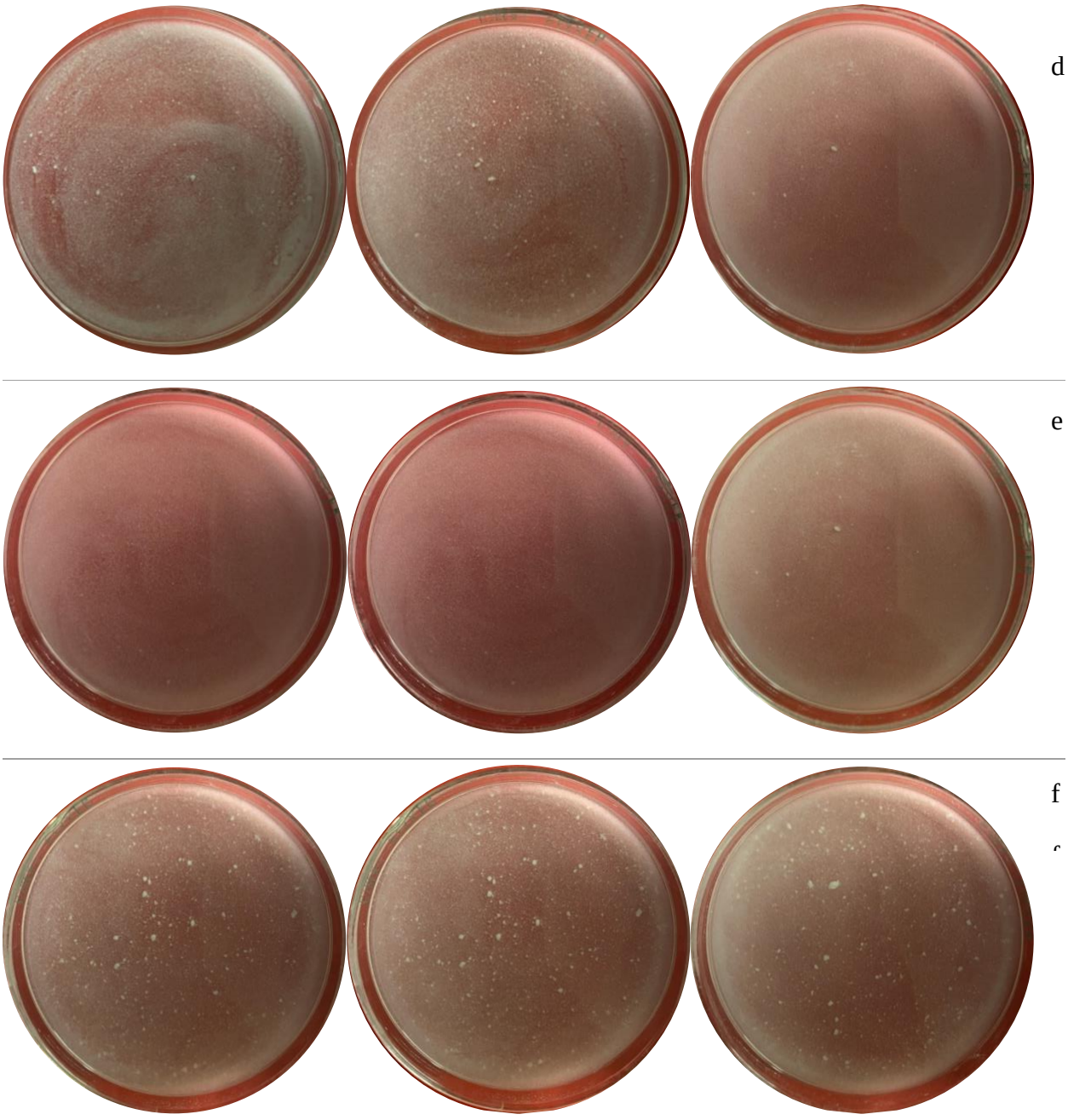


Figure F 1: Images displaying grains in diluted samples (1:10) of yogurts produced from mixes with 9% MSNF heated at 70 (a), 75 (b), or 85 (c) or with 12% MSNF heated at 70 (d), 75 (e), or 85 (f) for 30 min. One image was randomly selected for each replication. Grains with a diameter ≥ 1 mm were enumerated with ImageJ Software.