HIGH PRESSURE AND ULTRASONIFICATION TECHNOLOGIES FOR MANUFACTURING YOGURT

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of SUBBA RAO GURRAM find it satisfactory and recommend that it be accepted.

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Abstract

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Nonthermal processing is a rapidly growing field of research and industry use for production of safe foods and modification and/or improvement of quality. It is expected that this trend will grow, as consumers want minimally processed foods of natural flavor that are free from additives and preservatives. High hydrostatic pressure (HHP) and ultrasonification are two promising nonthermal processing technologies studied in this research for manufacturing low fat probiotic yogurt and improving the viability of probiotics in yogurt.

Yogurt was manufactured using heat, HHP, and a combined treatment of HHP and heat. The effect of ultrasonification on the physicochemical, rheological, textural, and microstructure of low fat probiotic yogurt were studied. The combined application of HHP and thermal treatment resulted in yogurt gels with improved physicochemical characteristics and water holding capacity over heat or HHP alone. The HHP and heat combined treatment resulted in yogurt gels with improved consistency indices over gels obtained from thermally treated milk. The starter and inoculation rate that provided different fermentation pathways also affected the consistency index and texture

properties. Rheological behavior differences of yogurts varied according to the treatment used, and were attributed to structural phenomena of casein micelles. The combined HHP and heat milk treatments exhibited small rounded micelles that tended to fuse and form small irregular aggregates in association with clumps of dense amorphous material, which resulted in improved gel texture and viscosity.

Ultrasonification was used to rupture yogurt bacteria to improve the viability of probiotics in yogurt. The probiotics grew better in sonicated culture yogurt compared to unsonicated culture yogurt, indicating increased availability of nutrients for the probiotics, which can be attributed to β-galactosidase availability. Sonicated starter yogurts presented lower syneresis compared to the control yogurts during storage. Ultrasonification improved the viability of probiotics by two log cycles at the end of storage period. The reduction of viability beyond the 24^{th} day can be attributed to the lowering of pH. Overall, the results suggest that ultrasonification can possibly improve the viability of probiotics and quality of yogurt. Finally, both HHP and ultrasonification are potentially promising nonthermal processing technologies that can be selected for manufacturing yogurt to improve quality and viability of probiotics.

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CHAPTER ONE

High hydrostatic pressure and ultrasonification applications in yogurt processing

1.1 History of making yogurt

Fermentation is one of the oldest methods practiced by human beings for the transformation of milk into products with an extended shelf life (Tamime and Robinson, 1999) and fermented dairy products have been consumed for nutrition and maintenance of good health for a very long time (Vinderola & Reinhemier, 1999). Although there are no records available regarding the origin of yogurt, the belief in its beneficial influence on human health and nutrition has existed in many civilizations.

Food historians generally agree that yogurt and other fermented milk products were discovered accidentally by Neolithic people living in Central Asia. Since at least 5000 B.C., yogurt has been a staple food for people in the Middle East, especially in Turkey. These foods occurred naturally due to local climate and primitive storage methods. Although the evolution of this process is intuitive, the production of yogurt soon became an established pattern of preservation, and sine the early 1900s, defined microorganisms have been used to prepare many fermented dairy products. Yogurt is formed by the slow lactic acid fermentation of milk lactose by the themophillic lactic acid bacteria *Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus*. These yogurt starter culture microorganisms play an important role in developing acid and the right flavor during the production of yogurt. The flavor is mainly developed as a result of

complex biochemical reactions initiated by the yogurt starter cultures and it varies from species to species; this characteristic is reflected in the end product (Tamime and Robinson, 1999).

Typically yogurt is characterized by a smooth, viscous gel, with an acetaldehyde (green apple) flavor. Some of the varieties around the world are stirred or drinkable yogurt, frozen yogurt, smoked yogurt, strained yogurt, sundae-style, dried yogurt, and yogurt cheese (Tamime and Robinson, 1999). Commercial yogurt production increased rapidly in Europe early in the twentieth century after Dr. Eli Metchnikoff published a book on his advocacy of regularly consuming cultured milks, especially yogurt, for the "Prolongation of Life" (Metchnikoff, 1908). Later, in 1939, yogurt was successfully introduced on a commercial scale into the U.S. in New York City. In general the world-wide interest for yogurt is related to its nutritional and health benefits.

1.2 Thermal treatment

The application of heat to milk has long been practiced traditionally to kill pathogens. In some rural communities where the scale of yogurt manufacturing is small, milk is heated in a cooking pot and the production of the yogurt takes place in the same container (Tamime and Robinson, 1999). The heat treatment of milk is one of the most important processing parameters affecting the physicochemical, rheology, texture, and microstructure of yogurt. Also, maintaining uniform temperature during incubation is a critical factor for good yogurt manufacturing.

During heat treatment of milk the main changes that occur are denaturation and aggregation of whey proteins with caseins and fat globules. The amount of whey protein associated with fat globules is lower compared to the amounts that are bound to casein micelles (Corredig and Dalgleish, 1999). Corredig and Dalgleish, (1999) also showed that under strong heating conditions (90 °C for 60 min) mainly two interactions occur between caseins and proteins: (a) a direct interaction of β-lactoglobulin with casein micelles via k-casein binding; (b) a reaction between two whey proteins (α-lactoglobulin and β-lactoglobulin) which act as an intermediate cross linking agent between the casein micelles. Whey proteins are bound to casein micelle through disulphide linkages and hydrophobic interactions (Law, 1996). During gelation, the casein micelles thus form branched chains rather than clusters, which occur in unheated milk gels (Barantes et al., 1996). Figure 1 shows the formation of yogurt using heat treatment compared to unheated milk (Aguilera and Stanley, 1999). The yogurt gel is formed as casein micelles gradually aggregate with the denatured whey proteins, forming a chain matrix. Tamime ad Robinson, (1999) also reported that yogurt prepared with unheated or inadequately heated, milk is characterized by poor texture, weak gel and increased susceptibility to whey off.

Yogurt used to be made from whole milk concentrated by boiling. In the modern industrial world, yogurt is made from whole milk, skim milk, homogenized whole milk, low fat milk, skim milk with or without non-fat dry milk solids, stabilizers/thickeners, hydrocolloids, and flavoring materials such as fruit, fruit syrups, and sugar (Fox and McSweeney, 1998). The functionality of hydrocolloids is demonstrated by their ability to

bind water, react with milk constituents (proteins), and stabilize the protein network, preventing free movement of water. Bhullar et al., (2002) also reported that addition of WPC favors firmness and viscosity.

A lot of work has been published on the heat effects upon yogurt manufacturing. The temperatures of heating milk for yogurt manufacturing generally vary from 75 °C for 1 to 5 min to 95 °C for 5 to 10 min. However, other time temperature combinations are also used, such as high temperature short time (HTST) or ultra high temperature (UHT) treatments (Sodini et al., 2004). The rheological and microstructural properties of acid milk gels from unheated milk are very different from those of severely heated milk gels (Lucey et al., 1998; Lucey et al., 1999). Insufficient heating will result in weak bodied yogurt gels, while excessive heating will lower gel strength and also result in grainy textured yogurt with a tendency towards syneresis (Sodani et al., 2004).

Most studies have shown that heating the milk base increases the water holding capacity (WHC) of yogurt (Van Marle, 1998; Mottar et al., 1989; Augustin et al., 1999; Barrantes et al., 1996). However Dannenberg and Kessler, (1988) stated that when denaturation and complex formation has reached a maximum, a further increase in the severity of the heat treatment of the milk does not improve the water holding capacity of the yogurt gel. This phenomenon was also observed by Lucey et al., (1998) in yogurts obtained from heated milk at 83 °C for 30 min and highly heated milk at 93 °C for 30 min.

Many studies have reported that gel firmness is increased due to heat treatment. Mottar et al., (1989) reported an increase of 71 % in yogurt hardness in UHT treated milk yogurt gels compared to conventional heating (90 °C for 10 min) milk yogurt gels. But for skim milk, Savello and Dargan (1995) reported that gel firmness of UHT (140 °C for 4 s or 16 s) skim milk fortified with 5 % protein was significantly lower than that of vat-heated (82 °C for 20 min) skim milk yogurt gels. Dannerberg and Kessler, (1988) reported that yogurt gel firmness was strongly dependent on the amount of β-lactoglobulin denaturation in milk due to heat. However, they also reported that the protein confirmation is destroyed at high temperatures and the parameters typical for denaturation process were not found at temperatures above 90 °C. Viscoelastic properties of chemically acidified gels are strongly influenced by heating of milk. Lucey et al., (1999) and Cho et al., (1991) reported considerable increases in firmness in the heated and non heated milk yogurt gels. Heating milk above 80 °C resulted in an increase in the pH of gelation, a reduction in the gelation time and a marked increase in the storage modulus compared to unheated milk (Lucey et al., 1999). When milk is heated to high temperatures, whey proteins are almost completely denatured and some of the denatured whey proteins associate with the casein micelles, which results in increased cross-linking with in the gel that leads to the quality of yogurt (Singh and Creamer, 1992).

1.3 Nonthermal treatment

Traditionally, foods have been preserved using heat treatment. Heat is by far the most widely used technology utilized to inactivate microbes in foods (Farkas 1997). Despite the effectiveness achieved by thermal processing, heat causes nutritional and sensory

deterioration in food. The processing of foods is becoming more sophisticated and diverse, in response to the growing demand for quality foods.

During the last decade many consumers in North America and Europe have modified their nutrition concepts and their food habits for health reasons, with a reduction in the amount of fat, sugar, salt, cholesterol and certain additives. In the past, food science was concerned about developing foods for human survival; now the focus has shifted a bit in order to include other factors such as high quality, health, nutrition, environment, minimal process, and organic products, to name a few.

The increase in demand for 'fresh' like or more natural foods has promoted the search for novel nonthermal processing technologies that are capable of inactivating food-borne pathogens while minimizing deterioration in food quality. These new technologies inactivate microorganisms chemically or enzymatically by essentially physical means, which also introduces many more possibilities without heat for pasteurization or sometimes with heat for sterilization. Some of the promising nonthermal processing technologies are high pressure processing, pulsed electric fields, ultrasonification, and irradiation.

1.4 High hydrostatic pressure processing

The effects of high hydrostatic pressure processing on biological materials and organisms in food were first reported more than a century ago, when Hite (1899) successfully treated raw milk and reported that high pressure could be used for the preservation of milk. However, due to requirement of more suitable equipment and high equipment and

maintenance costs, high pressure research in food science almost stopped for about 80 years. After advances were made in the availability of suitable equipment and its applications in the chemical, ceramic and metallurgical industries during the 1970s and 1980s, there was renewed interest in the possibility of HHP in foods (Hinrichs et al., 1996). The main areas of interest regarding HHP as a novel food processing technology include (Stewart et al., 2006):

A. Inactivation of microorganisms

B. Modification of biopolymers, e.g., protein denaturation, gel formation and enzyme activation or inactivation; and

C. Quality retention, especially in terms of flavor and color.

From early 1990's with the development of suitable equipment, interest in the HHP treatment of various food products re-emerged. HHP offers unique advantages over the traditional thermal treatments, as it mostly exerts antimicrobial effects without changing the sensory and nutritional quality of foods. There is a wealth of fundamental and applied research information on HHP in dairy products (Harte et al., 2007; Huppertz et al., 2006 a, b; Lopez-Fandino, 2006 a, b). HHP may also induce the gelation of milk concentrates at low temperature and neutral pH in the absence of any coagulating enzyme or gelling agent (Kumeno et al., 1993; Velez-Ruiz et al., 1998). Most of these HHP applications are mainly used to extend the shelf life, improve the rheology and texture, and/or to create functional dairy products.

1.5 High hydrostatic pressure induced changes in constituents of milk

High hydrostatic pressure (HHP) processing has a significant effect on different constituents of milk. Many authors reported disruption of casein micelles, distribution of different proteins and minerals, and unfolding of milk proteins by HHP (Huppertz et al., 2002, Harte et al., 2003; Needs et al., 2000; Lopez-Fandino et al., 1998; Lee et al., 1996). Huppertz et al., 2002 reported that the main effects are primarily on casein micelles and whey proteins, resulting in increased pH and reduced color (Hunter L-value) and turbidity of milk following HHP treatment.

A large number of factors, e.g., temperature, time, micelle concentration, pH, additives and pre-treatment of casein micelles affect both the disruption of casein micelles and reformation of casein particles under pressure. Under pressure, solubilization of micelle calcium phosphate leads to disruption of casein micelles with increasing pressure and time (Gebhart et al., 2005; Huppertz et al., 2006; Orlien et al., 2006) and in un-concentrated milk, micelle disruption is complete at 400 MPa. At 250 and 300 MPa, reformation of casein particles from disrupted micelles occurs, but this process does not occur at lower or higher pressures (Harte et al., 2003). Gebhart et al., 2005 and Orlien et al., 2006 reported that casein micelle disruption decreases with increasing temperature. As a result of the aforementioned changes, properties of casein micelles in HP-treated milk differ considerably from those in untreated milk.

The whey proteins, α-lactalbumin (α-la) and β-lactoglobulin (β-lg), are also influenced significantly under high pressure (Huppertz et al., 2006a and Lopez-Fandino 2006b). The sequence of events under pressure involves a reversible pressure-induced unfolding of the β-lactoglobulin molecule, leading to exposure of its free sulphydryl group. This sulphydryl group can subsequently undergo irreversible sulphydryl-disulphide interchange reactions with proteins, including whey proteins, caseins, or proteins of the milk fat globule membrane.

1.6 High hydrostatic pressure for yogurt manufacturing

Consumers increasingly demand convenience foods of the highest quality in terms of natural flavor freedom from emulsifiers, stabilizers, and preservatives. Due to this demand HHP (100-1000MPa) is slowly being adopted by the food industry and is of increasing interest for use in the dairy industry. HHP can alter the structure of proteins, inactivate enzymes, and inactivate microorganisms, but the basic mechanisms involved are only partially understood (Hummer et al., 1998). HHP of milk before fermentation in the production of yogurt resulted in increased solid-like behavior and whey retention properties of the yogurt, with other properties unaffected by the HHP treatment (Needs et al., 2000; Ferragut et al., 2000; Harte et al., 2002). Johnston et al., 1993 reported that acid set gels made from high pressure processed skim milk showed an improved rigidity and gel breaking strength, and a greater resistance to syneresis with increasing pressure and treatment time. Coagulation of milk started at a higher pH and yielded a stronger gel than untreated milk (Desobry-Banon et al., 1994). These changes were then supported by the

theory of micelle disruption into smaller casein micelle clusters or aggregates by HHP (Famelart et al., 1997; Harte et al., 2002).

Yogurt made from pressure treated milk showed higher storage modulus, but yielded more readily to large deformation compared to heat treated milk yogurt (Needs et al., 2000). But Harte et al., 2002 did not find significant differences in the yield stress of yogurts made from heat treated and high pressured treated milk. However, Johnston et al., (1994) reported improved hydrodynamic properties and viscosity when the milk was treated for one hour in the 100 to 600 MPa pressure range. Yogurt made from high pressure treated ewe's milk (200 to 500 MPa, 10 to 55 ºC, 15 min) showed higher firmness with increasing pressure and an additional significant increase was observed at 55 ºC. Heat treated milk yogurt showed increased levels of syneresis compared to the high pressure treated milk yogurts during storage (Ferragut et al., 2000).

1.7 Ultrasonification

Ultrasonification is the use of ultrasound to enhance or alter chemical reactions. Ultrasound has proven to be a very useful tool in enhancing the reaction rates in a variety of reacting systems (Thompson and Doraiswamy, 1999). It has successfully increased the conversion, improved the yield, changed the reaction pathway, and/or initiated the reaction in biological, chemical, and electrochemical systems (Thomson and Doraiswamy, 1999). In the past two decades, most of the research has been done by chemists and physicists who have found that the chemical and some mechanical effects

of ultrasound are a result of implosive collapse of bubbles. The interest in ultrasound and cavitational effects dates back over 100 years. In 1927, Loomis first reported the chemical and biological effects of ultrasound (Richards and Loomis, 1927; Wood and Loomis, 1927). Over the years, several theories like acoustic cavitation and bubble dynamics (Neppiras, 1980), rectified diffusion (Crum, 1984), stable cavities (Cum et al., 1992), and transient cavitation have been proposed for ultrasound by many scientists. Two competing cavitation theories exist: the hot spot theory, the electrical theory to explain the chemical effects due to cavitation (Margulis, 1985). The hot-spot theory postulates that when the bubbles cavitate, localized hot spots are formed, which reach temperatures and pressures in excess of 5000 K and 500 atm. The electrical theory postulates that an electrical charge is created on the surface of a cavitation bubble, forming enormous electrical field gradients across the bubble that are capable of bond breakage upon collapse (Margulis, 1985).

Ultrasound has attracted considerable interest in food science and technology due to its promising effects in food processing and preservation. A vast amount of work has been published on the ultrasonification effects on various food systems. Table 1 shows some of the applications of ultrasound in food processing.

The production of yogurt is an increasingly important process in the food processing dairy industry. Recently the dairy industry has shown tremendous interest in developing and producing low fat yogurt with live and active probiotic cultures. Nonthermal processing technologies may contribute to the dairy field. Technologies like high pressure processing and ultrasound are worth of research to improve the physicochemical,

rheological characteristics of low fat probiotic yogurt. These technologies might offer yogurt with better sensory quality and lower levels of stabilizers, emulsifiers, and other additives. Very little research has been done on the viability of probiotics in yogurt using both thermal and nonthermal processing technologies, so if proven effective, these technologies will provide a novel approach to the dairy industry.

1.8 Dissertation outline

This dissertation is presented in seven chapters, where this literature review is the first. Chapter two investigates the effects of milk treatments on the physicochemical characteristics and probiotic cell counts of yogurt using high pressure processing. Chapter three investigates the rheological and textural properties of low fat yogurt processed by high pressure, heat and combined heat, and high pressure processing. Chapter four describes the microstructural differences among the low fat yogurts manufactured by high pressure, heat, and combined heat and high pressure processing. Chapter five investigates the effect of ultrasonification on the release of β-galactosidase enzyme for improving the viability of probiotics in yogurt. Chapter six investigates the shelf life and viability of probiotics in low fat yogurt using sonicated yogurt cultures. Finally, chapter seven presents the conclusions and recommendations for future research.

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Table 1. Various applications of ultrasonification in food processing

Mechanical Effects	References
Crystallization of fats and sugars, etc.,	Midler 1970; Acton and Morris, 1992; Stasiak and Dolatowski, 2007
Sugar diffusion	Stasiak, 2005
Degassing	Eskin, 1996
Destruction of foams	Khmelev et al., 2007
Extraction of flavorings	Zhao et al., 1991; Mason and Zhao, 1994
Filtration and drying	Senapati, 1991; Muralidhara et al., 1985; Boucher 1971; Fairbanks 1974
Freezing (Ice Cream processing)	Action and Morris, 1992 ; (Zheng and Sun, 2006)
Mixing and homogenization	Singser and Beal, 1960; Gaffney 1996
Precipitation of airborne powders	Gallego-Juarez, J.A. 1998
Tenderization of meat	Dolatowski and Stasiak, 2002; Jayasooriya et al., 2007
Chemical and Biochemical effects	
Bactericidal action	Earnshaw R.G., 1998
Effluent treatment	Sangave and Pandit et al., 2006
Modification of growth of living cells	Liu et al., 2003
Alteration of enzyme activity	Ley and Low, 1989; Wiltshire, 1992
Oxidation	Rosenfeld and Schmidt, 1984
Sterilization of equipment	Slapp, 1995

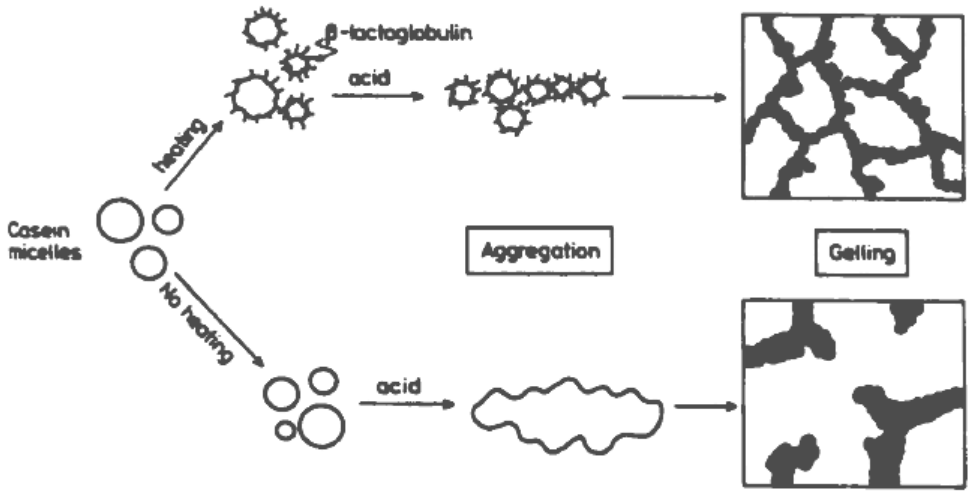

Figure 1. Yogurt gel formation after interaction between β-lactoglobulin and casein micelles (Aguilera and Stanley, 1999)

CHAPTER TWO

Effect of Milk Treatments on Acidification, Physicochemical Characteristics, and Probiotic Cell Counts in Low Fat Yogurt

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2.1 ABSTRACT

High hydrostatic pressure (HHP), 676 MPa for 5 min, Thermal treatment (TT), 85^0C for 30 min, and a combined treatment (HHP+TT) were used in the manufacturing of low fat yogurt. These processes were analyzed for their effects on acidification level, physicochemical characteristics, and probiotic bacteria. The processed milk was fermented with two different starter cultures at inoculation rates of 0.1 and 0.2%. All treated 12 samples were analyzed for fermentation time, pH, titratable acidity, water-holding capacity, syneresis, Hunter L*, a*, and b* values, as well as the viability of yogurt and probiotic bacteria. The treatments did not affect the growth of probiotic bacteria or the balance of strains (type of bacteria) in the starter culture; however, the level of inoculation influenced the fermentation time and most physicochemical properties of yogurt. The combined application of HHP and thermal treatment, when the inoculation level was 0.2%, resulted in yogurt gels with attractive physicochemical characteristics and high water-holding capacity. There was a decrease of 3 to 4 log reduction cycles in *L. acidophilus* when the pH dropped below 4.4 during milk fermentation. These results suggest that the use of combined HHP and heat could be a sound process to obtain higher quality and additive-free healthy and marketable low fat yogurt.

(**Key words:** high hydrostatic pressure, yogurt, and probiotics)

2.2 INTRODUCTION

Low fat or fat free yogurts with low calories have won popularity during the last decade. Traditionally, yogurt is made from the symbiotic growth of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. These organisms are claimed to offer some health benefits; however, they are not natural inhabitants of the intestine. These yogurt bacteria do not survive the gastric passage and colonize the gut. Hence, the recent trend is to add *L. acidophilus* and *Bifidobacterium* spp. to yogurt to overcome this limitation (Shah, 2000).

Several types of fermented dairy products that contain *L. acidophilus* are well established in the market in many countries. Products containing bifidobacteria are very popular in Japan, France, Germany, and USA, but are also produced in Canada, Italy, United Kingdom, and Brazil. In fact, almost 100 products containing these microorganisms are available on the market world-wide.

Stimulatory factors (pyruvate, HCO_3, adenine, guanine, adenosine, formate etc.,) are released by the yogurt starter culture bacteria during the incubation period. The growth association between *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in yogurt starter cultures could be described as symbiosis (mutually beneficial to each other). *L. delbrueckii* ssp. *bulgaricus* releases nutrients (i.e., amino acids) useful to *S. thermophilus* because of its proteolytic nature and *S. thermophilus* produces formic acid (Formate), which promotes the growth of lactobacilli (Tamime and Robinson, 1999).

Probiotic bacteria grow slowly in milk because of a lack of proteolytic activity. To improve their growth, it is common to add yogurt bacteria to reduce the fermentation time. However, *L. delbrueckii* ssp. *bulgaricus* also produces lactic acid during refrigerated storage, known as post-acidification, which causes loss of viability of probiotic bacteria (Shah, 2000). Therefore, different types of processing methods have been explored.

Among the novel technologies for food preservation, high hydrostatic pressure (HHP) is receiving a great deal of attention. The application of HHP to milk for yogurt preparation could be an alternative to the use of additives, which can adversely affect the taste, flavor, aroma, and mouth feel of yogurt (Ancos et al., 2000). Additional healthy aspects include maintenance of good health, stabilization of microbial ecology in the gut, reducing the risk of colon cancer, increased immune response, improvement in lactose malabsorption for lactose intolerant people, and reduction in concentration of cholesterol in blood plasma. Thus, an additive-free product is more favorable and will increase the consumption. Even more challenging would be to produce low fat and nonfat yogurts that do not whey-off during storage, without using stabilizers (Lucey and Singh, 2002).

HHP processing of milk before fermentation has been successfully used to manufacture low fat set-type yogurt (12% total solids) with a creamy thick consistency, requiring no addition of polysaccharides (Moorman et al., 1996). The yogurts presented increased solid-like behavior and whey retention properties of the yogurt, with other properties unaffected by the HHP treatment (Needs et al., 2000; Ferragut et al., 2000; Harte et al.,

2002). HHP was successfully used to prevent post-acidification on already fermented yogurt (Ancos et al., 2000).

Harte et al. (2003) found that the combined use of thermal treatment (85ºC, 30 min) and HHP (676 MPa, 5 min) assures extensive whey protein denaturation and micelle disruption. Although reaggregation of casein submicelles occurs during fermentation, the net effect of combined HHP is the improvement of yogurt yield stress and reduction of syneresis. However, little information is available concerning the growth of probiotic bacteria in high hydrostatic pressured milk. Therefore, the present research was undertaken to evaluate the effect of high hydrostatic pressure processing on acidification, physicochemical characteristics, and the growth of probiotic cell counts in low fat yogurt.

2.3 MATERIALS AND METHODS

Skim milk (0.0 – 0.2% fat and 9.17 – 9.20% total solids) was purchased from the Washington State University (WSU) Dairy Creamery and fortified with skim milk powder (less than 1% fat, 97% total solids) to increase the total solids to 14%. The fortified milk was then subjected to thermal treatments at 85ºC for 30 min. Milk was cooled in a water bath to 43ºC for the yogurt preparation.

2.3.1 Pressure treatment

Samples of fortified milk were placed in plastic bags and sealed. Pressure treatments were carried out using an isostatic pressure system (Engineered Pressure Systems, Inc., Haverhill, MA., USA) with a chamber size of 0.10 m diameter and 0.25 m height. The

medium for hydrostatic pressurization was 10% Hydrolubric 123B oil/water solution (Haughton International Inc., Valley Forge, PA). Samples were subjected to high hydrostatic pressure (HHP) at 676 MPa for 5 min at room temperature. Pressure was achieved within 4 to 5 min and the depressurization took less than 1 min.

2.3.2 Yogurt preparation

The processed milk (thermal, HHP or combined) was inoculated (0.1% or 0.2% v/v) with two different freeze-dried probiotic yogurt starter cultures (YO MIX 236 or DPL ABY 611) supplied by Rhodia Inc. (Madison, WI, USA) and Danisco USA Inc. (Milwaukee, WI, USA), respectively. These starter cultures are a mixture of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp *bulgaricus*, *Lactobacillus acidophilus*, and *Bifidobacterium longum.* The fermentation was carried out at 43ºC. Each fermentation process was monitored by continuous recording of pH values to measure the acidification rates during fermentation until the pH value reached 4.6 ± 0.1. The yogurt was cooled to 20ºC in an ice bath and then stirred with a mechanical mixer for 30 seconds according to a standardized protocol and stored at 4ºC for 15 to 16 hours. The experimental design of different treatments is summarized in Table 1.

2.3.3 Yogurt analysis

Total solids content was measured by drying the sample in a vacuum oven at 70ºC for 24 h (Case et al., 1985). Titratable acidity was measured by Dornic (ºD) and converted to percentage of lactic acid (ºD = 0.1 % of lactic acid) to a pink endpoint using a phenolphthalein indicator (Instituto Adolfo Lutz, 1976). The pH value was measured

using a digital 420 A pH meter (Orion Research Inc., Boston, MA, USA). All tests were carried out in triplicate.

Color (L*, a* and b*) of the milk before and after treatments and color of yogurt was studied using a Minolta CM-2002 Spectrophotometer (Minolta Camera Co., Tokyo, Japan). The measure of lightness L* (0-100) represents the black to white, a* (-100 to 100) green to red, and b* (-100 to 100) blue to yellow. Milk and yogurt samples (20 g) were held in small glass Petri dishes with flat, optically transparent sides and 10 mm thickness. Measurements were taken in triplicate at room temperature.

Water-holding capacity was evaluated by subjecting the yogurt to centrifugation at 15000 X G for 15 minutes at 20ºC (Harte et al., 2003). Ten grams of yogurt sample was evaluated using a Beckman J2-HS centrifuge (Beckman Instruments Inc., Seattle, WA, USA). Water-holding capacity was expressed as the percentage of pellet weight relative to the original weight of the sample:

$$WHC(\%) = \left[1 - \frac{Weight\ of\ whey\ after\ centrifugation}{Weight\ of\ yogurt}\right] \times 100$$

Susceptibility of yogurt to syneresis was determined using a drainage method. Yogurt samples were transferred into a funnel fitted with a qualitative paper Whatmann No. 5. The volume of the whey collected over 4 h at 4ºC was measured in a 25 mL graduated cylinder (Hassan et al., 1996 b).

Cell count enumerations of yogurts were analyzed after 7 days of storage at 4ºC. Yogurt samples of 1 mL were added to 9 mL sterile tryptone diluent (0.1% v/v). Appropriate dilutions were made and subsequently pour-plated in duplicate onto selective media. The International Dairy Federation Standard 117B (IDF, 1997) was used to enumerate *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp *bulgaricus.* Streptococci and lactobacilli were enumerated on M 17 agar with lactose after aerobic incubation at 37ºC for 48 h and MRS agar with glucose after anaerobic incubation at 37ºC for 72 h, respectively. *Bifidobacterium* were enumerated on MRS with glucose plus diclhoxacilin solution, lithium chloride and cistein chloride after anaerobic incubation at 37ºC for 72 h (Chr. Hansen, 1999). *Lactobacillus acidophilus* was counted using MRS agar with maltose after anaerobic incubation at 37ºC for 72 h (IDF, 1995). The results were expressed as colony-forming units per gram of yogurt (CFU/mL yogurt).

2.4 RESULTS AND DISCUSSION

Table 2 shows the titratable acidity in the milk bases, the fermentation time, pH value, total solids content, water-holding capacity (WHC), and syneresis of yogurt. Milk acidity varied from 28.48 to 30.97 ºD (Dornic degrees) for all treatments. Starter culture YO MIX 236 showed a higher acidification rate, reaching the final pH in 4 to 5 hours, according to the treatment, while the fermentation time for the DPL ABY 611 was at least 5h. This difference could be explained by the higher population of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* compared to those found in the starter culture DPL ABY 611 (see Table 6). The balance of strains in the culture and the level of inoculation affected the yogurt fermentation, as shown by pH curves in Figures 1 and 2.

Østile et al. (2003) found very different profiles of metabolites during fermentation, and showed the importance of controlling fermentation time since probiotic strains produced different amounts of metabolic products according to fermentation time. At the end of fermentation the pH value of yogurt varied from 4.48 to 4.70, showing suitable fermentation control. The pH value of fermented milk products tended to decrease during storage due to post-acidification, a result of starter culture activity (Brandão, 1995). The developed titratable acidity of yogurt ranged from 111.28 to 144.26ºD (1.11 to 1.44% lactic acid), and the average final value of titratable acidity was 123.92ºD (1.23% lactic acid). The total solids varied from 13.11 to 15.10% and syneresis was between 6 and 16.5%. Such variations were typical for these types of experiments because of their different conditions during treatment of the milk and fermentation of the yogurts.

The water-holding capacity (WHC) of yogurts was determined using the drainage tests by centrifugation, and varied from 25.59 to 32.87%, although the mechanical stability of the protein network under G-forces (15000G) was tested much more extensively than for those under normal storage. The effect of milk treatment, culture type, and inoculation rate was studied by ANOVA (Analysis of Variance). There was no difference between yogurt prepared with heat and heat combined with HHP treatments, the effect of starter and inoculation was highly significant ($p<0.01$). Using the starter culture YO MIX 236, WHC was higher in yogurts prepared with milk treated with heat or combined heat and HHP treatments, while yogurts fermented from the starter culture DPL ABY 611 and heated milk presented the higher WHC. However, the combined heat and HHP milk treatments before fermentation and use of a 0.1% inoculation rate (for both cultures) led

to attractive rheology and texture properties in yogurt, which presented a creamy thick consistency requiring no addition of stabilizers (data not shown).

There are few studies about the effect of high hydrostatic pressure on the physical properties of yogurt. Ferragut et al. (2000) showed that a high pressure treatment of ewe's milk improved firmness and WHC of corresponding yogurts. An increased number of network strands in pressurized milk gels explains the higher gel strength and improved WHC (Johnston et al, 1993). Harte et al. (2003) reported that yogurts made from HHP (676 MPa, 30 min) treated fortified milk exhibited the highest whey retention properties, while yogurts made from other treatments (except raw milk) exhibited lower whey retention values that were not significantly different from each other.

Most studies have shown that the heating of the milk base increases the WHC of yogurt. Lucey et al. (1998) and Parnell-Clunies et al. (1987), in their analysis of yogurt's microstructure, suggested that the branched, less coarse structure of yogurts made from heated milk could immobilize large volumes of the liquid phase, thus enhancing the WHC. Dannenberg and Kessler (1988) suggested that a large denaturation of β-lactoglobulin reduced the capacity of micelles to coalesce during fermentation, which resulted in the formation of a network composed of casein micelle chains of immobilizing large volumes of water. Whey protein denaturation and further aggregation to κ-casein are mainly responsible for the marked increase of WHC, firmness, and apparent viscosity of acid gels made from heated milks (Cho et al., 1991), but the mechanisms are not entirely understood. Becker and Puhan (1981) reported that in 63 yogurt samples made from skimmed milk, 15 showed a whey layer on the surface after

14 days of storage, especially in yogurts containing low total solids, however, yogurt made from whole milk did not show any whey separation. Increasing the total solids or protein content leads to a higher concentration of casein particles, which reinforces the protein matrix density and improves the WHC of the gel (Sodini et al., 2004).

The effects of treatments on the milk and yogurt are reflected in changes to the color values (Tables 4 and 5). HHP treated milk had lower L*, a*, and b* values than either heat and combined heat and HHP treated milk. Yogurt and heat treated milk had higher values of L*, a*, and b* due to changes in the light-scattering properties of milk. The disruption of micelles under high pressure caused a significant change in the appearance of the milk, which was quantified by measuring the color. Heat treatment also affected these characteristics. The decrease of L* (lightness) and increase of greenness (-a*) and yellowness (+b*) were also observed by Gervilla et al. (2001) when ewe's milk was treated by HHP. Harte et al. (2003) observed high L* values (increased whiteness) in milk subjected to HHP followed by thermal treatment, which could be explained by the reaggregation of disrupted micelles. The authors also found that HHP treatment reduced the lightness of raw or thermally treated milks; a small decrease in color was observed when milk was subjected to HHP at > 300 MPa for 5 min.

Needs et al. (2000) reported similar results and described colors values of HHP treated milk as translucent and greenish. Warming the HHP milk to 43ºC increased L* and a*, but b* remained unchanged, whereas HHP samples had larger ΔE than heated milk at all stages.

The effect of variables on the lactic acid bacteria cell counts are reported in Table 6. The effect of milk treatment, culture type, and inoculation rate was studied by ANOVA. The effects of the starter culture and inoculation rate were highly significant ($p<0.01$) on the count of *Streptococcus thermophilus*, while the milk treatment did not affect their growth. *Lactobacillus delbrueckii* ssp *bulgaricus* counting differs only between starter cultures; yogurts prepared with any milk treatment and different inoculation rate showed similar results. The variables did not affect the counting of *Lactobacillus acidophilus* and *Bifidobacterium longum* in yogurt samples. These results suggested that milk treatment, besides HHP can alter the structure of casein and whey protein, it did not affect the lactic bacteria growth. The counts after 1 week of preparation were $1.00x10^4$ to $1.05x10^7$ CFU/mL for *Bifidobacterium longum*, $9.00x10^5$ to $4.55x10^7$ CFU/mL for *L. acidophilus*, $1.60x10^6$ to $2.61x10^9$ CFU/mL for *L. delbrueckii* ssp *bulgaricus*, and $2.50x10^7$ to $5.75x10^9$ CFU/mL for *S. thermophilus*. These ranges depended on the experimental conditions and the starter culture used, in which *S. thermophilus* predominated in all treatment preparations.

Culture YO MIX 236 showed a higher population of traditional yogurt bacteria (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus)* than DPL ABY 611. It was observed that when *L. delbrueckii* ssp. *bulgaricus* population is higher (10^8 or 10^9), the viability of *B. longum* was around 10^5 or 10^4. This different strain association between cultures could explain the lower viability of probiotic bacteria in yogurts made with cultures YO MIX 236.

Several factors have been claimed to affect the viability of both yogurt and probiotic cultures in fermented milk products. The viability depends on the strains used, interaction between species present, culture conditions, production of hydrogen peroxide by yogurt bacteria, final acidity of the product, concentration of lactic and acetic acid (Shah, 2000), oxygen content in the product, and permeation through the package (especially for *Bifidobacterium* spp). Although *L. acidophilus* and bifidobacteria tolerate acid, a rapid decline in their numbers in yogurt has been observed under acidic conditions (Shah and Jelen, 1990; Lankaputhra and Shah, 1995). *Bifidobacteria* are not as acid tolerant as *Lactobacillus acidophilus*. The growth of *Lactobacillus acidophilus* ceases below 4.0 and for *Bifidobacteria* ssp. is retarded below pH 5.0 (Shah, 1997). Post-acidification is found to cause loss of viability of probiotic bacteria (Shah et al., 1995).

Beal et al. (1999) reported that final pH significantly influenced bacterial concentrations. *L. bulgaricus* concentrations were higher in yogurts with final pH at 4.4 than at pH 4.8, which indicates that *L. bulgaricus* was more resistant to acidic conditions but growth of *S. thermophilus* growth had already stopped at 4.8. The pH effect on *S. thermophilus* was related to a slight decrease in cell concentrations between pH 4.8 and 4.4. The greater tolerance of *L. bulgaricus* to low pH was in agreement with previous observations.

In order to exert positive therapeutic effects, the yogurt and probiotic bacteria must be viable, active, and abundant. It has been suggested that these microorganisms should be present in a food at a minimum level of 10^6 CFU/g or the daily intake should be about 10^8

CFU/g (Shah, 2000; Vinderola et al., 2000). From a health point of view, the starter culture DPL ABY 611 gave better results in producing higher probiotic bacterial count.

Beal et al. (1999) studied the combined effect of culture conditions and storage time on acidification and viscosity of stirred yogurt. The yogurt bacteria grew from $1.1x10^6$ - $2.6x10^6$ CFU/mL to $1.1x10^8$ - $5.8x10^8$ CFU/mL for *L. bulgaricus* and from $1.5x10^6$ - $3.4x10^7$ CFU/mL to $3.1x10^8$ - $6.1x10^9$ CFU/mL for *S. thermophilus*, depending on experimental conditions and strain used. Bacterial concentrations were influenced by storage time, final fermentation pH, strain association, and incubation temperature.

Comparing the two starter cultures used during fermentation (0.1 and 0.2%), it was noticed that the microorganisms multiplied more in yogurts with lower levels of inoculation for DPL ABY 611 culture (Table 6) and in most cases the bacterial count was higher when the inoculation rate was 0.2% for YO MIX 236 culture. These results are supported by Dave and Shah (1997), who studied the effect of starter culture concentration (0.05, 0.1, 0.15, and 0.2%) on the viability of yogurt and probiotic bacteria using commercial starter cultures. These authors also found that *Lactobacillus delbrueckii* ssp. *bulgaricus* remained viable for longer periods in yogurt prepared with less inoculum, however for *L. acidophilus,* if the pH of yogurt dropped below 4.4 at the time of fermentation, there was a 3 to 4 log cycle decrease. For bifidobacteria, the count dropped to $< 10^6$ log CFU/g in yogurt with lower concentration of inoculum.

Østile et al. (2003) studied the growth and metabolism of selected probiotic bacteria, and reported that the initial viable cell counts were between 7.7 and 8.51 log CFU/mL and above 8.7 – 9.18 log CFU/mL after 16 h of incubation. The *L. acidophilus* strains produced the highest amount of lactic acid, while bifidobacteria strains produced the lowest amount after 48 h of incubation. However, the acetic acid levels were higher in milk inoculated with bifidobacteria strains. All strains produced acetaldehyde, but the amount produced by *L. acidophilus* was much higher than for the bifidobacteria strains.

Because bifidobacteria are affected by environmental conditions, Clark et al. (1993) studied the survival of *B. infantis*, *B. adolescentis*, *B. longum*, and *B. bifidum* under acidic conditions and reported that *B. longum* survived the best. The results clearly show (Table 6) that the count of *Bifidobacterium longum* in starter culture DPL ABY 611 for both 0.1% and 0.2% inoculations is high compared to starter culture YO MIX 236 for all the treatments. Thus, selection of appropriate strains on the basis of acid and bile tolerance would help improve viability of these probiotic bacterial strains.

Overall, viability of probiotic bacteria can be improved by appropriate selection of acid and bile resistant strains, by two-step fermentation, micro-encapsulation, stress adaptation, incorporation of micronutrients such as peptides and amino acids, and sonification of yogurt bacteria (Shah, 2000). The slow growth of bifidobacteria in milk may be improved by the addition of growth-promoting substances like yeast extract or pepsin-digested milk (Rasic, 1983). Østile et al. (2003) reported that *L. acidophilus* and *Bifidobacteria* strains showed satisfactory growth for the production of a probiotic

fermented milk when tryptone was used as a supplement. The growth of bifidobacteria is stimulated in human milk because of the presence of a bifidus factor (Scardovi, 1986) identified as the substance N-acetyl-D-glucosamine, which contains saccharides and is lacking in cow's milk (Kurmann, 1988). This work showed the importance of selecting the right starter culture with the right combination of probiotic strains for low fat yogurt using different treatments of High Hydrostatic Pressure processing. This research adds support to the results of prior studies on the influence of milk treatment on fermentation time and yogurt acidity, which are very important for the survival of Probiotic bacteria.

2.5 CONCLUSIONS

This study has shown that the application of HHP for a short time, combined with thermal treatment produced yogurt gels with attractive physicochemical characteristics and high water-holding capacity. Furthermore, the milk treatments did not affect the growth of probiotic bacteria and the balance of strains in the starter culture, whereas it was found that the level of inoculation affected the yogurt fermentation and properties overall. This work has proven that the use of combined heat and HHP for treatment of milk before yogurt fermentation could be an alternative processing method for manufacture of high quality yogurt products with no addition of stabilizers and thickeners.

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Table 1. Experimental design of different treatments

Run	Culture type	Inoculation	Treatment
1	DPL ABY 611	0.1%	Heat
2	DPL ABY 611	0.1%	HHP
3	DPL ABY 611	0.1%	HHP + Heat
4	DPL ABY 611	0.2%	Heat
5	DPL ABY 611	0.2%	HHP
6	DPL ABY 611	0.2%	HHP + Heat
7	YO MIX 236	0.1%	Heat
8	YO MIX 236	0.1%	HHP
9	YO MIX 236	0.1%	HHP + Heat
10	YO MIX 236	0.2%	Heat
11	YO MIX 236	0.2%	HHP
12	YO MIX 236	0.2%	HHP + Heat

Heat – 85ºC for 30 min.

HHP – High hydrostatic pressure – 676 MPa for 5 min.

Table 2. The effect of milk treatment on acidification, fermentation time, and physicochemical characteristics of yogurts fermented from starter culture YO MIX 236.

	0.1%			0.2%		
	heat	HHP	HHP + heat	heat	HHP	HHP + heat
Milk acidity ºD	29.14	30.80	28.48	29.47	29.47	29.14
Fermentation time h	4:15	5:00	4:15	5:15	4:00	4:15
Yogurt pH	4.54	4.56	4.50	4.60	4.56	4.57
Yogurt acidity ºD	126.97	123.50	140.29	116.58	114.89	119.03
Total solids %	15.10	13.88	14.26	14.16	14.34	14.95
Water-holding capacity %	26.87	27.34	30.15	31.66	28.47	30.86
Syneresis %	13.00	16.50	14.00	15.50	12.50	11.0

Table 3. The effect of milk treatment on acidification, fermentation time, and physicochemical characteristics of yogurts fermented from starter culture DPL ABY 611.

	0.1%			**0.2%**		
	heat	HHP	HHP + heat	heat	HHP	HHP + heat
Milk acidity	30.14	30.97	28.81	29.08	29.73	29.08
Fermentation time h	5:10	5:30	5:00	5:00	5:10	5:15
Yogurt pH	4.68	4.59	4.48	4.70	4.65	4.60
Acidity ºD	111.28	133.13	144.26	112.91	127.22	116.94
Total solids %	14.12	14.11	14.44	13.11	14.90	14.26
Water holding capacity %	32.87	27.02	30.07	26.57	25.59	25.80
Syneresis %	6.00	11.00	12.00	14.00	9.00	12.50

Table 4. Color profile of milk (before and after treatments) and color of yogurt fermented from starter culture YO MIX 236.

	0.1%				**0.2%**			
Before treatment	L	a	b	ΔE	L	a	b	ΔE
Heat	52.39	-2.66	5.14	15.12	52.93	-2.64	4.92	15.36
HHP	53.17	-2.37	4.40	15.19	52.99	-2.58	4.62	15.27
HHP + Heat	53.37	-2.51	4.41	15.36	56.16	-2.78	5.24	18.09
After treatment								
Heat	54.76	-1.18	7.33	18.16	54.56	-2.09	5.38	16.85
HHP	40.45	-3.54	-1.04	4.95	35.47	-3.16	-0.47	7.95
HHP + Heat	54.51	-2.35	4.71	16.35	53.92	-2.63	4.76	16.02
Yogurt								
Heat	78.17	-1.23	10.75	24.36	60.24	-1.18	7.20	22.48
HHP	63.61	-1.50	6.52	25.04	54.52	-1.34	6.42	17.46
HHP + Heat	66.71	-1.27	9.17	29.03	61.10	-1.06	5.96	22.74

Table 5. Color profile of milk (before and after treatments) and color of yogurt fermented from starter culture DPL ABY 611.

	0.1%				**0.2%**			
Before treatment	L	a	B	ΔE	L	a	b	ΔE
Heat	51.05	-2.55	4.42	13.64	41.72	-1.68	2.91	8.80
HHP	52.22	-2.31	4.33	14.41	52.72	-2.89	4.54	15.00
HHP + Heat	53.27	-2.51	4.41	15.36	53.00	-2.85	4.34	15.18
After treatment								
Heat	53.73	-1.49	6.00	53.73	41.70	-1.39	3.86	9.18
HHP	40.82	-3.36	-0.94	40.82	36.22	-4.55	-1.87	7.18
HHP + Heat	54.51	-2.35	4.71	16.35	55.41	-1.92	5.04	17.33
Yogurt								
Heat	57.65	-0.80	8.02	20.89	62.30	-1.17	7.63	24.46
HHP	60.49	-1.45	6.38	22.26	64.10	-1.41	7.59	25.98
HHP + Heat	71.48	-1.43	8.56	29.81	65.10	-0.98	7.86	26.99

Table 6. Lactic acid bacteria counts in yogurts fermented from starter cultures YO MIX 236 and DPL ABY 611 (CFU/mL).

		0.1%			**0.2%**		
		heat	HHP	HHP + heat	heat	HHP	HHP + heat
YO MIX 236	*B. longum*	1.00E+06	2.06E+05	6.05E+04	5.80E+04	8.80E+05	1.00E+04
	L. acidophilus	9.65E+05	1.85E+06	9.05E+05	2.11E+06	2.80E+06	2.70E+06
	L. bulgaricus	9.90E+07	4.73E+08	1.16E+09	1.04E+09	2.18E+09	2.61E+09
	S. thermophilus	5.75E+09	2.75E+09	2.28E+09	2.54E+09	4.43E+09	3.20E+09
DPL ABY 611	*B. longum*	1.56E+06	7.90E+06	8.75E+06	1.05E+07	1.25E+06	3.85E+06
	L. acidophilus	3.10E+06	1.42E+07	1.65E+07	4.55E+07	1.30E+06	7.00E+06
	L. bulgaricus	1.60E+06	8.68E+07	2.30E+07	1.00E+07	1.07E+07	1.25E+07
	S. thermophilus	6.24E+07	1.03E+09	9.00E+08	8.10E+08	2.50E+07	7.60E+07

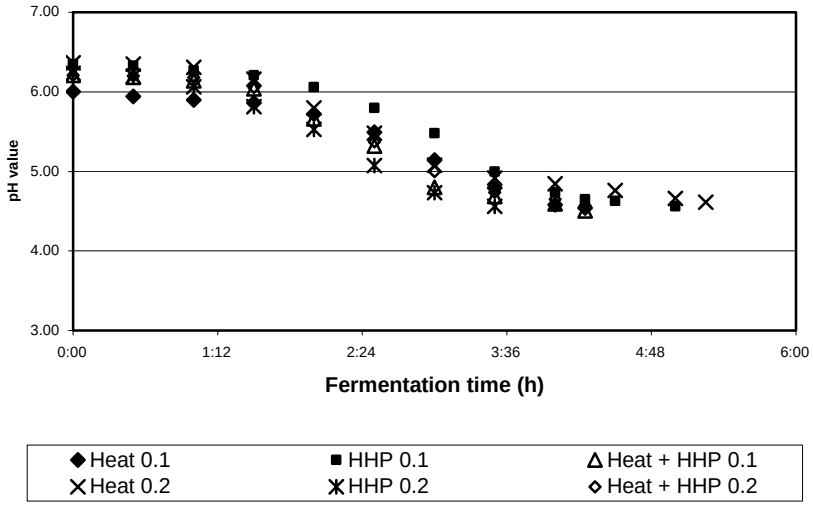

Figure 1 – pH curves during the fermentation of yogurt with culture YO MIX 236.

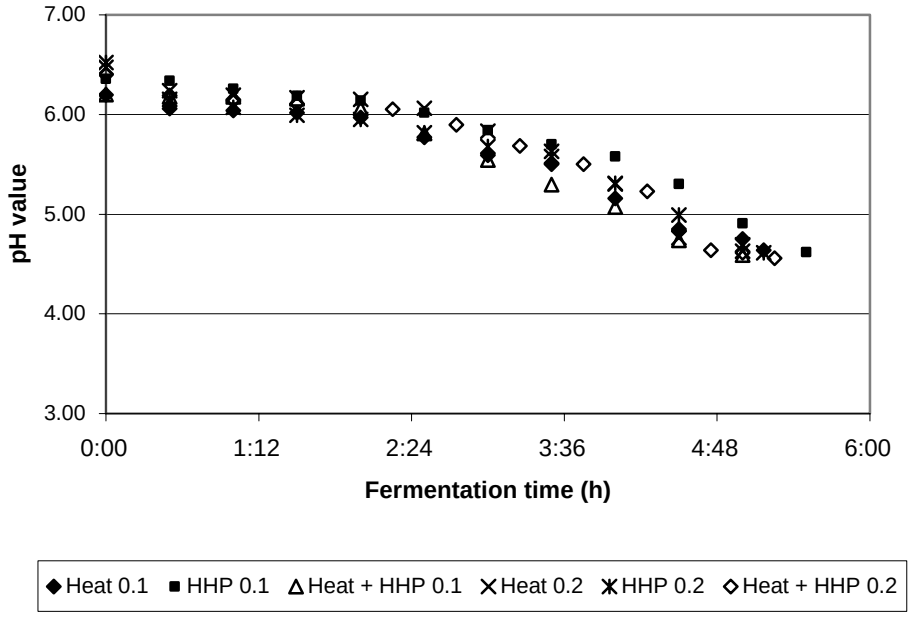

Figure 2 – pH curves during the fermentation of yogurt with culture DPL ABY 611.

CHAPTER THREE

Effect of High Hydrostatic Pressure Processing on Rheological and Texture Properties of Probiotic Low Fat Yogurt Fermented by Different Starter Cultures

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3.1 ABSTRACT

The effect of milk processing on the rheological and textural properties of probiotic low fat yogurt (fermented by two different starter cultures) was studied. Skim milk fortified with skim milk powder was subjected to three treatments: thermal treatment at 85ºC for 30 min; high hydrostatic pressure at 676 MPa for 5 min; and combined treatments of high hydrostatic pressure (676 MPa for 5 min) and heat (85^{o}C for 30 min). The processed milk was fermented using two different starter cultures containing *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp *bulgaricus*, *Lactobacillus acidophilus,* and *Bifidobacterium longum* at inoculation rates of 0.1 and 0.2%. Rheology parameters were determined and a texture profile analysis was carried out. Yogurts presented different rheological behavior according to the treatment used, which could be attributed to structural phenomena. The HHP and heat combined treatment resulted in yogurt gels with higher consistency index values than gels obtained from thermally treated milk. The type of starter culture and inoculation rate, providing different fermentation pathways, also affected the consistency index and texture properties significantly. The combined HHP and heat milk treatments before fermentation, and an inoculation rate of 0.1% (for both cultures), led to desirable rheology and texture properties in yogurt, which presented a creamy and thick consistency requiring no addition of stabilizers.

(**Key words:** high hydrostatic pressure, yogurt, rheology, texture, and probiotics)

Abbreviation key: HHP = high hydrostatic pressure.

3.2 INTRODUCTION

In recent years, low calorie and low fat foods have won popularity among consumers. Yogurt, a fermented dairy product, has gained special prominence and economic importance due to its high nutritional value and health benefits. The consumption of yogurt has steadily increased over the last 30 years in the United States (Economic Research Service, 2002) and in other parts of the world.

Fermented dairy products have been consumed for nutritional reasons and maintenance of good health for a long time (Vinderola and Reinheimer, 1999). The food industry has noticed this shift, and during the last few years there has been a fast growth in the market of diet and functional foods, including fermented dairy products. The quality of fermented dairy products depends on the food's texture and body, because the amount of solids is very low. Therefore, physical properties of cultured milk are major criteria for quality assessment. For instance, the most important textural characteristics of yogurt are firmness and the ability to retain water (Hassan et al., 1996 b). Physical properties of cultured milk are also affected by many other factors, including composition and heat treatment, mechanical handling of coagulum, and the type of culture (Hassan et al., 1996 a).

Probiotics are beneficial live microorganisms which when given to human beings through food (functional foods) affect the host beneficially. Probiotics are beneficial because they produce enzymes that help the body digest food. They also produce B-complex vitamins and, in cases of diarrhea, help in the neutralization of pathogenic microorganisms

responsible for infections. Probiotic yogurt occupies a very satisfactory position in the dairy products market, and there is a clear trend to increase its consumption in the next few years. Additional healthy aspects, like an additive-free product, will make this increase much more favorable. Therefore, the type of culture is one of the most critical factors influencing the texture and rheological properties of yogurt, making selection of the appropriate culture of great importance (Vlahopoulou and Bell, 1993).

For example, total solids content can affect the type of yogurt. During fermentation of milk into yogurt, the pH falls to around 4.4 and the destabilized micelles aggregate into a three-dimensional matrix in which whey is trapped (Rawson and Marshall, 1997). The use of stabilizers to improve texture and reduce whey separation is common. Other strategies to increase the total solids content include the addition of milk solids and/or whey protein concentrate (Mistry and Hassan, 1992).

High hydrostatic pressure processing has been a promising non thermal food processing method in many countries. The small-scale production of pressurized foods has become a reality in Japan (fruit-based products, and other foods), France (orange juice), and the USA (avocado spread). Large volume pressure vessels (500 liters) are currently available for such products from manufacturers. For example, high pressure-treated milk has been successfully used to manufacture a low fat set-type yogurt (12% total solids) with a creamy, thick consistency that requires no addition of polysaccharides (Moorman et al., 1996).

Harte et al. (2003) reported that yogurt made from milk subjected to HHP (400-500 MPa) and thermal treatment (85ºC for 30 min) showed increased yield stress, resistance to normal penetration, and elastic modulus, while having reduced syneresis, compared to yogurts made from thermally treated milk and from raw milk. Thus, the use of HHP offers microbiologically safe and additive-free low fat yogurt with improved characteristics, such as reduced syneresis, better texture, increased shelf life, and high nutritional and sensory quality (Trujillo et al., 2002; Harte et al., 2003). For instance, it has been reported that HHP improves acid coagulation of milk without detrimental effects on important quality characteristics such as taste, flavor, vitamins, and nutrients (Trujillo et al., 2002).

Although a certain amount of attention has been directed towards the sensory properties of probiotic yogurt, most publications have focused on the health aspects. Little information is available concerning the growth of probiotic bacteria in high hydrostatic pressured milk. Moreover, there is limited published information concerning the technological production of fermented probiotic dairy products and the rheological and texture properties of these microorganisms in high hydrostatic pressured milk.

This study will allow researchers to improve the textural properties of traditional yogurt and to develop novel varieties with improved functional properties. Specific objectives of this study were to determine and compare the effects on the textural and rheology properties of superior quality stirred probiotic yogurt prepared with different probiotic cultures.

3.3 MATERIALS AND METHODS

3.3.1 Heat treatment

Skim milk (0-0.2% fat and 9.17-9.20% total solids) was purchased from the Washington State University (WSU) Dairy Creamery and was fortified with skim milk powder to increase the total solids to 14%. The fortified milk was then subjected to thermal treatments at 85ºC for 30 min using a plate heater with magnetic stirrer. Milk was cooled in a water bath to 42ºC for the yogurt preparation.

3.3.2 Pressure treatment

Samples of fortified milk (700 mL) were placed in polyethylene plastic bags and heat sealed. Pressure treatments were carried out using an isostatic pressure system (Engineered Pressure Systems, Inc., Haverhill, MA, USA) with a chamber size of 0.10 m diameter and 0.25 m height. The medium for hydrostatic pressurization was 5% Mobil Hydrasol 78 water solution. Samples were subjected to high hydrostatic pressure (HHP) at 676 MPa for 5 min at room temperature, according to previous research of Harte et al., 2002. Targeted pressure was achieved in 4 to 5 min and depressurization took less than 1 min.

3.3.3 Yogurt preparation

Processed milk (thermal, HHP or submitted to both treatments) was inoculated (0.1% or 0.2% v/v) with two different freeze-dried probiotic yogurt starter cultures (YO MIX 236 or DPL ABY 611) supplied by Rhodia Inc. (Madison, WI, USA) and Danisco USA Inc. (Milwaukee, WI, USA), respectively. These starter cultures consisted of a mixture of

Streptococcus thermophilus, *Lactobacillus delbrueckii* ssp *bulgaricus*, *Lactobacillus acidophilus*, and *Bifidobacterium longum*. The fermentation was carried out at 43ºC until the pH value reached 4.6 ± 0.1. The yogurt was cooled to 20ºC in an ice bath and then stirred with a mechanical mixer for 30 seconds using a standardized procedure in all experiments. The cooled yogurt was then poured into 100 mL cups and stored at 4ºC for 15-16 hours. Stirred yogurt samples were withdrawn from storage for rheology and texture evaluation.

3.3.4 Rheological and texture properties

The effect of combined HHP and thermal treatment was studied and compared with the other two methods individually, by determining the rheological properties (yield stress, consistency index, and flow behavior index) and textural properties (TPA analysis) in order to obtain a high quality probiotic yogurt with less syneresis and longer shelf life. All determinations were carried out in triplicate.

Total solids content was measured by drying the sample in a vacuum oven at 70ºC for 24 h (Case, Bradley Jr. and Williams, 1985). The pH was measured using a digital 420 A pH meter (Orion Research Inc., Boston, MA, USA).

Rheological properties were measured using a Physica rheometer, model 320 (Paar Physica USA, Inc., Glen Allen, VA, USA). The measurements were made at 10ºC using concentric cylinders (CC27). The temperature-control was maintained by water circulation from an external water bath through the jacket surrounding the rotor and cup assembly. Shear rates ranging from 0.1 to 300 s^{-1} (with logarithmic scale increased at

every 10 s) under programmed upward and downward curves were used, and corresponding shear stress data was obtained. The rheological parameters were obtained at shear rates ranging from 0.1 to 103 s^{-1} using Origin Software 5.0 version (Northampton, MA) and adjusted by the Herschel-Bulkley model.

Texture analyzer, TA-XT2 Texture (Stable Micro Systems, Texture Technologies, Scarsdale, NY, USA), was used to evaluate the texture profiles with a 2 kg compression load cell. The analysis was carried out through a double compression test using an aluminum cylinder (P/50, diameter 50 mm). The cylinder penetrated 35% of strain on the surface of the coagulum, and the crosshead speed was 1 mms^{-1} for 12 s. Three replicate samples (70g of yogurt) were prepared at 5ºC for each type of yogurt. Szczesniak et al., (1963) showed that the textural attributes or parameters resulted from TPA force-time curve are well correlated with sensory evaluation.

Szczesniak et al. (1963) defined chewiness as the energy required to masticate a solid food and gumminess as the energy to disintegrate a semi-solid food. Typical parameters quantified were cohesiveness (the extent to which a material can be deformed before it ruptures), hardness (the force necessary to attain a given deformation), springiness or elasticity (the rate at which the deformed material returns to its undeformed state after removal of deforming force), and adhesiveness (the work necessary to overcome the attractive forces between the surface of the yogurt and the surface of other material with which it comes in contact) (Rawson and Marshall, 1997).

3.3.5 Statistical Analysis

All experiments (Table 1) were repeated in triplicate on individual yogurt samples. Statistical analyses were performed using a randomized block design, using SAS Statistical Software (Carey, SAS Institute, Inc., NC, USA) by Tukey's pair wise comparisons at the 99% confidence level.

3.4 RESULTS AND DISCUSSION

The shear stress and shear rate relationships (upward and downward curves) of the yogurt determined using the Herschel - Bulkley model is shown in Tables 2 and 3. These products could be characterized as non-Newtonian fluids with thixotropic flow behavior resulting from the structural breakdown during the shearing cycle. This is observed by the difference between the upward and downward curves of the shear rate/stress relationship of the yogurts when applying the Herschel - Bulkley model. These results were consistent with those reported in the literature for yogurt.

The yogurts presented different rheological behavior according to the treatment used ($p<0.01$), which can be attributed to structural phenomena. The differences could also be explained by a different capacity of the protein to interact with casein micelles. Denatured whey proteins, obtained by heating process, are an important cross-linking agent. Samples prepared with milk treated by HHP combined with heat using 0.1% of DPL ABY 611 culture presented the higher consistency index, however there is no significant difference between heat and HHP treatments alone. The type of culture and

inoculation rate, which provided different fermentation pathways, also affected the consistency index significantly ($p<0.01$).

Yield stress (upward curves) showed no significant difference ($p<0.01$) between culture types (DPL ABY 611 or YO MIX 236), although treatment and inoculation rate differed significantly ($p<0.01$) from the others. Yogurt prepared with milk treated by HHP combined with heat using 0.1% of DPL ABY 611, showed the highest yield stress.

For both cultures of yogurts prepared, the consistency index decreased when increasing the concentration of culture. These results agree with those obtained by Saxelin et al. (1999). They reported that probiotic strains combined with *S. thermophilus* and *L. bulgaricus* reduced viscosity compared with the yogurt culture alone.

During heat treatment of milk, the main change that occurs is denaturation and aggregation of whey proteins with caseins, via κ-casein binding, and fat globules (Corredig and Dalgleish, 1999). Complexation of β-lactoglobulin with κ-casein gives the casein micelles a hairy or spiky appearance. During gelation, the casein micelles thus altered form branched chains rather than clusters, the latter being common in curd made from unheated milk (Barrantes et al., 1996). Cross-linking or bridging of denaturated whey protein associated with the casein micelles results in an increase in numbers and strength of bonds between protein particles (Lucey et al., 1997).

High hydrostatic pressure (HHP) can alter both structures of casein and whey proteins. The denaturation of whey protein by HHP was reported by Datta and Deeth (1999), Gaucheron et al. (1997), and Trujillo et al. (2002). An increase in the viscosity of β-lactoglobulin stabilized emulsions following HHP, including the generation of gel-like characteristics, was reported by Dickinson and James (1998), while α-lactoalbumin showed more resistance to pressure denaturation (Hinrichs and Kessler, 1997). The application of HHP at room temperatures to skim milk leads to a decrease in the mean hydrodynamic diameter of casein particles, with a decrease in milk turbidity and lightness, and an increase in viscosity of the milk (Johnston et al., 1992). The presence of small particles would explain the decrease in the apparent lightness (Gaucheron et al., 1997). Needs et al. (2000), in a microstructure study, also observed in pressure treated milk held at 4ºC that the micelles were fragmented, forming small irregularly shaped particles, which are often formed into clumps and chains. During yogurt preparation, the irregular micelle fragments in milk changes to round, separate, and homogeneous compact micelles (Harte et al., 2003), but he also observed that HHP treatment alone (676 MPa, 5 min) is not suitable for promoting whey protein denaturation and further aggregation of β-lactoglobulin with casein in order to obtain a cream, thick consistency with no addition of stabilizers.

The HHP and heat combined treatment of milk and fermentation with 0.1% and 0.2% of DPL ABY 611 and 0.1% of YO MIX 236 resulted in yogurt gels with higher consistency index than gels obtained from thermally treated milk. In another study, yogurt gels prepared from HHP at 676 MPa for 30 minutes showed equivalent rheological curves,

compared with yogurt gels obtained from heated milk. Yogurt gels prepared from HHP for shorter times (676 MPa, 5 min) exhibited weak structured gels (Harte et al., 2002). In this study, the results showed the synergistic effect of combined treatment. Furthermore, some differences could be related to the fermentation process. The gel firmness of the yogurt depended on the starter culture (DPL ABY 611 or YO MIX 236), which modified the gel properties. The viscous characteristics of the acid gel are increased when texturing starters are used because of the interaction of exopolysaccharides (EPS) with the casein network (Sodini et al, 2004). However, Hassan et al. (1996b), Hess et al. (1997), and Rohm and Kovac (1994) observed a decrease in firmness when using a texturing starter. Further, Beal et al. (1999) found that strain association, temperature, and final pH had significant effects on yogurt viscosity. The texturing character of *S. thermophilus*, for instance, increased with decreasing temperature and final pH. Dannenberg and Kessler (1988) also found that yield stress of skimmed milk yogurt was related to the extent of whey protein denaturation; the higher the level of denaturation, the higher the number of labile bounds in the gel structure.

Tables 4 and 5 show the results obtained using the TA-XT2 texture analyzer in measuring the textures of different yogurt samples prepared under the same protocol. Texture of stirred yogurt is the result of both acid aggregation of casein micelles and production of exopolysaccharides by ropy strains during incubation (Cerning, 1995).

The texture profile was different according to the treatment, culture type, and inoculation rate used. This observation was confirmed by a statistical analysis, comprising ANOVA

and a multiple comparison of means (data not shown). Yogurts prepared with milk treated by HHP combined with heat presented more hardness ($p<0.01$). Combined effects of HHP and heat resulted in a high level of protein denaturation. Dannenberg and Kessler (1988) reported that yogurt gel firmness was strongly related to the level of β-lactoglobulin denaturation for up to 60% denaturation. Between 60 and 90% β-lactoglobulin denaturation, the effect of heating intensity became less evident, and therefore significant differences were observed above 90%. Additionally, severe heating intensities involving more than 90% denaturation of β-lactoglobulin led to a slight reduction in the firmness of the yogurt gel.

It was also observed that all results of consistency index (K) showed high correlation with hardness ($r^2>0.83\%$ for yogurts fermented by 0.1% starter culture and $r^2>0.76\%$ for yogurts fermented by 0.2% starter culture). Yogurt made from combined treatments using 0.1% starter culture showed higher yield stress and consistency index values which are can be clearly correlated with its high hardness and gumminess.

The milk treatment and starter culture also had a significant effect on gumminess of yogurt ($p<0.01$), however the inoculation rate showed no major differences. Yogurt prepared with combined HHP and heat fermented by YO MIX 236 culture, showed the higher values for gumminess. On the other hand, the gumminess was correlated only with yield stress of yogurts fermented by 0.1% starter culture, $r^2>0.92\%$, independently of milk treatment.

After fermentation the pH value of yogurts varied from 4.48 to 4.70. Oliveira et al. (2002) reported similar values during the manufacture of lactic beverage containing probiotic starter cultures. However, the pH value of fermented milk products tended to decrease during storage due to post-acidification, a result of starter culture activity. In the case of yogurt, if pH reaches below 4.0, syneresis becomes evident due to curd contraction owing to the reduction of hydration of water (Brandão, 1995).

Furthermore, the pH value has an influence on the viability of probiotic cultures in fermented milk. The survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in Argentinean yogurt was studied during refrigerated storage by Vinderola et al. (2000). The authors found that a decrease of pH reduced the viable cell count of these microorganisms. Thamer and Penna (2004) reported similar results. The highest probiotic microorganism populations were observed in dairy beverages with lower acidity.

Although *Lactobacillus acidophilus* tolerates acidity, a rapid decrease in their number has been observed under acidic conditions (Shah and Jelen, 1990; Lankaputhra and Shah, 1995). *Bifidobacteria* are not as acid tolerant as *Lactobacillus acidophilus*. The growth of the latter microorganism ceases below 4.0, while the growth of *Bifidobacteria* ssp. is retarded below pH 5.0 (Shah, 1997). Thus, in order to obtain a higher population of *Bifidobacteria*, Almeida et al. (2001) standardized the pH value of probiotic fermented dairy beverages above 5.0.

The aggregation strength in yogurt is also related to the yogurt's total solids and pH value (Tables 4 and 5). The increase in the hardness of yogurt observed at low pH could be explained by the effect of pH on the electric charge of casein, as suggested by Harwalkar and Kalab (1986). These researchers reported an increase of 20% in gel firmness when the final pH was decreased from 4.50 to 3.85. They assumed it was caused by the higher intramicellar repulsion due to the increase of the positive charge of casein at lower pH, below the isoelectric point (pI) of caseins. This would tend to swell the casein particles, resulting in an increased rigidity of the milk gel. However, they observed larger pores in the protein network at low pH. It reduced intermolecular interactions, which resulted in the formation of an open structure more susceptible to forming grains and a lumpy texture when gel is stirred. Such a porous structure also makes the whey separation easier Harwalkar and Kalab (1986).

The effects of treatments on milk and yogurt are also reflected in changes to the color values. HHP treated milk had lower L*, a*, and b* values than either heat and combined heat and HHP treated milk (data not shown). Yogurt and heat treated milk had higher values of L*, a*, and b* due to changes in the light-scattering properties of milk. The disruption of micelles under high pressure caused a significant change in the appearance of the milk, which was quantified by measuring the color. Heat treatment also affected these characteristics. The decrease of L* (lightness) and increase of greenness (-a*) and yellowness (+b*) were also observed by Gervilla et al. (2001) when ewe's milk was treated by HHP. Harte et al. (2003) observed high L* values (increased whiteness) in milk subjected to HHP followed by thermal treatment and related to reaggregation of

disrupted micelles. The HHP treatment reduced the lightness of raw or thermally treated milks and a small decrease in color was observed when milk was subjected to HHP. Complementary studies, regarding the effect of milk treatment on acidification, physicochemical characteristics, probiotic cell counts and microstructure of probiotic low fat yogurt were conducted by Penna et al., 2006 a, b.

An interesting relationship between acidification and texture was observed for culture DPL ABY 611; the lower the amount of starter culture the higher the hardness and adhesiveness. Starter culture YO MIX 236 showed the opposite behavior. The duration of the fermentation had a positive effect on texture development irrespective of final pH. The slower the acidification, the longer the fermentation time and the higher the viscosity. This emphasizes that the textural properties of yogurt may be governed by the duration of fermentation. Results of Beal et al. (1999) and Garcia-Garibay and Marshall (1991) support this proposition. It could be explained by the firmer structure of the gel resulting from acid coagulation at low pH. So the different fermentation times related to the various experimental conditions may affect product viscosity.

3.5 CONCLUSIONS

The milk treatment before yogurt fermentation significantly affected the rheology and texture properties of probiotic yogurts. Starter culture and the inoculation rate that governs the fermentation also modified the gel properties. Combined HHP (676 MPa for 5 min) and heat (85ºC for 30 min) treatment of milk before fermentation and a 0.1%

inoculation rate (for both cultures) led to attractive rheology and texture properties. The combined HHP and heat treated yogurt presented a creamy and thick consistency requiring no addition of stabilizers.

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Table 1. Experimental design of probiotic low fat yogurt preparation

Run	Culture type	Inoculation	Treatment
1	DPL ABY 611	0.1%	Heat
2	DPL ABY 611	0.1%	HHP
3	DPL ABY 611	0.1%	HHP + heat
4	DPL ABY 611	0.2%	Heat
5	DPL ABY 611	0.2%	HHP
6	DPL ABY 611	0.2%	HPP + heat
7	YO MIX 236	0.1%	Heat
8	YO MIX 236	0.1%	HHP
9	YO MIX 236	0.1%	HPP + heat
10	YO MIX 236	0.2%	Heat
11	YO MIX 236	0.2%	HHP
12	YO MIX 236	0.2%	HPP + heat

Heat – 85ºC for 30 min.

HHP – High hydrostatic pressure – 676 MPa for 5 min.

Table 2. Flow parameters of yogurt prepared with culture DPL ABY 611 using 0.1% and 0.2% of starter culture, using the Herschel-Bulkley model. The shear rates ranged from 0.1 to 300 s^{-1} and the measurements were made at 10ºC.

	τ_0 (Pa)	K (Pa.s^n)	n	R^2	τ_0 (Pa)	K (Pa.s^n)	n	R^2
0.1%	Upward curves				Downward curves			
Heat	2.297	1.539	0.724	0.996	0.855	0.222	0.969	0.990
HHP	0.851	1.995	0.611	0.986	0.060	0.100	0.980	0.994
HHP + heat	3.428	4.569	0.507	0.980	1.581	0.271	0.949	0.986
0.2%	Upward curves				Downward curves			
Heat	2.073	2.072	0.624	0.9937	1.310	0.283	0.881	0.9859
HHP	0.646	0.371	0.886	0.9971	0.064	0.135	0.978	0.9912
HHP + heat	3.083	2.132	0.651	0.9861	0.862	0.206	0.953	0.9932

τ_0 – Yield stress (Pa); K – Consistency index (Pa.s^n); n – Flow behavior index (dimensionless); R^2 – Determination coefficient.

Table 3. Flow parameters of yogurt prepared with culture YO MIX 236 using 0.1% and 0.2% of starter culture, using the Herschel-Bulkley model. The shear rates ranged from 0.1 to 300 s^{-1} and the measurements were made at 10ºC.

	τ_0 (Pa)	K (Pa.s^n)	n	R^2	τ_0 (Pa)	K (Pa.s^n)	n	R^2
0.1%		Upward curves				Downward curves		
Heat	1.158	1.269	0.840	0.9946	0.115	0.019	1.412	0.9937
HHP	1.646	4.276	0.537	0.9814	0.617	0.145	0.944	0.9880
HHP + heat	3.355	4.320	0.564	0.9929	1.380	0.713	0.689	0.9728
0.2%		Upward curves				Downward curves		
Heat	3.227	3.106	0.651	0.9931	1.627	1.022	0.664	0.9719
HHP	1.743	0.368	0.984	0.9938	0.849	0.144	0.967	0.9899
HHP + heat	2.054	1.928	0.653	0.9934	1.152	0.349	0.788	0.9792

τ_0 – Yield stress (Pa); K – Consistency index (Pa.s^n); n – Flow behavior index (dimensionless); R^2 – Determination coefficient.

Table 4. Probiotic Yogurt DPL ABY 611 texture profile evaluated using the TA-XT2 Texture Analyzer, Total Solids, and pH value.

	0.1%			0.2%		
	Heat	HHP	HHP + heat	Heat	HHP	HHP + heat
Hardness	28.52	24.15	46.14	28.14	23.68	32.15
Fracturability(g)	5.42	5.16	5.49	4.96	5.28	5.24
Adhesiveness (g.s)	-41.24	-13.76	-129.37	-25.32	-7.93	-51.10
Springiness	0.96	0.98	0.90	0.98	3.08	0.94
Cohesiveness	0.76	0.81	0.66	0.76	0.93	0.72
Gumminess	21.59	19.49	30.67	21.31	21.94	23.12
Resilience	0.27	0.34	0.15	0.31	0.34	0.25
Total Solids	14.12	14.11	14.44	13.11	14.90	14.26
pH	4.68	4.59	4.48	4.70	4.65	4.60

Table 5. Probiotic Yogurt YO MIX 236 texture profile evaluated using the TA-XT2 Texture Analyzer, Total Solids, and pH Value.

	0.1%			0.2%		
	Heat	HHP	HHP + heat	Heat	HHP	HHP + heat
Hardness	28.11	35.26	44.28	40.53	22.66	46.84
Fracturability(g)	5.42	6.16	5.86	5.33	5.82	6.69
Adhesiveness (g.s)	-17.54	-90.72	-113.06	-74.43	-24.92	-112.63
Springiness	1.00	0.92	0.92	0.94	4.18	0.94
Cohesiveness	0.80	0.70	0.67	0.71	1.08	0.68
Gumminess	22.56	24.81	29.56	28.96	24.57	31.80
Resilience	0.34	0.20	0.15	0.20	0.51	0.17
Total Solids	15.10	13.88	14.26	14.16	14.34	14.95
pH	4.54	4.56	4.50	4.60	4.56	4.57

CHAPTER FOUR

Effect of High Hydrostatic Pressure Processing on Microstructure of Probiotic Low Fat Yogurt

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4.1 ABSTRACT

Skim milk fortified with skim milk powder was subjected to three treatments: thermal treatment at 85ºC for 30 min, high hydrostatic pressure at 676 MPa for 5 min, and combined treatments of heat and high hydrostatic pressure. The processed milk was fermented by using two different starter cultures containing *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus,* and *Bifidobacterium longum*. The microstructure of heat-treated milk yogurt had fewer interconnected chains of irregular shape casein micelles, forming a network that enclosed the void spaces. On the other hand, microstructure of HHP yogurt had more interconnected clusters of densely aggregated protein of reduced particle size, with an appearance more spherical in shape, exhibiting a smoother more regular surface and presenting more uniform size distribution. The combined heat and HHP milk treatments led to compact yogurt gels with increasingly larger casein micelles clusters interspaced by void spaces, and exhibited a high degree of cross-linking. The rounded micelles tended to fuse and form small irregular aggregates in association with clumps of dense amorphous material, which resulted in improved gel texture and viscosity.

Key words: high hydrostatic pressure, yogurt, probiotics, and microstructure

Abbreviation key: SEM = scanning electron microscopy, **TEM** = transmission electron microscopy, **HHP** = high hydrostatic pressure

4.2 INTRODUCTION

High hydrostatic pressure (HHP) processing technology has recently received considerable attention among food researchers. Derived from material sciences, which includes ceramics, super alloys, artificial diamond, etc., high pressure technology (100 to 1000 MPa) is of increasing interest for use in biological and food systems, primarily because it permits microbial inactivation at low or moderate temperature. The small-scale production of pressurized foods has become a reality in Japan (fruit-based products and other foods), France (orange juice) and the USA (avocado spread), but large volume (500 liters) pressure vessels for large-scale production are also available from manufacturers. For example, high pressure treated milk has been successfully used to manufacture low fat set-type yogurt (12% total solids) with creamy thick consistency, requiring no addition of polysaccharides (Moorman et al*.,* 1996).

Previous studies have shown the various effects of high pressure on the constituents and properties of milk (Thom et al., 2002). In one study, it was found that the primary structure remains intact during high pressure processing (Mozhaev et al., 1994). However, Hendrickx et al. (1998) reported that at high pressures, hydrogen bonds can rupture leading to irreversible denaturation and changes in the tertiary structure of proteins. The combined effect of HHP and thermal treatments has also been studied. Fortified low fat milk, for example, exhibited improved elastic modulus and yield stress as well as reduced syneresis in yogurts (Harte et al., 2003).

In milk, HP causes the casein micelles to disintegrate into smaller (diameter) casein particles, with a decrease in milk turbidity and lightness and an increase in milk viscosity (Johnston et al., 1994). Furthermore, the pressure-induced dissociation of the colloidal calcium phosphate and denaturation of serum proteins in milk may change, improving its technological properties (López-Fandiño et al., 1996). In addition to microbial destruction, the effects of HP on protein structure and mineral equilibrium suggest different applications for dairy products.

It has been reported that HHP improves acid coagulation of milk without detrimental effects on important quality characteristics, such as taste, flavor, vitamins, and nutrients (Trujillo et al., 2002). Harte et al. (2003) reported that yogurt made from milk subjected to HHP (400-500 MPa) and thermal treatment (85ºC for 30 min) showed increased yield stress, resistance to normal penetration, and elastic modulus, while having reduced syneresis, compared to yogurts made from thermally treated milk and raw milk. Thus, the use of HHP offers microbiologically safe and additive-free low fat yogurt with improved performances, such as reduced syneresis, high nutritional and sensory quality, novel texture, and increased shelf life (Trujillo et al., 2002; Harte et al., 2003).

Low calorie skimmed or half-skimmed yogurts have won popularity during the last decade. Probiotic yogurt occupies a very satisfactory position in the dairy products market, and there is a clear trend to increase its consumption in the next few years. Additional health aspects, for instance an additive-free product, will make this increase in consumption much more favorable. However, it is more challenging to produce low fat

and nonfat yogurt products that do not whey-off during storage without using stabilizers (Lucey and Singh, 2002).

Yogurt is formed during the slow lactic fermentation of milk lactose by the thermophilic lactic acid bacteria, *Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus,* and can have probiotics added, mainly *Lactobacillus acidophilus* and *Bifidobacterium*. These bacteria are good to have in the formulation because of the many advantages to the consumer. The first two, *Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus,* are needed to convert milk to yogurt, while *Lactobacillus acidophilus* and *Bifidobacterium* are added because of their functional and health-promoting properties. To be truly effective, the probiotics must be alive in yogurt when consumed. Effective yogurt contains at least 100 to 1000 million live bacteria per mL.

Yogurt has been known for its nutraceutical, therapeutic, and probiotic effects such as digestion enhancement, immune system boosting, anticarcinogenic activity, and reduction of serum cholesterol. Stirred yogurt is prepared by breaking the set gel and then filling the product into retail containers. In this type of yogurt, a combination of high solids content and the addition of both fruit and stabilizers give the manufacturer several options for controlling the texture and physical properties of yogurt (Lucey, 2002). The potential advantages of using probiotic bacteria include improvement in lactose digestion, reduction of bacterial carcinogenic enzymes and the incidence of diarrhea, stimulation of the immune system, and prevention of infections in the digestive tract. Probiotics act beneficially in many ways, for example they produce enzymes that help the body digest

food, they produce B-complex vitamins, and in cases of diarrhea, they help in the neutralization of pathogenic microorganisms responsible for infections. Probiotic yogurt occupies a very satisfactory position in the dairy products market, and there is a clear trend to increase its consumption in the next few years.

The structural properties and the stability of yogurt are quite complicated and a number of factors greatly influence the results, factors both related to chemical composition and processing conditions (Olsen, 2002). Casein micelle is a poly-condensation or polymerization model that envisages two cross-linking routes for assembly of the micelle. They are cross-linked by individual caseins through hydrophobic regions of the caseins and bridged involving colloidal calcium phosphate. The formation and integrity of the micelle is viewed as being controlled by a balance between attractive and repulsive forces in casein micelles, i.e., localized excess of hydrophobic attraction over electrostatic repulsion (Horne, 1998). Whey separation and several rheological changes have been implicated to excessive rearrangements of particles making up the gel network before and during gel formation (Lucey, 2001).

The microstructure of the protein matrix varies, depending upon protein content, heat treatment of the mix (Harwalkar and Kalab, 1986), and the presence or absence of milk fat, thickening agents (stabilizers), and bacterial exopolysaccharide (Kalab et al., 1983; Schellhaass and Morris, 1985; Teggatz and Morris, 1990). However, heat treatment of milk does not prevent whey separation and may even increase it, at least in model glucono-δ-lactone (GDL) induced gels (Lucey et al., 1998 a). Heat treatment increases

the rigidity of yogurt gels, which is an important texture attribute, but it is not very effective in preventing whey separation in milk incubated at extremely high temperatures.

The combined effect of heat and high hydrostatic pressure (HHP) on the microstructure of probiotic yogurt gels, and a comparison to heat and HHP alone, do not appear to have been reported. Therefore, the objective of this study was to investigate the combined effect of milk treatment on the microstructure of probiotic yogurt gels, and to understand the yogurt microstructure more fully to establish some relationship between treatment and the causes of physical defects.

4.3 MATERIALS AND METHODS

4.3.1 Heat treatment

Skim milk (0.0 – 0.2% fat and 9.17 – 9.20% total solids) was purchased from the Washington State University (WSU) Dairy Creamery and fortified with skim milk powder (0.0 to 1% fat and 97% total solids) to increase the total solids content to 14%. The fortified milk was then subjected to thermal treatments at 85ºC for 30 min. Milk was cooled in a water bath to 42ºC for the yogurt preparation.

4.3.2 Pressure treatment

Samples of fortified milk were placed in plastic bags and sealed. Pressure treatments were carried out using an isostatic pressure system (Engineered Pressure Systems, Inc., Haverhill, MA, USA) having a chamber size of 0.10 m diameter and 0.25 m height. The medium for hydrostatic pressurization was 3% Hydrolubric 123B water solution.

Samples were subjected to high hydrostatic pressure (HHP) at 676 MPa for 5 min at room temperature. Pressure was achieved in 4 to 5 min and the depressurization took less than 1 min.

4.3.3 Yogurt preparation

The processed milk (thermal, HHP and combined) was inoculated (0.2% v/v) with two different freeze-dried probiotic yogurt starter cultures (YO MIX 236 and DPL ABY 611) supplied by Rhodia Inc. (Madison, WI, USA) and Danisco USA Inc. (Milwaukee, WI, USA), respectively. These starter cultures are a mixture of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus*, and *Bifidobacterium longum*. The fermentation was carried out at 43^{o}C, which is the optimum temperature for the starter culture bacteria. Each fermentation process was monitored by continuous recording of pH values to measure the acidification rates during fermentation until the pH value reached 4.6 ± 0.1. The yogurt was cooled to 20ºC in an ice bath and then stirred with a mechanical mixer for 30 seconds according to a standardized protocol, and stored at 4ºC for 15-16 hours. The experimental design of yogurt preparation is summarized in Table 1.

4.3.4 Microstructure analysis

Transmission electron microscopy: Microstructure of probiotic yogurt was determined by transmission electron microscope (Joel EX). Yogurt samples (5 ml) were kept overnight in 2% glutaraldehyde, 2% paraformaldehyde, and 0.05M PIPES buffer at 4°C for fixation, then rinsed three times with 0.05M PIPES buffer for 10 minutes each, and rinsed two times with phosphate buffer for 10 minutes each. Following, the samples were post-

fixed in 2% osmium tetroxide (Sigma Chemical Co., St. Louis, MO, USA) for one and a half hours, rinsed twice with 0.05M phosphate buffer for 10 minutes each, and dehydrated with increasing concentrations of acetone each (30, 50, 70, 95%, 3 times with 100%). Finally, the samples were infiltrated with a solution containing 1:1 acetone and Spurr's epoxy resin (Sigma Chemical Co.) and held overnight at room temperature. Samples were changed to 100% Spurr's resin, and hardened in oven for 24 hours at 70°C and cut into thin sections (60 to 90 nm). The grids with samples were stained with 4% uranyl acetate and Sato's lead stain and examined with a transmission electron microscope, Joel 1200 EX JEM (Joel Ltd., Akishima, Japan) operating at 80 kV.

Scanning electron microscopy: Yogurt samples were kept overnight in 2.0% glutaraldehyde and 2% paraformaldehyde, and 0.05 M PIPES buffer at 4ºC for fixation. These samples were rinsed three times with 0.05M PIPES buffer for 10 minutes each, and rinsed two times with phosphate buffer for 10 minutes each. Following, samples were post-fixed in 2% osmium tetroxide (Sigma Chemical Co., St. Louis, MO, USA) for one and a half hour, rinsed twice with 0.05M phosphate buffer for 10 minutes each and dehydrated with increasing concentrations of ethanol each (30, 50, 70, 95%, 3 times at 100%), and then dried using Critical Point Drying method with a Samdri PVT 3D (Tousimis Research Corporation, Rockville, MD, EUA), with liquid carbon dioxide. Dry sections were fractured with a blade and fragments mounted on aluminum stubs, and gold-coated in vacuum using a Hummer V Sputtering device (Technics, Munich, Germany) in an argon atmosphere at 60-70 militorr. Microstructures of yogurts were

examined with a scanning electron microscope, a Hitachi S-570 (Hitachi, Tokyo, Japan) operating at 20 KV.

4.4 RESULTS AND DISCUSSION

The scanning electron micrographs and transmission electron micrographs of the yogurt gels made with heat, HHP, and combined HPP and heat treatments with 0.2% culture inoculation rates are presented in Figures 1 and 2. The use of different starter cultures led to no differences in organization of the gel network.

Yogurt consists of a coarse network composed of casein particles linked in clusters or chains to form a three-dimensional network. The heat-treated milk yogurt microstructure (Figures 1A and B) is composed of chains of casein micelles, forming a network enclosing the void spaces, some of which contain only the aqueous phase of the yogurt, while others enclose the bacterial cells. These results were consistent with those reported by Kalab et al. (1983).

Figures 2A and B show that the micelles are less interconnected and exhibit irregular shapes with large pores. The protein network appears in dark gray and void spaces in white. The water phase is retained in the network and syneresis is due to whey separation from mainly the larger pores (Olsen, 2002).

Several authors have also shown a marked effect of milk-base heating on the structure of yogurt and milk gels (Harwalkar and Kalab, 1986; Lucey et al. 1999). Gels made from heated milk exhibited a finer and more continuous branched kind of network, characterized by small void spaces (Parnell-Clunies et al., 1987). The finer microstructure

of yogurt from heated milk can be attributed to the decrease of micelle size after heating. But it is likely ascribed to the bridging capacity of denatured whey proteins. The appendages between β-lactoglobulin and κ-casein are involved in bridging protein particles and reduces the formation of dense clusters, as observed in the gels of unheated milks (Lucey et al, 1998b). High heat treatment of milk causes unfolding and aggregation of whey protein, some of which interact with casein micelles involving κ-casein (Singh, 1995; Smits and van Brouwershaven, 1980). These whey proteins appear as appendages or filaments on the micellar surface in electronic micrographs (Kalab et al, 1983). Denatured whey protein could act as bridging material by interacting with the whey proteins and would increase the number and strength of bonds between protein particles. While denatured whey proteins are known to affect the formation of acid milk gels (Lucey et al., 1997), the mechanism by which they affect the rheological properties are not adequately explained. The denatured whey protein load on the casein micelle and degree of whey protein aggregation, both at the casein micelle surface and in the serum phase, are two major areas requiring elucidation (Walsh-O'Grady et al. 2001).

HHP yogurt microstructure exhibited protein clusters with some pores and more interconnected clusters of densely aggregated protein particles. Also, the starter culture cells are observable (Figures 1C and D). Pressure treatment considerably reduced particle size, with an appearance different from the micelles in the heat-treated milks. They are more spherical in shape, exhibit a smoother more regular surface, and present more uniform size distribution (Figures 2C and D) as well as some spikes on casein micelle surfaces, as reported by Garcia-Risco et al. (2000). Needs et al. (2000) suggested that

yogurt made with pressured treated milk presented differences in particle size, surface area, and degree of association because many close micelle-micelle bonds or interactions were established. The use of high hydrostatic pressure to introduce denaturation, aggregation, and gel formation of milk proteins has been studied by many researchers (Famelart et al., 1997; Ancos et al., 2000; Needs et al., 2000; Walsh-O'Grady et al., 2001; Harte et al., 2003). The behavior of protein under pressure is governed by the principle of Le Chatelier (Balny and Masson, 1993), which implies that any reaction accompanied by a decrease in volume is enhanced by an increase in pressure and vice-versa. Hence, hydrophobic interactions and ionic effects are liable to disruption by high pressure, while the formation of hydrogen bonds is favored by high pressure (Cheftel, 1992). Since these bonds contribute to protein conformation and structural interactions in solution, any changes associated with them will result in modifications to the overall structure of the protein matrix. Covalent bonds, on the other hand, appear not to undergo any changes during high pressure treatment. High pressure treatment of milk induced a partial and irreversible dissociation of casein micelles, even after pressure release. The simultaneous dissociation of casein micelles and whey protein unfolding and the possibility of disulphide bond formation between the denatured whey proteins and the caseins could lead to the formation of a range of interaction products, which on pressure release may reverse to a more aggregate state. Structure development during acidification of casein/whey protein mixture would be different from the structure developed during acidification of casein/whey protein complexes formed through the introduction of pressure-induced whey protein into an intact micellar casein suspension at room temperature (Walsh-O'Grady et al., 2001). Although HHP treatment of milk may affect

the Maillard reaction or the mutarotation equilibrium of lactose, the effect of HHP on lactose has not been studied thus far (Huppertz et al., 2002).

The combined HHP and heat milk treatments led to compact yogurt gels with increasingly larger casein micelles clusters interspaced by void spaces, and exhibited a high degree of cross-linking. The streptococci and lactobacilli are easily distinguished (Figures 1E and F). Stirring of the yogurt during preparation resulted in formation of large areas of separated whey and a denser protein network, as described by Hassan et al. (2003). In pressure treated milk, the rounded micelles often formed into small irregular aggregates in association with clumps of dense amorphous material (Figures 2E and F), in agreement with data reported by Needs et al. (2000). The differences in structure of yogurt could be related to different degrees of denaturation of whey protein caused by accumulated treatments. Polymerization of β-lactoglobulin due to exposure of –SH groups and SH/SS interchange under HHP has been reported by Funterberger et al. (1997). The cross-linking capacity of denatured whey played a key role in yogurt structure, contributing to an increase in the degree of bridging between protein particles. García-Risco et al. (2000) reported that pressurization and heat led to a progressively lower proteolytic degradation, which is also very interesting for yogurt shelf life. The casein micelles of heat treatment milk showed superficial filamentous appendages that appear to inhibit the fusion of the particles of casein. The micelles tend to fuse and form a dense network, which resulted in improved gel texture and viscosity (Krasaekoopt et al., 2003). Casein micelles of yogurt gels prepared from HHP milk were round and homogeneous in size with mean diameters of 200 nm (Harte et al., 2002). The median

particle size in heated yogurt gels prepared with casein alone was found to be between 750 and 850 nm, while yogurt gels prepared with casein and β-lactoglobulin showed particles ranging between 350 and 500 nm (Famelart et al., 2004). Tedford and Schaschke (2000) reported the structural changes to β-lactoglobulin were induced by the combined effects of pressure (55 and 100MPa) and temperature (35 and 75ºC) and the molecular structure of β-lactoglobulin can be affected following treatment at pressures as low as 55-100 MPa in combination with temperature. For irreversibly disruption of the molecular structure at both the secondary and tertiary level sufficient energy can be applied. The exact mechanism and extent by which combined pressure and temperature results in folding are not known. A model of acid gelation of heated milk and HHP milk was proposed by Famelart et al. (2004) and Harte et al. (2002), respectively. Soluble heat-induced aggregates occur as thread-like particles in heated milk, and colloidal heat-induced aggregates are present. They both interact at pH ~ 5.5, leading to the first increase in elastic modulus (G'). Then casein-casein interactions take place at pH ~5.0, leading to the second increase in G'. With the decrease of pH, the casein-casein interactions take place. HHP treatment causes extensive micelle disruption into smaller casein aggregates or sub-micelles. The aggregation of small sub micelles would result in compact aggregates of smaller size, as the isoeletric point is reached during the fermentation process. It is hypothesized that the formation of S-S bonds between partially denatured β-lactoglobulin and κ-casein in the surface of sub-micelles would also promote the formation of smaller micelles, acting as a physical barrier to aggregation. The combined HHP and heat milk treatment followed by fermentation exhibited a dense and

homogeneous micelle distribution with high water holding capacity. A schematic diagram of the effects of heat treatment and HHP of milk is shown in Figure 3.

4.5 CONCLUSIONS

The results of this study showed that the use of HHP to treat milk before fermentation affected the microstructure of probiotic yogurts. The microstructure of heat-treated milk yogurt was composed of fewer interconnected chains of irregular shape casein micelles, forming a network that enclosed the void spaces, while the microstructure of HHP treated yogurt exhibited more interconnected clusters of densely aggregated protein with reduced particle size, appearing more spherical in shape and exhibiting a smoother more regular surface and more uniform size distribution. The combined heat and HHP milk treatments led to compact yogurt gels with increasingly larger casein micelles clusters interspaced by void spaces, and exhibited a high degree of cross-linking. The rounded micelles tended to fuse and form small irregular aggregates in association with clumps of dense amorphous material, which resulted in improved gel texture and viscosity. Therefore, the combined HPP and heat treatment before fermentation would be a better process for a uniform consistent microstructure with better texture and physical attributes.

4.6 ACKNOWLEDGEMENTS

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Table 1. Experimental Design of Low Fat Yogurt Preparation

Run	Culture type	Inoculation	Treatment
1	DPL ABY 611	0.2%	Heat
2	DPL ABY 611	0.2%	HHP
3	DPL ABY 611	0.2%	HPP + Heat
4	YO MIX 236	0.2%	Heat
5	YO MIX 236	0.2%	HHP
6	YO MIX 236	0.2%	HPP + Heat

Heat – 85ºC for 30 min.

HHP – High hydrostatic pressure – 676 MPa for 5 min.

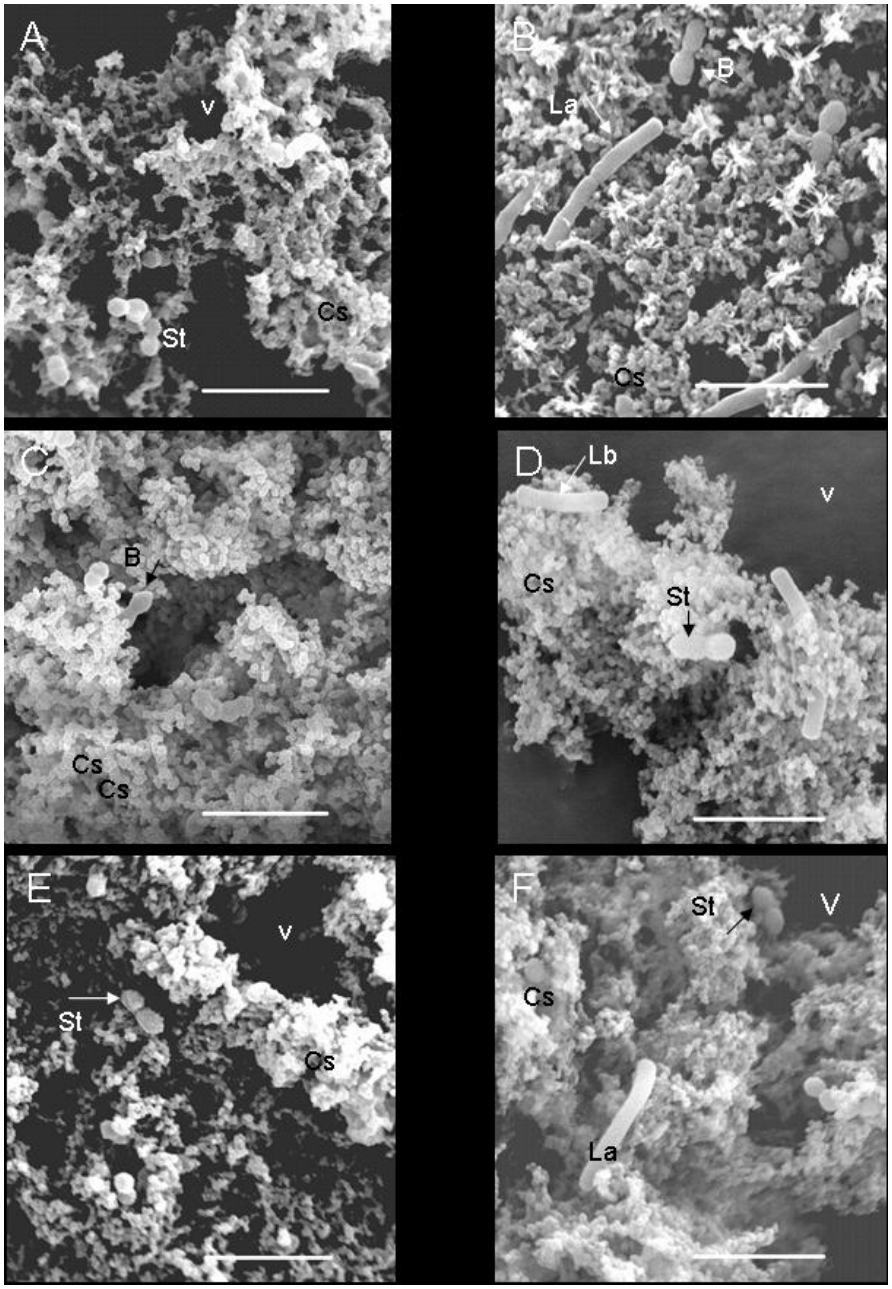

Figure **1 -** Scanning Electron Microscopy (SEM) micrographs of yogurt fermented with starters YO MIX 236 (A, C, E) and DPL ABY 611 (B, D, F) with different treatments: A and B - Heat, C and D - HPP, E and F - HPP + Heat. Magnification 6 K. Scale bar 5µm. St – *Streptococcus thermophilus*, Lb – *Lactobacillus delbrueckii* ssp *bulgaricus*, La – *Lactobacillus acidophilus*, B – *Bifidobacterium longum*, v – void space, cs – casein.

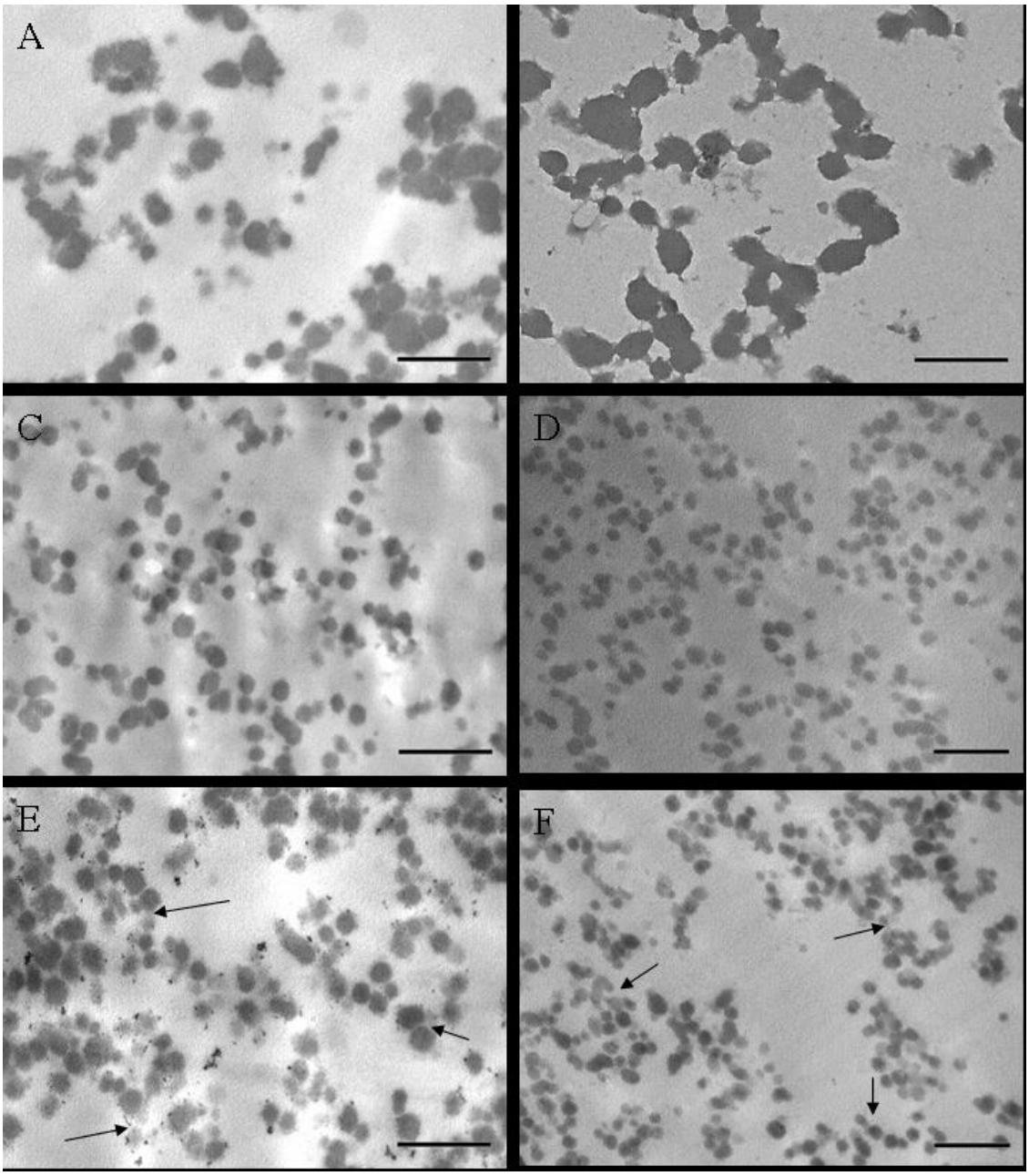

Figure 2 - Transmission Electron Microscopy (TEM) micrographs of yogurt fermented with starters YO MIX 236 (A, C, E) and DPL ABY 611 (B, D, F) with different treatments: A and B - Heat, C and D - HPP, E and F - HPP + Heat. (arrow) filamentous projections form long-range bridges between micelles . Magnification 25K. Scale bar 1000 ηm.

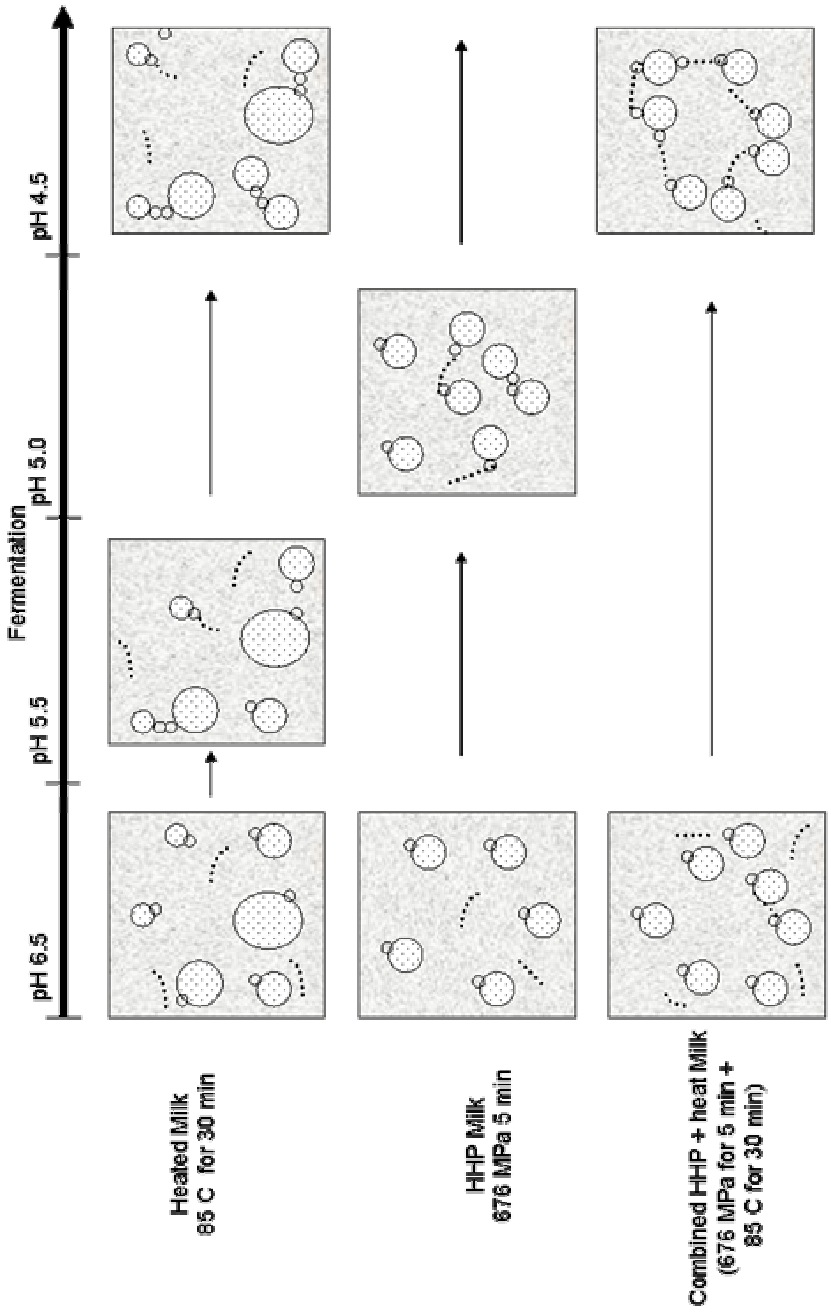

Figure 3 - Schematic diagram of the effect of Heat, HPP, and combined HHP + Heat of casein micelle microstructure.

; ; - Casein micelle, whey protein, and κ-casein aggregates

CHAPTER FIVE

Ultrasonification for release of β-Galactosidase Enzyme from Yogurt Bacteria to improve the Viability of Probiotics

Subba Rao Gurram, Ralph P. Cavalieri, Stephanie Clark, Barry G. Swanson, and Gustavo V. Barbosa-Cánovas

5.1 ABSTRACT

Probiotic bacteria are a mixed culture of microorganisms, which when consumed by humans in yogurt, are beneficial. To be effective, yogurt must contain at least 10 million live probiotic bacteria per milliliter. Yogurt with live and active cultures occupies a satisfactory position in the dairy market, and there is a trend to increase yogurt sales in the next few years.

Ultrasonification was used to rupture yogurt bacteria for enhanced viability of probiotic bacteria. Two selected cultures, ABY611 and YoMix 236, containing *Streptococcus thermophilus* and *Lactobacillus delbruekii ssp. bulgaricus* and probiotics, *Lactobacillus acidophilus* and *Bifidobacterium longum* were used in this study. The yogurt cultures were sonicated using an ultrasonic processor at 24 kHz for 3, 4, and 5 min. A thermocouple was used to monitor the temperature throughout the experiments. The ultrasonic treatment was kept constant at 100 % amplitude for all treatments. Sonicated and unsonicated yogurt starter cultures (control) were selected for making yogurt.

Physicochemical and rheological characteristics, enzymatic activity, microstructure, and probiotics viability of yogurt samples were studied. *β-galactosidase* (β-Gal) activity

increased due to ultrasonification. The β-Gal activity significantly increased 4.73 times in sonicated yogurt samples compared to 3.28 times in unsonicated yogurt samples. The viability of probiotics increased by two log cycles in sonicated yogurt samples compared to one-half log cycle in unsonicated yogurt samples. This research suggests that the probiotics grow healthier in sonicated yogurt samples than in unsonicated yogurt samples, suggesting the availability of more nutrients for the probiotics due to more β-Gal availability.

The ultrasonification technique, where the yogurt bacteria are ruptured to release more β-*Gal,* will enable manufacturers to utilize lower inoculation levels to reach beneficial levels of probiotics in yogurt. Also, sonicated starter cultures potentially extend the shelf life of yogurt by extending the life span for probiotics.

Key words: Yogurt, Ultrasonification, β-Galactosidase Enzyme, Probiotics.

5.2 INTRODUCTION

Many consume yogurt because of live and active cultures (probiotics) effects. The health benefits of probiotic bacteria first came to the attention of the general public in 1908, when Dr. Elie Metchnikoff, a Russian biologist, wrote the book "*The Prolongation of Life*". Metchnikoff (1908) suggested that consumption of fermented milk with *Lactobacillus acidophilus* bacteria was beneficial for gastrointestinal health, as well as for the promotion of longevity. India's Ayurvedic writings, dating back to 6,000 BC, indicate that regular consumption of cultured dairy products led to a long and healthy life (Natren, 2005).

The use of cultured dairy products is common in many areas of the world where lactose malabsorption is common (Gallangher et al., 1974 and Kretchmer, 1972). Yogurt, culture containing fluid drinks, and some brands of cheese are the products claimed to have probiotics around the world. Yogurt is formed by the slow lactic fermentation of milk lactose by the thermophilic lactic acid bacteria, *Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus* and can have added probiotics: *Lactobacillus acidophilus* and *Bifidobacterium* species. The first two are needed to convert milk to yogurt and the later two are often added because of their health promoting properties. Effective yogurt contains at least 10 million live probiotic bacteria per mL (National Yogurt Association). Probiotic bacteria are a mixed culture of microorganisms, which when applied to humans, affect the host beneficially. It is widely accepted by the research community that the probiotic microbes have a powerful beneficial influence on the host

by improving the balance of microflora in the gut (Tannock, 1999). The main sources of probiotic bacteria are conventional dairy products, dietary supplements and medicinal foods.

Yogurt has been attributed nutraceutical, therapeutic and probiotic effects, such as digestion enhancement, immune system boosting, anticarcinogenic activity and reduction of serum cholesterol (Analie Lourens-Hattingh and Bennie C. Viljoen, 2001). The potential advantages of using probiotic bacteria include improvement in lactose digestion, reduction of bacterial carcinogenic enzymes and the incidence of diarrhea, stimulation of the immune system and prevention of infections in the digestive tract (Modler, 1990; Hughes and Hoover, 1991). Probiotics act beneficially because they produce enzymes that help the body digest food, they produce B-complex vitamins, and in cases of diarrhea, they help in the neutralization of pathogenic microorganisms responsible for infections (Mittal and Garg, 1992; Ishibashi and Shimamura, 1993). Probiotic yogurt occupies a very satisfactory position in the dairy products market, and there is a clear trend to increase its consumption in the next few years (Agri-Food Canada, 2002).

The survival of probiotic bacteria in yogurt is affected by several factors, including low pH (Hood and Zottola, 1988), hydrogen peroxide produced by yogurt bacteria (Gilliard and Speck, 1977), and oxygen content in the product and oxygen permeation through the package (Schioppa et. al., 1981; Hull et. al., 1984; Ishibashi and Shimamura, 1993; Lankaputhra and Shah, 1994). The pH of yogurt may decline as low as 3.28 during storage after 31 days (Lourens and Viljoen, 2002). A rapid decrease in *L. acidophilus*

number was observed under acidic conditions (Lankaputhra and Shah, 1994). Bifidobacteria ceases to grow below pH 4.0 (Shah, 1997). Yogurt bacteria are also assumed to be responsible for the death of probiotic bacteria (Shah and Jelen, 1990). β-Galactosidase hydrolyzes a portion of lactose in milk produced by yogurt bacteria, reducing post-acidification. Probiotic bacteria, Lactobacillus acidophilus and Bifidobacterium, utilize glucose and galactose, products of lactose hydrolysis for their growth. So improving the β-Gal by rupturing the yogurt bacteria will improve the viability of probiotic bacteria.

The main objective of our research is to rupture yogurt bacterial cells by ultrasonification to release their intracellular β-Gal to potentially improve the viability of probiotic bacteria in yogurt.

5.3 MATERIALS AND METHODS

Two selected yogurt cultures were sonicated and analyzed for the amount of β-galactosidase activity. Sonicated and unsonicated cultures and probiotic cultures were used to make yogurt and enumerations of the cultures were done to evaluate the viability of yogurt and probiotic bacteria. Physicochemical characteristics and microstructure of sonicated and unsonicated yogurts were analyzed.

5.3.1 Yogurt and Probiotic Cultures

Two selected yogurt cultures, YoMix236 and ABY611, were supplied by Rhodia, Inc. (Madison, WI, USA) and Danisco USA, Inc. (Milwaukee, WI, USA) respectively. These

starter cultures consisted of individual *Streptococcus thermophilus* and *Lactobacillus delbruekii ssp bulgaricus*. Probiotic cultures containing individual *Lactobacillus acidophilus* and *Bifidobacterium longum* and also a mixture of *Lactobacillus acidophilus* and *Bifidobacterium longum* were obtained from Danisco USA Inc. (Milwaukee, WI, USA). Frozen culture (100 g of each culture) was mixed in 1000 mL of pasteurized milk and working stocks of 100 mL were prepared and stored at -21 ºC for experiments.

5.3.2 Ultrasonification Treatment

A Hielscher USA Inc. (Ringwood, NJ) ultrasonic processor model UP400S (400 W, 24 kHz) with a 22 mm diameter probe was used. A 500 ml double-walled vessel (8 cm internal diameter and 13.5 cm depth) was used as a treatment chamber. The temperature was established and kept constant via a refrigerated bath (VWR Scientific Model 1166, Niles IL). A k-type thermocouple was used in the treatment chamber to monitor the temperature (t± 0.5 ºC) throughout the experiments. The ultrasound wave was kept constant at 100 % amplitude (120 mm) in all treatments. A magnetic stirrer was used inside the vessel to assure the homogeneity of the samples throughout the sampling. The treatment times were 3, 4, and 5 min for both yogurt cultures, and samples were taken at 0, 3, 4 and 5 min time intervals to prepare yogurt and estimate the enzymatic activity.

5.3.3 Yogurt preparation

Yogurt was prepared using skim milk fortified with skim milk powder to standardize the desirable total solids (14 %). The milk was held in plastic bags at 4 ºC for 2 h and then

subjected to thermal treatment (85 ºC for 30 min). After heat treatment, the milk was cooled in an ice bath and maintained at 43 °C for yogurt preparation. The processed milk was inoculated (0.1 %) with sonicated or unsonicated yogurt cultures, and probiotic cultures (0.1 %). Fermentation was carried out at 43 ºC and stopped when the pH value reached 4.6. The yogurt was cooled rapidly to 20 ºC and immediately stored at 4 ºC for 36 h, and then analytical evaluations were carried out.

5.3.4 Physicochemical characteristics

Fermentation time is considered as the time required for the pH to decrease to an end point (4.4 to 4.6), presupposing that the required quality properties have been developed (Soukoulis et al., 2007). The pH value was measured using a digital 420 A pH meter (Orion Research Inc., Boston, MA, USA). Water holding capacity was evaluated by subjecting the yogurt to centrifugation at 15,000xg for 15 min at 20 ºC (Harte et al., 2003). Ten grams of yogurt sample was evaluated using a Beckman J2-HS centrifuge (Beckman Instruments Inc., Seattle, WA, USA). Water holding capacity was expressed as the percentage of pellet weight relative to the original weight of the sample:

$$WHC(\%) = \left[1 - \frac{Weight\ of\ whey\ after\ centrifugation}{Weight\ of\ yogurt}\right] \times 100$$

Susceptibility of yogurt to syneresis was determined using a drainage method. Yogurt samples were transferred into a funnel fitted with a qualitative paper Whatmann No. 5. The volume of the whey collected over 4 h at 4 ºC was measured in a 25 mL graduated cylinder (Hassan et al., 1996). All tests were carried out in triplicate.

5.3.5 Enzymatic Activity

β-*Galactosidase* enzymatic activity was evaluated for both sonicated and unsonicated yogurt cultures. Ten grams of frozen culture was mixed with distilled water to 100 ml in a volumetric flask. To 1 ml of above solution, 5 ml of 0.005M Ortho-nitrophenyl-β-D-galactopyranoside (ONPG) in 0.1 N phosphate buffer, pH 7.0 was added. One ml aliquots of the diluted samples were incubated with 5 ml of ONPG solution at 37 ºC for 15 min. The reaction was stopped by adding 2.5 ml of 1M cold sodium carbonate solution. Absorbance was measured at 420 nm using 8452A diode array spectrophotometer (Hewlett-Packard Palo Alto, CA). *β-gal* activity was estimated as the amount of enzyme liberating one micromole of O-nitrophenol from ONPG per minute per gram of sample at 37 ºC (Mahoney et al., 1975 and British Pharmacopoeia, 2002). Samples were taken before and after sonification for *β-gal* activity.

5.3.6 Scanning electron microscopy

Disposable 15 ml plastic conical test tubes containing sonicated and unsonicated yogurt cultures were centrifuged at 1500 rpm for 5 min at 4 ºC. The samples were transferred to disposable 1.5 ml sterile plastic microcentrifuge tubes. 0.5 ml of a solution of glutaraldehyde (2 %) paraformaldehyde (2 %) in 0.1 M phosphate buffer (pH 7.2) was added to each microtube and the fixation process was allowed to proceed for 24 h at 4 ºC. After that, the fixation solution was washed for 5 min with phosphate buffer (0.1M) followed by two consecutive 10 min washes with cacodylate buffer (0.1M). The post-fixed procedure consisted of adding 2 % osmium tetroxide in cacodylate buffer (0.1M) at

4 ºC for 24 h. Each sample was washed three times with cacodylate buffer (0.1M) for 10 min each time.

Dehydration of samples was achieved with serial dilutions of ethanol (30 %, 50 %, 60 %, 70 %, 95 %, and 100 %). Each solution was maintained in contact with the sample for 10 min, and the last solution (100 % ethanol) was used three consecutive times. After the dehydration with ethanol, the second dehydration procedure with hexamethyldisilazane (HMDS) was carried out with the samples. Consecutive 15 min contact with ethanol/acetone/HMDS solutions at different ratios (1:0:0, 1:1:0, 0:1:0, 0:1:1, 0:0:1, 0:0:1) were used. Air drying was used as a final step, leaving the micro-centrifuge tubes with an open lid inside of a hood for at least one night. The samples were then mounted onto aluminum stubs, and gold plating was used as a final step to view on a Hitachi S-570 (Japan, Tokyo) scanning electron microscope (SEM) operating at 30 kV.

5.3.7 Microbiology

Enumerations of yogurt and probiotic cultures were carried out according to the standard International Dairy Federation (IDF) protocols. Cell count enumerations of yogurts were analyzed after 7 d of storage at 4 ºC. Yogurt samples of 1 mL were added to 9 mL sterile tryptone diluent (0.1 % v/v). Appropriate dilutions were made and subsequently pour-plated in duplicate onto selective media (Table 1). The International Dairy Federation Standard 117B (IDF, 1997) was used to enumerate *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp *bulgaricus.* Streptococci and lactobacilli were enumerated on M 17 agar with lactose after aerobic incubation at 37 ºC for 48 h and MRS agar with glucose after anaerobic incubation at 37 ºC for 72 h, respectively. *Bifidobacterium* were

enumerated on MRS with glucose plus diclhoxacilin solution, lithium chloride and cistein chloride after anaerobic incubation at 37 ºC for 72 h (Chr. Hansen, 1999). *Lactobacillus acidophilus* was counted using MRS agar with maltose after anaerobic incubation at 37 ºC for 72 h (IDF, 1995). The results were expressed as colony forming units per milliliter of yogurt and then statistically analyzed for viability of probiotics.

5.3.8 Statistical Analysis

All the experiments were done in triplicate. Statistical analysis was performed using SAS software. Significant differences were defined at $P < 0.05$.

5.4 RESULTS AND DISCUSSION

Different theories have been proposed to improve the viability of probiotics; selection of appropriate starter cultures, acid resistant strains, two-step fermentation, micro-encapsulation, stress adaptation, and incorporation of micronutrients such as peptides and amino acids (Shah, 2000). This research study shows that ultrasonification, a nonthermal technology, can be a feasible technology for improving the viability of probiotics.

β-*Gal* activity of *Lactobacillus Bulgaricus* (LB) for both YoMix236 and ABY611 cultures, increased significantly due to sonification (figures 1 and 2). In the case of *Streptococcus thermophilus* (ST), as the sonification time increased from 3 to 8 min, the β-*Gal* activity increased but not as significantly as in the case of LB. This might be because of ST's coccus (round) shape, which is the most stable structure among different shapes. The β-*Gal* activity of LB increased significantly but stopped increasing

significantly after 4 min of sonification (Figures 1 and 2). The use of different starter cultures did not result in significant differences in the β-*Gal* activity.

The viability of yogurt cultures before and after sonification at different time intervals is shown in Table 1. The decrease in live yogurt cultures after sonification demonstrates that sonification injured or killed bacteria. Due to sonification, ABY611 culture had higher injury or shock compared to the YoMix236. Bacteria were reduced by two to three log cycles after sonification for 3 min and four log cycles after 4 min of sonification. But the optimum level for maximum *β-Gal* and effective yogurt bacteria was attained at 4 min, which was used for yogurt manufacturing for the subsequent experiments.

Physicochemical characteristics of yogurt, namely fermentation time, pH, total solids, water holding capacity (WHC) and syneresis are shown in the Table 2. The fermentation time varied between 5.5 h to 6.1 h. Unsonicated cultures took less time to reduce the pH from 6.5 to 4.6 compared to the sonicated cultures. Because of greater viability, the time differences in fermentation can be attributed to the initial higher counts of ST in unsonicated yogurt culture compared to the sonicated cultures (Table 3), high metabolic activity of yogurt cultures (Haque et al. 2001), and to the different stains of bacteria (Lin and Chien, 2007). ABY611 strain showed a higher acidification rate, reaching the final pH in 5.2 to 5.5 h, while the fermentation time for YoMix236 was above 5.5 h. Østile et al. (2003) found very different profiles of metabolites during fermentation, and showed the importance of controlling fermentation time since probiotic strains produce different

amounts of metabolic products according to fermentation time. The balance and the type of strains in the sonicated culture also affected the fermentation time. At the end of the fermentation the pH varied from 4.56 to 4.58, however the pH values tend to decrease after storage due to post-acidification, a result of starter culture activity (Brandao, 1995).

The syneresis varied from 12.00 to 14.75 %, with no clear trend in syneresis between the sonicated and unsonicated yogurt samples. The syneresis of yogurt using Yomix236 increased from 12 % for unsonicated yogurt to 13.60 % for sonicated cultures but in the case of ABY611 the syneresis of yogurt decreased in sonicated yogurt 13.73 % compared to unsonicated yogurt syneresis 14.75 %. It is not clear whether these differences are caused due to sonification or other experimental parameters. Penna et al., 2006 had similar variations and stated that these differences might be due to differences in treatment of milk, fermentation conditions, and differences in yogurt culture strains.

The water holding capacity of sonicated and unsonicated yogurts varied from 26.32 to 31.62 %. Water holding capacity was higher in unsonicated culture yogurt for both the starter cultures, compared to sonicated culture yogurt. There are no studies that have reported the effects of sonification on water holding capacity in yogurt. Penna et al., (2006) reported that the water holding capacity was improved using high pressure processing compared to just the normal thermal process and attributed this increase to the increased number of network strands in pressurized gels.

Table 3 shows the β-Gal activity during yogurt manufacturing using sonicated and unsonicated yogurt cultures. The β-Gal activity increased 4.73 times, from 0.49 to 2.32

for sonicated cultures, but the activity increased only 3.28 times, from 0.21 to 0.69 in 6 h of fermentation time in unsonicated cultures, respectively using YoMix236 starter culture. For ABY611 culture, the *β-Gal* activity increased 3.30 times, from 0.63 to 2.08 in sonicated cultures but the activity increased only 2.28 times, from 0.38 to 0.87 in 6 h of fermentation time in unsonicated culture, respectively. The *β-Gal* activity was higher, by 3.36 times in sonicated yogurt compared to the unsonicated yogurt for YoMix236, and by 2.39 times in sonicated yogurt compared to the unsonicated yogurt for ABY611. This increase in *β-Gal* activity can be attributed to the increase in probiotic organisms in yogurt because the more the *β-Gal* activity provided higher nutrients.

The growth of probiotic organisms in yogurt before and after fermentation is shown in table 4 and 5 for YoMix236 and ABY611, respectively. The bacterial counts after one week of yogurt preparation using YoMix236 were $8.40x10^6$ to $9.05x10^9$ CFU/mL for ST, $3.47x10^6$ to $1.42x10^8$ CFU/mL for LB, $4.55x10^5$ to $3.27x10^8$ CFU/mL for LA, and $9.33x10^5$ to $4.43x10^9$ CFU/mL for BL. These ranges depend on the experimental conditions and the starter culture used. ST counts were higher by one to two log cycles in unsonicated yogurt compared to sonicated culture yogurt. LB cell counts were also equal or higher in unsonicated yogurt compared to sonicated yogurt. The partially injured cells might have shown decay after fermentation, which led to the improvement in the viability of probiotic organisms. The probiotic counts were higher in the presence of sonicated starter culture by one log cycle for LA and by 4 log cycles for BL. These results show that the probiotics grow better along with sonicated yogurt cultures than with unsonicated yogurt cultures, indicating the availability of more nutrients for the probiotics. This

phenomenon may be due to the availability of more nutrients, which is due to more *β-Gal* activity, because of the rupture of yogurt bacteria.

Several factors have been shown to affect the viability of both yogurt and probiotic cultures. The viability depends on the strains used, interaction between species present, culture conditions, final acidity of yogurt, oxygen content in yogurt and permeation of oxygen through the package. Lankaputhra and Shah (1995) observed a drastic decline in the probiotic bacteria under acidic conditions. The bacterial counts after one week of yogurt preparation using ABY611, were $3.23x10^6$ to $1.40x10^9$ CFU/mL for ST, $4.20x10^5$ to $2.45x10^8$ CFU/mL for LB, $5.34x10^6$ to $2.45x10^8$ CFU/mL for LA, and $4.70x10^6$ to $8.30x10^8$ CFU/mL for BL. ST counts were higher by two log cycles in unsonicated culture yogurt compared to sonicated culture yogurt after fermentation. LB cell counts were higher by one to two log cycless in unsonicated culture yogurt compared to sonicated culture yogurt. The results show that the probiotics grow better by more than one log cycle in sonicated culture yogurt compared to unsonicated culture yogurt.

In order to exert positive therapeutic effects, the yogurt and probiotic organisms must be viable, active and abundant. It has been suggested that these organisms should be present in a food at a minimum level of 10^6 CFU/mL or the daily intake should be about 10^8 CFU/mL (Vinderola et al., 2000). From a health point of view, the starter culture, Yomix236 showed better probiotic counts compared to the ABY611 culture in the sonicated culture yogurt. Dave and Shah (1997) reported notable differences in the viability of probiotic organisms stored at 4 and 10 ^oC in glass and plastic containers in different commercial yogurts. They reported less than five log cycles of Bifidobacteria

and equal to five log cycles for Lactobacillus acidophilus in commercial yogurts stored at 4 ^{o}C in plastic cups and attributed these differences to strains, production of hydrogen peroxide, acid concentration, and also the storage temperature. Bacterial strains are also influenced by the fermentation time, pH, strain association and incubation temperature, however they concluded that oxygen might have played a major role for the viability of probiotics.

Figure 3 and 4 show the microstructure of ST and LB before and after sonification for 3 min, 4 min and for 5 min. ST did not exhibit visible rupture or damage to the bacterial cells, but in the case of LB the cells shrank and the surface appears wrinkled, and some damage to the cells can be clearly observed, indicating the effect of sonification. At 5 min of sonication, more broken LB cells were observed, which confirms the damage of yogurt bacterial cells. This phenomenon is not visible in ST, which can be justified by its round and spherical shape, which is more stable structure to mechanical or physical stresses compared to the long cylindrical shape of LB. Cell count enumerations also confirm that the LB cell counts are less compared to the ST after 4 min of sonication treatment, which can be attributed to the stable shape of ST (Table 4 and 5).

5.5 CONCLUSIONS

Both starter cultures, ABY 611 and YoMix 236 showed similar patterns of increase of β-Galactosidase enzymatic activity and a notable difference in the viability of probiotic organisms after sonification. The results demonstrate that the probiotics grow better in yogurts made with sonicated starter cultures than in unsonicated starter cultures,

suggesting the availability of more β-Gal. The results show that for both yogurt starter cultures the probiotics grow better, by more than one log cycle, during fermentation when sonicated cultures were used. Thus ultrasonification, a nonthermal technology, is promising in the dairy industry. This research showed promising results for its application to improve the viability of probiotics in yogurt.

5.6 ACKNOWLEDGEMENTS

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Table 1. Viability of yogurt bacteria before and after sonication at different time intervals

Type of culture		Time interval (minutes)			
		0	3	4	5
S. thermophilus	ABY611	4.30E+09	1.81 E+07	3.23 E+06	1.40 E+04
	YoMix236	3.48E+10	2.40 E+08	8.40 E+06	3.45 E+05
L. bulgaricus	ABY611	1.33 E+09	5.31 E+07	4.20 E+05	8.30 E+04
	YoMix236	3.60 E+10	6.80 E+07	3.47 E+06	7.60 E+05

The units of starter culture counts are colony forming units per milliliter.

Table 2. Physicochemical characteristics of yogurts made from sonicated and unsonicated yogurt cultures.

Characteristic	YoMix236		ABY611	
	sonicated	**unsonicated**	**sonicated**	**unsonicated**
Total solids %	14.15±0.12^{ab}	13.97±0.08^{b}	14.36±0.11^{a}	14.05±0.07^{b}
Fermentation time, h	6:10±0.05^{b}	5:28±0.08^{a}	5:30±0.05^{a}	5:12±0.05^{a}
Yogurt pH	4.56±0.002^{a}	4.58±0.01^{a}	4.60±0.01^{a}	4.59±0.02^{a}
WHC %	26.32±0.18^{d}	28.53±0.31^{c}	30.11±0.26^{b}	31.62±0.57^{a}
Syneresis %	13.60±0.21^{b}	12.00±0.15^{c}	13.73±0.38^{b}	14.75±0.49^{a}

$^{a-d}$ Different letters within a row and between the columns indicate significant differences ($p<0.05$) exist and the values after ± indicate standard deviations.

Where, WHC – Water holding capacity

Table 3. β-Galactosidase activity* during yogurt manufacturing using sonicated and unsonicated yogurt cultures

Time (hours)	Yogurt Cultures in Yogurt (Probiotics added)			
	YoMix236		ABY611	
	sonicated	**unsonicated**	**sonicated**	**unsonicated**
0	0.49±0.03[b]	0.21±0.02[a]	0.63±0.01[b]	0.38±0.05[a]
6	2.32±0.15[b]	0.690.03±[a]	2.08±0.11[b]	0.87±0.02[a]

[a-b] Different letters within a row and between the columns indicate significant differences ($p<0.05$) exists and the values after ± indicate standard deviations.

*μmole of O-nitrophenol from ONPG per minute per gram

Table 4. Growth of Probiotics in the presence of sonicated (4 min) and un-sonicated yogurt cultures for YoMix236 (fermented until the pH reached 4.6 – approx. 5 to 6 h)

Type of culture	Sonicated cultures		Unsonicated cultures	
	Before fermentation	After fermentation	Before fermentation	After fermentation
ST	8.40 E+06[b]	6.05 E+07[b]	5.12 E+07[b]	9.05 E+09[a]
LB	3.47 E+06[c]	2.21 E+07[b]	3.33 E+06[c]	1.42 E+08[a]
LA*	4.55 E+05[c]	3.27 E+08[a]	4.16 E+07[b]	8.20 E+07[ab]
BL*	6.32 E+06[b]	4.43 E+09[a]	8.74 E+06[b]	9.33 E+05[c]

[a-c] Different letters within a row indicate significant differences ($p<0.05$) exist.
* Probiotics (LA and BL) were not sonicated.

Where,

ST – *Streptococcus thermophilus*
LB – *Lactobacillus delbruekii ssp. bulgaricus*
LA – *Lactobacillus acidophilus*
BL – *Bifidobacterium longum*

Table 5. Growth of Probiotics in the presence of sonicated (4 min) and un-sonicated yogurt cultures for ABY 611 (fermented until the pH reached 4.6 – approx. 5 to 6 h)

Type of culture	Sonicated cultures		Unsonicated cultures	
	Before fermentation	After fermentation	Before fermentation	After fermentation
ST	3.23 E+06[c]	1.20 E+07[b]	4.50 E+06[c]	1.40 E+09[a]
LB	4.20 E+05[c]	8.30 E+06[b]	5.60 E+06[b]	2.45 E+08[a]
LA*	5.34 E+06[c]	2.45 E+08[a]	3.40 E+07[b]	8.20 E+07[b]
BL*	4.70 E+06[b]	8.30 E+08[a]	4.70 E+06[b]	1.80 E+07[a]

[a-c] Different letters within a row indicate significant differences ($p<0.05$) exist.
* Probiotics (LA and BL) were not sonicated.

ST – *Streptococcus thermophilus*
LB – *Lactobacillus delbruekii ssp. bulgaricus*
LA – *Lactobacillus acidophilus*
BL – *Bifidobacterium longum*

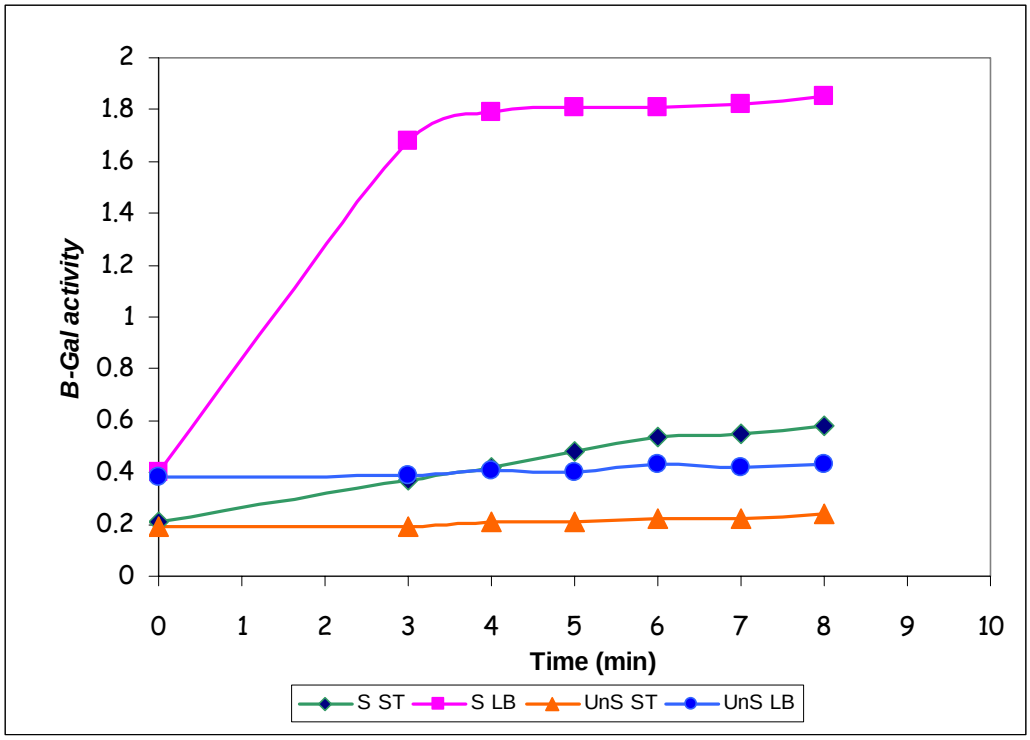

Figure 1 : *β-Galactosidase* enzymatic activity* of sonicated and unsonicated yogurt cultures, YoMix236.

S ST – Sonicated *Streptococcus thermophilus*
S LB – Sonicated *Lactobacillus delbruekii ssp. bulgaricus*
Un ST – Unsonicated *Streptococcus thermophilus*
Un LB – Unsonicated *Lactobacillus delbruekii ssp. bulgaricus*

* *β-Galactosidase* enzymatic activity is estimated as the amount of active enzyme liberating one micromole of O-nitrophenol from ONPG per minute per gram of sample at 37 ºC.

Sonification conditions: power = 400 W, frequency = 24 kHz

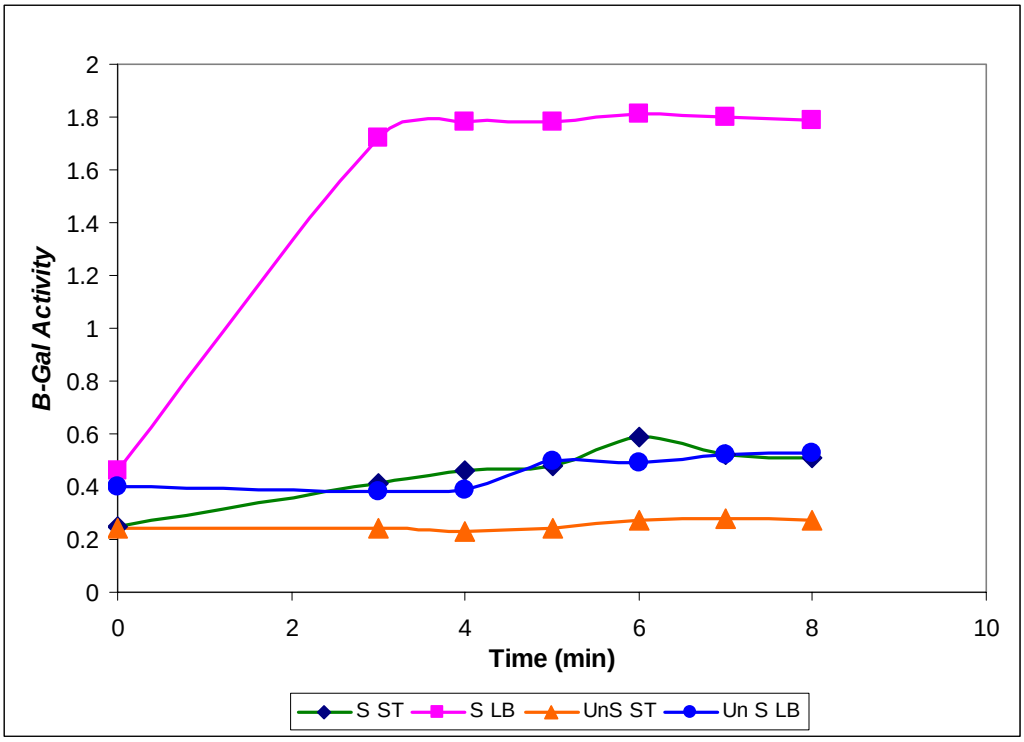

Figure 2: *β-Galactosidase* enzymatic activity* of sonicated and unsonicated yogurt cultures, ABY611.

S ST – Sonicated *Streptococcus thermophilus*
S LB – Sonicated *Lactobacillus delbruekii ssp. bulgaricus*
Un ST – Unsonicated *Streptococcus thermophilus*
Un LB – Unsonicated *Lactobacillus delbruekii ssp. bulgaricus*

* *β-Galactosidase* enzymatic activity is estimated as the amount of active enzyme liberating one micromole of O-nitrophenol from ONPG per minute per gram of sample at 37 ºC.

Sonication conditions: power = 400 W, frequency = 24 kHz

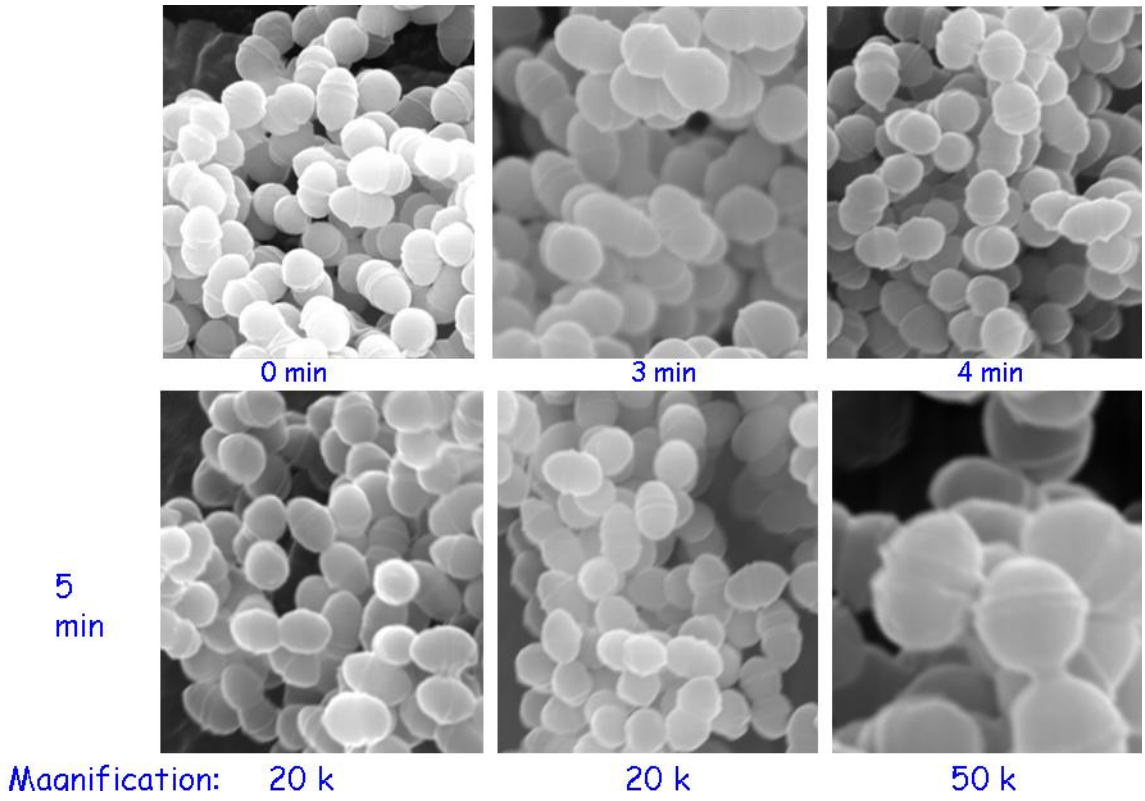

Figure 3: Scanning Electron Micrographs of yogurt culture *Streptococcus thermophilus* before and after sonication.

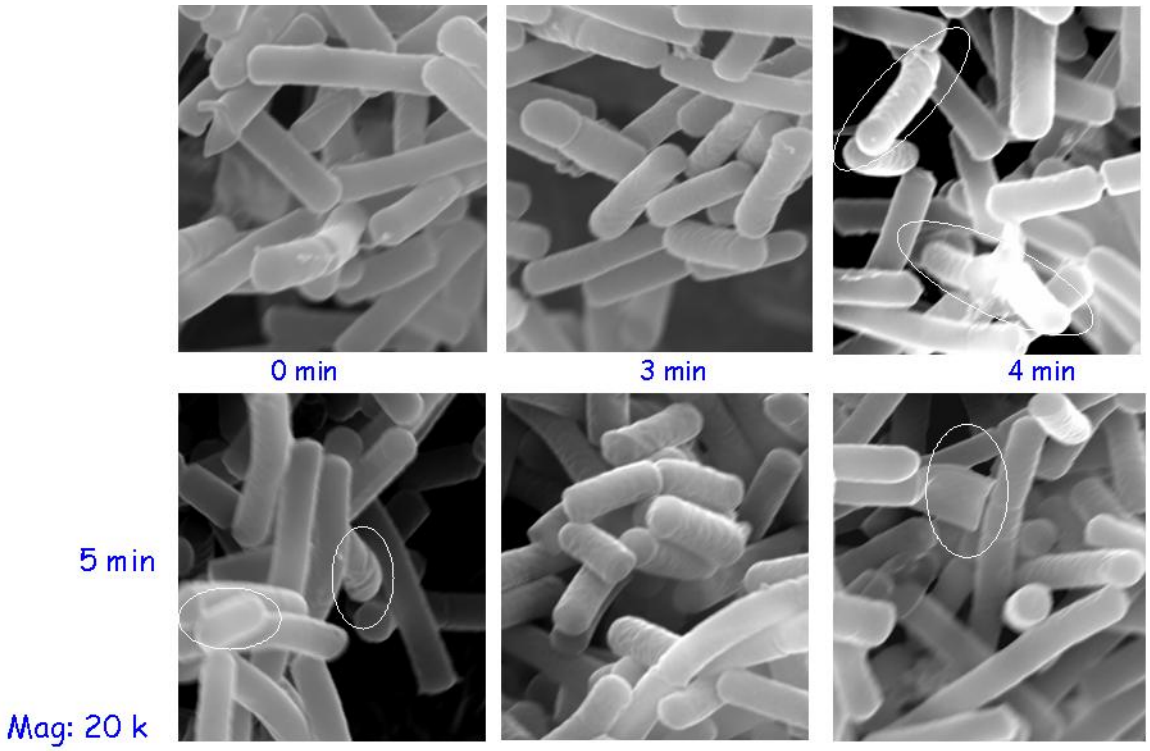

Figure 4: Scanning electron micrographs of yogurt culture, *Lactobacillus delbruekii ssp. bulgaricus* before and after sonication.

CHAPTER SIX

Effect of storage on the rheological characteristics and viability of probiotics of stirred yogurt manufactured with sonicated starter cultures

Subba Rao Gurram, Ralph P. Cavalieri, Stephanie Clark, Barry G. Swanson, and Gustavo V. Barbosa-Cánovas

6.1 ABSTRACT

The influence of starter culture sonification on yogurt rheological and physicochemical characteristics during storage was analyzed. The viable probiotics in sonicated and unsonicated starter culture yogurts were evaluated. Storage times of 1, 8, 16, 24, 32 d at 5 ºC were chosen for analysis. Two starter cultures, ABY611 and YoMix236, having *Streptococcus thermophilus* and *Lactobacillus bulgaricus,* were selected for yogurt manufacturing. The pH of sonicated and unsonicated starter culture yogurts varied from 4.6 to 3.98 during the 32 d storage period. Sonification of cultures significantly reduced the post acidification in yogurt for both starter cultures. Significant differences were not observed in water holding capacity of yogurts, but there was a decreasing trend during the storage times. Yogurts made from sonicated starter cultures had less syneresis compared to the control yogurts. During storage, yogurts with sonicated starter culture had two log cycles more probiotics compared to yogurts made from unsonicated starter cultures. In general, the probiotics declined after the 24^{th} day, and this can be attributed to the significant decrease in pH of the yogurts. Textural properties including hardness, adhesiveness, springiness, and gumminess, were evaluated for the yogurts. There was an overall decrease of these quality parameters for yogurts during the storage period. Rheological flow curves were fitted to an Herschel-Bulkley model and, in general, the

yield stress and consistency indices increased for the first week and a gradual decrease was observed for both sonicated and unsonicated starter yogurts. Overall, ultrasonification may improve the viability of probiotics and quality characteristics of yogurt.

Keywords: yogurt, probiotics, rheology.

6.2 INTRODUCTION

Food promotes the well-being and health of human beings, and, at the same time, reduces the risk of diseases. Fermented dairy products are consumed for nutrition and maintenance of good health. The food industry noticed this trend and during the last few years there was rapid growth in the market of low fat and functional foods. Also, the dairy industry is continuously looking for new technologies to improve and produce high quality dairy products.

The main source of probiotics is conventional dairy products, dietary supplements and medicinal foods. Yogurt, culture containing fluid drinks, and some brands of cheese are the products claimed to have probiotics. Yogurt is made by the slow lactic fermentation of milk lactose by the thermophilic lactic acid bacteria, *Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus*. Probiotics such as *Lactobacillus acidophilus* and *Bifidobacterium* are often added. The first two are needed to convert milk to yogurt and the latter two are being added because of their health promoting properties. For therapeutic benefits, the minimum level of probiotic bacteria in yogurt has been

suggested at least 10^6 live probiotic bacteria per milliliter (Speck, 1978). National Yogurt Association (NYA) of the United States specifies 10^6 cfu/mL of lactic acid bacteria at the time of manufacture, as a prerequisite to use the NYA 'Live and Active Culture' logo on the containers of products (National Yogurt Association, 2005). Probiotic bacteria are a mixed culture of microorganisms, which when consumed by humans, affect the host beneficially. Probiotics in yogurt has nutraceutical and therapeutic effects, such as digestion enhancement, immune system boosting, anticarcinogenic activity and reduction of serum cholesterol (Analie Lourens-Hattingh and Bennie C. Viljoen, 2001). It is also widely accepted by the research community that probiotic microbes have a powerful beneficial influence on the host by improving the balance of microflora in the gut (Tannock, 1999).

The survival of probiotic bacteria in yogurt is affected by several factors like low pH (Hood and Zoottola, 1988), hydrogen peroxide produced by yogurt bacteria (Gilliard and Speck, 1977), and oxygen content in the product and oxygen permeation through the package (Schioppa et al., 1981; Hull et al., 1984; Ishibashi and Shimamura, 1993; Lankaputhra and Shah, 1994). The pH of yogurt declined as low as 3.28 during storage after 31 days (Lourens and Viljoen, 2002) and a rapid decrease in *L. acidophilus* number was observed under acidic conditions (Lankaputhra and Shah, 1994). Bifidobacteria ceases to grow below pH 4.0 (Shah, 1997). Yogurt bacteria are also assumed to be responsible for the death of probiotic bacteria (Shah and Jelen, 1990).

β-Galactosidase (β-gal) or lactase released from starter cultures and is used to hydrolyze lactose in milk. The products of lactase hydrolysis, glucose and galactose, could be used by probiotics to improve the shelf life of probiotics. So increasing the amount of β-gal by rupturing the yogurt bacteria will improve the viability of probiotic bacteria. Another advantage of this process is that it will reduce the amount of lactose for yogurt bacteria, which are responsible for lowering the pH during storage (Shah and Jelen, 1990, Hood and Zoottola, 1988). Activity of β-gal will be increased several times by cell lysis induced by ultrasonification (Citti, 1965). Probiotic bacteria, *Lactobacillus acidophilus* and *Bifidobacterium* can utilize glucose and galactose, products of lactose hydrolysis, for their growth and the viability of probiotic bacteria can be improved.

Quality attributes such as texture, consistency, firmness, and flow properties are essential characteristics and quality parameters of yogurt; all these parameters can be related to sensory acceptability and consumer satisfaction (Vélez-Ruiz and Barbosa-Cánovas, 1997). Yogurt is a time dependant non-Newtonian pseudoplastic material. In quality determinations during storage, most of the works used power law and Herschel-Bulkley (H-B) models. Stirred yogurt is a complex time dependent shear thinning viscoelastic fluid. To express the flow of yogurt in a quantitative way, the more applied model is the H-B model and is given by (Ibarz and Barbosa-Cánovas, 2003):

$$\tau = \tau_0 + k\,\gamma^n$$

Where,

τ is the shear stress (Pa) and τ_0 is the yield stress (Pa)

k is the consistency index ($Pa.s^n$) and γ is the shear rate (s^{-1})

n is the flow behavior index (dimensionless) and $0 \leq n \geq 1$

The main purpose of this research was to determine the shelf life of probiotics when grown with sonicated yogurt starter cultures and to characterize the rheological and textural properties of yogurt made using the sonicated yogurt cultures.

6.3 MATERIALS AND METHODS:

Two selected yogurt cultures containing *Streptococcus thermophilus* and *Lactobacillus delbrukii spp. bulgaricus* and probiotics, *Lactobacillus acidophilus* and *Bifidobacterium* (frozen cultures supplied by Danisco USA Inc.) were used. Frozen cultures (100 g of each culture) was mixed with 1000 mL pasteurized milk and working stocks of 100 mL were prepared and stored at -21 ºC for experiments. Ultrasonification treatment was carried out to rupture and activate the yogurt culture before manufacturing yogurt. Yogurt was made using the sonicated and unsonicated yogurt cultures, in triplicate, and analyzed for physicochemical, textural, rheological, and shelf life of probiotics. The statistical significance of differences between treatments was determined by ANOVA using the general linear model (GLM). The level of significance was set at $P < 0.05$.

6.3.1 Ultrasonification Treatment

A Hielscher USA Inc. (Ringwood, NJ) ultrasonic processor model UP400S (400 W, 24 kHz) with a 22 mm diameter probe was used. A 500 ml double-walled vessel (8 cm internal diameter and 13.5 cm depth) was used as a treatment chamber. The temperature was set up and kept constant via a refrigerated bath (VWR Scientific Model 1166, Niles IL). A type-K thermocouple was used in the treatment chamber to monitor the

temperature (t± 0.5 ºC) throughout the experiments. The ultrasound wave was kept constant at 100 % amplitude (120 mm) in all treatments. A magnetic stirrer was used inside the vessel to assure the homogeneity of the samples throughout the sampling. Our previous results showed that the optimum ultrasonification time for the highest β-gal activity was 4 min and was used for both types of yogurt cultures. These sonicated and unsonicated cultures were used to prepare yogurt.

6.3.2 Yogurt preparation

Yogurt was prepared using skim milk fortified with skim milk powder to standardize the desirable total solids (14 %). The milk was held in plastic bags at 4 ºC for 2 h and then subjected to thermal treatment (85 ºC for 30 min). After heat treatment, the milk was cooled in an ice bath and then maintained at 43 ^{o}C for yogurt preparation. The processed milk was inoculated (0.1 %) with sonicated or unsonicated yogurt cultures, and probiotic cultures (0.1 %) were added. Fermentation was carried out at 43 ºC and stopped when the pH value reached 4.6. The yogurt was cooled rapidly to 20 ºC and immediately stored at 4 ºC for 36 h, and then analytical evaluations were carried out.

6.3.3 Physicochemical characteristics

Fermentation time is the time necessary to reach pH 4.6 in hours. The pH value was measured using a digital 420 A pH meter (Orion Research Inc., Boston, MA, USA). Water-holding capacity was evaluated by subjecting the yogurt to centrifugation at 15,000 x g for 15 min at 20 ºC (Harte et al., 2003). Ten grams of yogurt sample was evaluated using a Beckman J2-HS centrifuge (Beckman Instruments Inc., Seattle, WA,

USA). Water holding capacity was expressed as the percentage of pellet weight relative to the original weight of the sample:

$$WHC(\%) = \left[1 - \frac{Weight\ of\ whey\ after\ centrifugation}{Weight\ of\ yogurt}\right] \times 100$$

Susceptibility of yogurt to syneresis was determined using a drainage method. Yogurt samples were transferred into a funnel fitted with a qualitative paper Whatmann No. 5. The volume of the whey collected over 4 h at 4 ºC was measured in a 25 mL graduated cylinder (Hassan et al., 1996). All tests were carried out in triplicate.

6.3.4 Microbiological Analysis

Enumerations of yogurt and probiotic cultures were carried out according to the International Dairy Federation (IDF) standard protocols (Table 1). Yogurts are analyzed after 8, 16, 24 and 32 d of storage at 4 ºC. Yogurt samples (1 mL) was be added to 9 mL sterile tryptone diluent (0.1 % v/v). Appropriate dilutions are made and subsequently pour-plated in duplicate onto selective media (Table 1). Enumeration of probiotic microorganisms are also done as shown in Table 1.

6.3.5 Textural Properties

Texture measurements of yogurts were carried out on stirred samples using a TA-XT2 Texture Analyzer (Stable Micro Systems, Texture Technologies, Scarsdale, NY) with a 2 kg compression load cell. The analysis was carried out through a double compression test using an aluminum cylinder (P/50, diameter 50 mm). The cylinder penetrated 35 % of strain the surface of the coagulum, and the crosshead speed was 1 mm s^{-1}, during 12 s).

Four replicate samples (70 g of yogurt) were performed at 5 ºC for each type of yogurt. Typical parameters quantified were 'hardness' (the force necessary to attain a given deformation), 'springiness' (or elasticity which is the rate at which the deformed material goes back to its undeformed condition after the deforming force has been removed), 'adhesiveness' (work necessary to overcome the attractive forces between the surface of the yogurt and the surface of other material with which it comes in contact), and gumminess (the property or the state of being viscous) (Rawson and Marshall, 1997).

6.3.6 Rheological Properties

Rheological measurements were made at 10 ºC using a concentric cylinder Physica rheometer, model 320 (Paar Physica USA, Inc., Glen Allen, VA, USA). Shear rates ranging from 0.1 to 300 s^{-1} (with logarithm increase each 10 s) under programmed upward and downward curves were used, and corresponding shear stress data were obtained. The shear stress and shear stress data obtained from the rheometer were adjusted to the Herschel-Bulkley model to obtain the rheological characteristics: yield stress, consistency index, and flow behavior index.

6.4 RESULTS AND DISCUSSION

Physicochemical characteristics of yogurt made using sonicated yogurt starters and unsonicated yogurt starters were analyzed for 32 d. The pH, water holding capacity and syneresis of yogurt are shown in table 2.

6.4.1 pH

The pH of sonicated yogurt made using ABY 611 yogurt culture varied from 4.60 to 4.32 and for unsonicated yogurt was 4.59 to 3.98 during the 32 d of storage. The pH of sonicated yogurt made using YoMix236 yogurt culture varied from 4.56 to 4.28 and for unsonicated yogurt it varied from 4.58 to 4.03 during the 32 d of storage. It is evident that the sonification significantly reduced the extent of post acidification of yogurt for both types of starter cultures, which can be attributed to low activity of yogurt starter culture after yogurt fermentation. The pH of yogurt made from sonicated cultures was well maintained and did not drop significantly for up to 16 d and was still maintained at 4.32 for ABY611 and 4.28 for YoMix236 after 32 d of storage, respectively. However, the pH of yogurt made from unsonicated yogurt culture ABY611 dropped significantly by the 16^{th} d to 4.32 and below 4.0 by the 32^{nd} d, which was detrimental for the viability of probiotics (Table 3 and 4). Also, the pH of yogurt made from unsonicated yogurt culture YoMix236 dropped significantly to 4.36 by the 16^{th} d and to 4.03 by the 32^{nd} d of storage. This drop in pH over time can be explained by lactose in the yogurt being fermented to lactic acid (post acidification) by yogurt starter cultures that are still active. Similar observations of a decrease in pH of stirred yogurt over a storage period have been reported earlier (Briceno and Martinez 1995, Shah 1997, Aryana et al. 2006).

β-galactosidase released from yogurt starter cultures after sonification was possibly used to hydrolyze lactose to produce glucose and galactose, which was used by the probiotics to improve their viability.

6.4.2 Water Holding Capacity (WHC)

Water holding capacity (WHC) of yogurt made using sonicated and unsonicated yogurt starter cultures, during 32 d of storage is shown in Table 2. The WHC of yogurts varied from 30.88 to 23.3 % for ABY 611 and from 28.47 to 21.18 % for YoMix236 when tested under extensive G-forces (15,000xg) than those under normal storage. WHC of sonicated and unsonicated yogurt cultures did not show a clear trend but showed a gradual decreasing trend during the 32 d storage period. Yogurt made from unsonicated yogurt cultures showed a higher WHC compared to the sonicated yogurt, which can attributed to the casein aggregation to trap the serum phase within the protein matrix (Everett and McLeod, 2005). Harwalker and Kalab (1986) have shown that the WHC of yogurt made from reconstituted non fat dry milk was proportional to the total solids (TS) and at 20 % TS content the spontaneous whey drainage was stopped, which led to the enhancement of interactions between the casein particles. Barrantes et al., (1996) also showed that on an average yogurt (set-type) with milk fat had high WHC compared to yogurts made having vegetable oils. Skim milk standardized to 14 % TS was used for all the sonicated and unsonicated experiments but such variations were typical for these types of experiments because of their different conditions during treatment of the milk and fermentation of the yogurts.

Most studies have shown that the heating of the milk base improves WHC. Danneenberg and Kessler (1988) suggested that a large denaturation of β-lactoglobulin reduced the capacity of micelles to coalesce during fermentation, which explains low WHC compared to the unsonicated starter culture yogurts. But, whey protein denaturation and further

aggregation to κ-casein are mainly responsible for the marked increase of WHC, firmness, and apparent viscosity of acid gels made from heated milks (Cho et al., 1991), but the mechanisms are not entirely understood. Increasing the total solids or protein content leads to a higher concentration of casein particles, which reinforces the protein matrix density and improves the WHC of the gel (Sodini et al., 2004).

6.4.3 Syneresis

Syneresis of yogurt made by sonicated and unsonicated yogurt starter cultures during 32 days of storage time is shown in Table 2. Syneresis of yogurt varied from 7.8 to 19 % for ABY 611 starter culture and from 13.56 to 32 % for YoMix236 starter culture. YoMix236 yogurt samples showed higher syneresis compared to ABY611 starter culture during the entire storage period of 32 d. Also, the yogurts made from sonicated cultures showed lower syneresis compared to the unsonicated yogurt culture samples during the entire storage time. On the 16^{th} day of storage, there was significantly higher syneresis of the yogurt made from both types of unsonicated starter cultures. This can be attributed to the sudden drop in the pH, which has a significant effect on the physicochemical, sensory, rheological and textural properties of yogurt (Sodini et al. 2004).

Harwalker and Kaleb (1986) reported an increase in the rigidity of yogurt at a lower pH and explained that it could be due to the effect of pH on the electric charge of casein particles. They reported a 20 % increase in the gel firmness when the final pH was decreased from 4.50 to 3.85. They assumed that it was caused by intramicellar repulsions due to the increase of the positive charge on the casein particles at a lower pH. These

forces tend to swell the casein micelles increasing syneresis. Also, these forces reduced intercellular interactions, resulting in more open casein micelle structure, which is susceptible to form grains and also to give a lumpy structure when yogurt is stirred (Harwalker and Kaleb, 1986). This kind of loose casein micelle structure can make whey separation easier, which is directly proportional to the amount of syneresis. Thickness-in-mouth of a lower pH yogurt can be improved by using different varieties of starter cultures: EPS forming or a texturing starter or a mixture (Martin et al. 1999).

6.4.4. Yogurt and probiotic bacterial counts

The enumeration of yogurt starter and probiotic bacteria for both sonicated and unsonicated cultures for a storage period of 32 d are shown in Tables 3 and 4. The initial counts of ST and LB are higher in the unsonicated yogurt samples compared to the sonicated yogurt samples. This difference could possibly be due to the inactivation of cells by sonification, differences in starter culture, and the incubation time to reach pH of 4.6. In order to extend therapeutic effects, the yogurt and probiotics must be viable, active and abundant. It has been suggested that these microorganisms should be present in the food at a minimum level of 10^6 CFU/mL or the daily intake should be at least 10^8 CFU/mL. National Yogurt Association (NYA) states that companies can claim “Live & Active Cultures” on their packages if the refrigerated yogurt contains at least 100 million probiotics per milliliter and at least 10 million cultures per milliliter for the frozen yogurt at the time of manufacture.

Greater viability of cultures by one to two log cycles in the sonicated yogurt for both types of cultures was seen after 24 h of yogurt manufacture. After 32 d of storage the sonicated yogurt had two log cycles higher numbers in the probiotic counts compared to the unsonicated yogurt samples. For both ABY611 and YoMix236, the probiotic counts were higher compared to the starter cultures during the entire storage time for the sonicated yogurt samples. The probiotics counts showed a reduction after the 24^{th} d (ABY611) and after the 16^{th} d (YoMix236) of storage and this can be related to the drop in the pH of the samples (Table 2) and other factors that affect the viability of probiotics in general. Several factors have been claimed to affect the viability of probiotics cultures in fermented milk products. Although LA and BL tolerate acid, a rapid decline in their numbers in yogurt has been observed under acidic conditions (Lankaputhra and Shah, 1994).

The increase in the number of probiotics during manufacture and the viability of probiotics during storage were dependant on the species and the strain of associative yogurt bacteria (Dave and Shah, 1997). For both the cultures ABY611 and YoMix236, the yogurt starter culture counts were higher compared to the probiotics for the unsonicated yogurt samples, which ultimately affected the entire storage period for the growth of probiotics. The probiotic counts of the unsonicated yogurt samples fell below the general yogurt standards by the third week for both types of cultures. On the contrary, the probiotic counts in the sonicated yogurt samples were well maintained above the standards for contributing to the therapeutic effects for the entire storage period. These results are in agreement with the results of Dave and Shah (1997) that assessed the

viability of probiotics using four commercial yogurt starter cultures for a storage time of 35 d. This could be attributed to the release of β-galactosidase by sonification and to inactivate the yogurt cultures. The significant increase in the probiotics of sonicated yogurt samples can be due to the release of more β-galactosidase, which is used to convert lactose to glucose and galactose. The results show that probiotics have improved their viability and this can be due to the glucose and galactose released by hydrolysis and less acidic conditions (higher pH in sonicated samples).

6.4.5 Body and Texture

Body and texture is one of the most important components of yogurt quality and an essential factor for the description of mouth feel and overall acceptability. The maintenance of a uniform texture and particularly firmness among different units, processing dates and shelf life is a prime goal in yogurt production (Chanasattru et al., 2002). Textural attributes of yogurt made from sonicated and unsonicated starters for two different cultures for a storage period of 32 d are shown in Table 5. Sonicated culture yogurts showed higher gel strength compared to the unsonicated yogurt cultures. However there was an overall decrease during the entire storage period for both the starter culture types. There was a significant increase in the gumminess during the first week of storage compared to the rest of the storage period for both the starter culture types. These differences of textural attributes in the yogurt might be more related to the type of strains of the specific starter cultures than to the sonification. Rawson and Marshall (1997) also reported that yogurts made with ropy strains were the hardest (firmest) compared to non-ropy strains, suggesting that ropiness contributes to increased

firmness and also has more to do with the protein structure. Sodani et al., (2004) reported the effects of varying total solids, protein content, thickeners or enzymes in the milk base on textural properties. Increasing the total solids or protein content leads to a higher concentration of casein micelles and reinforces the protein matrix density, improving the texture, rheological, and WHC of yogurt. Also, fat plays a major role providing strength to the gel structure and reducing whey separation. Sodani et al., (2004) also claimed an increase of yogurt viscoelasticity and apparent viscosity by 20 to 60 % when fat in the milk base was increased. So standardization of different components within the basic ingredients is an important aspect of manufacturing sonicated or unsonicated yogurt for consistent and repeatable results. In the future, it would be interesting to see how sonification affects the yogurt characteristics and probiotics viability using the ropy and non-ropy structures.

6.4.6 Rheology

Rheology is an important quality aspect for stirred yogurt. In this study the rheological characterization of stirred yogurt manufactured using sonicated and unsonicated starter cultures were evaluated for a storage period of 32 d. The flow parameters of stirred yogurt made from sonicated and unsonicated ABY611 and YoMix236 starter cultures for the storage period are presented in Table 6. Time dependent shear thinning was evident for all the sonicated and unsonicated yogurt samples. Starter culture ABY611 showed that the yield stress and consistency index increased from 2.635 to 3.516 for the sonicated and 3.072 to 3.580 for the unsonicated during the first 8 d of storage but after that it showed a gradual decrease for the rest of the storage period. A similar trend was observed

for YoMix236 culture for both sonicated and unsonicated, except on the 16th d. This might be an outlier in the data (also R^2 = 0.971) as it is very typical for these kinds of experiments, especially in stirred yogurt. Similar results were reported by Lubbers et al., (2004), Tamine and Deeth, (1980), and Domagala et al., (2005). Lubbers et al., (2004) reported similar trends for their results for strawberry fat free stirred yogurt during a storage period of 28 d. They also reported that the apparent viscosity showed a significant increase during the storage time. However Domagala et al. (2005) reported a decrease in the apparent viscosity for yogurts during the storage period of 21 d. Therefore, highly standardized procedures are necessary in order to obtain reproducible results.

Applying constant shear rate for a specific period of time results in typical curves for viscosity versus time, and viscosity usually decreases at any time when the experiment is repeated with increased shear rate. Although an equilibrium viscosity is not achieved faster, the decrease in viscosity reduces with increased time of shear (O'Donnel and Buttler, 2002; van Marle et al., 1999). This phenomenon was physically visible and also observed with the results reported for both the sonicated and unsonicated yogurts (data not shown). Most authors analyze the rheological characteristics of stirred yogurt by increasing shear rate stepwise or by increasing shear rate linearly with time, followed by a decrease until the shear rate is 0 s^{-1}. Flow curves were fitted by means of either Power law or by Herschel-Bulkley models. However Rohm, (1992) reported that any equation coefficients obtained by regression analysis will depend heavily on the configuration of the test i.e., the acceleration of the shear rate due to the time dependant viscosity decay of stirred yogurt.

Yield stress is defined as the minimum shear stress required to initiate flow, frequently used to characterize stirred yogurt. For ABY611, sonicated yogurts showed lower yield stress compared to the unsonicated yogurt for the entire storage period, but in the case of YoMix236 starter sonicated yogurt showed higher yield stress when sonicated. These differences can be attributed to the type of specific strains, total solids, and protein content. Variation within the milk base among these components makes rheological characterization of yogurt a challenge. Also, measuring stirred yogurt's rheological behavior is very difficult due to sensitivity to sample preparation, sensitivity to shear, due to wall slip, and poor reproducibility (Yoon and McCarthy 2002). The sonicated yogurts showed a higher flow behavior index compared to the unsonicated yogurts for both types of starter culture. Overall, the flow behavior index decreased during the entire storage period for both ABY611 and YoMix236 sonicated and unsonicated yogurt samples, which are in agreement with the results of Lubbers et al., (2004) and Penna et al., (2006).

6.5 CONCLUSIONS

Sonification of yogurt cultures demonstrated that the viability of probiotics can be improved by two log cycles during a storage period of 32 d. Post-acidification of sonicated yogurt culture samples was not as high as unsonicated yogurt culture samples. pH was well maintained above 4.47 for 16 d in sonicated yogurt samples but dropped below 4.36 for the unsonicated culture yogurts after 8 d of storage. Yogurts made from unsonicated starters demonstrated high whey holding capacity compared to the sonicated yogurts. Syneresis showed a gradual increase during the entire storage for all the yogurts.

The partial inactivation of yogurt bacteria by sonification guaranteed two log cycles higher levels in probiotics in yogurt for the entire storage period. Textural and rheological properties were better in sonicated yogurt, but not significant, and showed a gradual decrease in quality during the storage regardless of treatment.

6.6 ACKNOWLEDGEMENTS

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Table 1. Selective media for enumeration of yogurt and probiotic microorganisms

Microorganism	Media	Incubation	Reference
Streptococcus thermophilus	M 17 agar	Aerobic 37ºC/48 h	IDF Standard 117B: 1997
Lactobacillus delbrueckii ssp. bulgaricus	MRS agar pH 5.4	Anaerobic 37ºC/72 h	IDF Standard 117B: 1997
Bifidobacterium spp.	MRS + glucose, dicloxacilin, lithium chloride and cistein chloride	Anaerobic 37ºC/72 h	CHR. Hansen, 1999
Lactobacillus acidophilus	MRS + maltose	Aerobic 37ºC/72 h	CHR. Hansen, 1999

Where, MRS = Methicillin-Resistant *Staphylococcus*

Table 2. Physicochemical properties of yogurts made from sonicated and unsonicated yogurt starter cultures ABY611 and YoMix236 during storage.

ABY 611	**Sonicated**					**Unsonicated**				
Days	**1**	**8**	**16**	**24**	**32**	**1**	**8**	**16**	**24**	**32**
pH	4.6±0.01^{a}	4.58±0.01^{ac}	4.55±0.02^{ac}	4.35±0.05^{b}	4.32±0.03^{b}	4.59±0.02^{a}	4.53±0.01^{c}	4.32±0.02^{b}	4.19±0.03^{d}	3.98±0.2^{e}
WHC, %	27.62±0.12^{ae}	30.26±0.09^{b}	26.73±0.21^{acef}	24.56±0.08^{cdef}	23.3±0.17^{df}	30.88±.25^{b}	29.67±0.31^{abe}	26.79±0.12^{e}	24.66±0.14^{f}	25.13±0.11^{ef}
Syneresis,%	7.8±0.03^{a}	10.5±0.21^{b}	13.6±0.13^{c}	17.7±0.69^{d}	18.2±0.19^{d}	8.0±0.01^{a}	11.2±0.48^{b}	16.8±0.52^{d}	18.5±0.31^{d}	19.0±0.32^{d}
YoMix 236	**Sonicated**					**Unsonicated**				
pH	4.56±0.02^{a}	4.51±0.05^{ab}	4.47±0.01^{b}	4.37±0.02^{c}	4.28±0.07^{de}	4.58±0.03^{a}	4.56±0.02^{a}	4.36±0.06^{cd}	4.22±0.04^{e}	4.03±0.01^{f}
WHC, %	26.57±0.22^{ab}	25.78±0.06^{a}	27.16±0.33^{b}	21.18±0.18^{c}	24.50±0.42^{d}	27.22±0.35^{b}	28.47±0.18^{e}	23.54±0.14^{f}	23.27±0.15^{f}	21.49±32^{c}
Syneresis,%	13.56±0.57^{a}	14.33±0.24^{ae}	16.92±0.15^{b}	28.45±0.29^{c}	31.6±0.07^{d}	14.63±0.32^{e}	16.72±0.21^{b}	25.88±0.05^{f}	29.4±0.12^{g}	32.0±0.57^{d}

$^{a\text{-}g}$ Different letters between the rows indicate significant differences ($p<0.05$) exist among the yogurts.

Where,

WHC = water holding capacity

Table 3. Enumerations of yogurt starter (sonicated and unsonicated) and probiotic bacteria in yogurt. The units are colony forming units per milliliter.

ABY611	**Sonicated**				
Days	**1**	**8**	**16**	**24**	**32**
ST	1.20E+07[b]	1.56E+07[b]	1.32E+07[b]	1.15E+07[b]	8.77E+06[c]
LB	8.31E+06[b]	9.36E+06[b]	4.53E+06[c]	8.50E+05[d]	5.10E+05[d]
LA	2.45E+08[a]	3.30E+08[a]	3.11E+08[a]	5.50E+07[b]	1.10E+07[b]
BL	8.30E+08[a]	9.80E+08[a]	7.22E+08[a]	2.70E+08[b]	8.54E+07[b]
ABY611	**Unsonicated**				
ST	1.40E+09[a]	2.33E+09[a]	1.51E+09[a]	1.07E+09[a]	9.88E+08[a]
LB	2.45E+08[a]	4.63E+08[a]	3.20E+08[a]	1.12E+08[b]	9.60E+07[b]
LA	8.20E+07[b]	9.50E+07[b]	1.30E+07[b]	5.70E+06[c]	1.73E+05[d]
BL	1.80E+07[c]	2.90E+07[c]	5.52E+06[d]	9.80E+05[d]	1.60E+05[e]

[a-e] Different letters between the rows (for each culture of sonicated and unsonicated) indicate significant differences ($p<0.05$) exist among the yogurts for the entire shelf life.

Where,

ST – *Streptococcus thermophilus*
LB – *Lactobacillus delbruekii ssp. bulgaricus*
LA – *Lactobacillus acidophilus*
BL – *Bifidobacterium longum*

and 1.20E+07 represents 1.20×10^7

Table 4. Enumerations of yogurt starter (sonicated and unsonicated) and probiotic bacteria in yogurt. The units are colony forming units per milliliter.

YoMix 236	**Sonicated**				
Days	**1**	**8**	**16**	**24**	**32**
ST	6.05E+07^{c}	9.40E+07^{bc}	6.32E+07^{c}	9.20E+06^{d}	2.96E+06^{d}
LB	2.21E+07^{d}	3.72E+07^{d}	8.76E+06^{e}	1.08E+06^{f}	6.20E+05^{g}
LA	3.27E+08^{a}	4.60E+08^{a}	3.78E+08^{a}	9.70E+07^{b}	2.50E+07^{c}
BL	4.43E+09^{a}	8.70E+09^{a}	5.20E+09^{a}	8.68E+08^{b}	7.24E+07^{c}
YoMix 236	**Unsonicated**				
ST	9.05E+09^{a}	9.76E+09^{a}	3.62E+09^{b}	9.60E+08^{b}	5.36E+08^{b}
LB	1.42E+08^{b}	3.78E+08^{a}	2.44E+08^{ab}	1.75E+08^{a}	9.90E+07^{c}
LA	8.20E+07^{bc}	9.88E+07^{bc}	4.60E+07^{c}	6.90E+06^{d}	8.70E+05^{e}
BL	9.33E+05de	2.74E+06^{d}	1.20E+06^{d}	6.30E+05^{e}	1.02E+05^{f}

$^{a-g}$ Different letters between the rows (for each culture of sonicated and unsonicated) indicate significant differences ($p<0.05$) exist among the yogurts for the entire shelf life.

Where,

ST – *Streptococcus thermophilus*
LB – *Lactobacillus delbruekii ssp. bulgaricus*
LA – *Lactobacillus acidophilus*
BL – *Bifidobacterium longum*

and 6.05E+07 represents 6.05 x 10^{7}

Table 5. Textural characteristics of yogurt made from sonicated and unsonicated yogurt starter cultures.

ABY 611	**Sonicated**					**Unsonicated**				
Days	**1**	**8**	**16**	**24**	**32**	**1**	**8**	**16**	**24**	**32**
Hardness	43.52^{a}	43.11^{a}	41.85^{ab}	40.22^{bc}	38.62^{c}	40.60^{b}	42.78^{a}	38.42^{c}	34.80^{d}	33.26^{d}
Adhesiveness	72.82^{ad}	112.56^{b}	44.23^{c}	66.80^{a}	79.81^{ad}	94.40^{d}	74.55^{ad}	121.79^{b}	68.59^{a}	7.86^{e}
Springiness	0.96^{a}	1.12^{ac}	3.38^{b}	1.36^{c}	0.94^{a}	0.95^{a}	0.98^{a}	4.13^{d}	1.18^{a}	1.06^{a}
Gumminess	22.47^{a}	24.79^{b}	21.90^{a}	20.09^{c}	21.16^{ac}	27.10^{de}	28.54^{cd}	25.37^{be}	26.67^{e}	23.18^{ab}
YoMix 236	**Sonicated**					**Unsonicated**				
Hardness	44.75^{a}	44.92^{ab}	43.26^{abc}	41.51^{bcd}	40.83^{cd}	39.51^{d}	44.32^{ab}	38.35^{d}	37.94^{d}	37.86^{d}
Adhesiveness	85.51^{ac}	65.23^{ad}	116.08^{be}	120.26^{ce}	74.82^{ad}	81.99^{ab}	39.65^{df}	122.92^{e}	59.88^{ad}	18.53^{f}
Springiness	0.94^{a}	0.94^{a}	1.23^{b}	0.98^{a}	1.07^{ab}	0.94^{a}	3.08^{c}	0.92^{a}	0.99^{a}	1.00^{a}
Gumminess	25.83^{a}	28.94^{b}	27.33^{ab}	27.93^{ab}	26.59^{ab}	26.43^{ab}	26.72^{ab}	25.46^{c}	21.6^{c}	21.59^{c}

$^{a\text{-}f}$ Different letters between the rows indicate significant differences ($p<0.05$) exist among the yogurts.

Units of
- Hardness: gram force
- Adhesiveness: gram force s^{-1}
- Springiness: dimensionless
- Gumminess: gram force

Table 6. Rheological parameters of yogurt manufactured from sonicated and unsonicated starter cultures of ABY611 and YoMix236 using H-B rheological model

ABY 611	**Sonicated**					**Unsonicated**				
Days	**1**	**8**	**16**	**24**	**32**	**1**	**8**	**16**	**24**	**32**
τ_0	2.64±0.07	3.52±0.11	2.18±0.05	1.36±0.18	1.21±0.13	3.07±0.03	3.58±0.06	2.49±0.02	2.19±0.35	2.09±0.12
k	1.69±0.04	2.41±0.05	2.12±0.02	1.89±0.04	1.86±0.05	2.08±0.01	3.23±0.02	2.79±0.02	2.33±0.06	2.27±0.01
n	0.88±0.02	0.78±0.04	0.64±0.05	0.65±0.08	0.55±0.01	0.87±0.01	0.69±0.03	0.55±0.001	0.60±0.04	0.52±0.01
R^2	0.998	0.986	0.99	0.992	0.986	0.997	0.991	0.989	0.994	0.971
YoMix 236	**Sonicated**					**Unsonicated**				
τ_0	3.38±0.23	4.06±0.08	3.02±0.05	2.67±0.14	2.62±0.31	2.85±0.01	2.99±0.03	3.64±0.52	1.89±0.15	1.66±0.08
k	1.98±0.03	2.24±0.04	2.22±0.01	2.61±0.01	2.47±0.07	1.28±0.01	2.95±0.11	2.88±0.05	2.56±0.03	2.36±0.02
n	0.85±0.01	0.86±0.02	0.76±0.08	0.60±0.02	0.64±0.01	0.78±0.02	0.70±0.09	0.66±0.02	0.86±0.05	0.62±0.01
R^2	0.977	0.986	0.988	0.996	0.972	0.993	0.986	0.971	0.987	0.994

τ_0 – Yield stress (Pa); k – Consistency index (Pa.s^n); n – Flow behavior index (dimensionless); R^2 –Coefficient of determination

CHAPTER SEVEN

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

The food industry is constantly seeking new "fresh-like" products with improved quality, extended shelf life, and few additives. A number of alternative nonthermal processing technologies are under research for delivering high quality products. High pressure processing is one of the promising technologies that was recently commercialized for some food products. A wide range of opportunities still exist and need to be explored. The present research explored the possibilities of manufacturing yogurt using two nonthermal processing technologies: high pressure processing and ultrasonification. The high pressure processing was used to manufacture low fat probiotic yogurt and the ultrasonification was used to improve the viability of probiotics in yogurt. The quality of yogurt was evaluated in both the cases.

The application of high pressure combined with thermal treatment produced yogurt gels with improved physicochemical characteristics compared to heat and high pressure treatments alone. Also, the milk treatments did not affect the growth of probiotic bacteria and the balance of strains in the starter culture. It was found that the level of inoculation affected the yogurt fermentation and physicochemical properties. High pressure can alter the structure of caseins and whey proteins. Denatured whey proteins, obtained by the heating process, are an important cross-linking agent. The yogurts manufactured presented different rheological behaviors according to the treatment used, which can be attributed to the structural phenomenon. Yogurts manufactured using combined high

pressure and heat showed improved consistency index implying that this process can be a potential processing method for manufacturing yogurt free of additives. In this study, the results demonstrated a synergistic effect of combined treatment. The gel firmness varied with type and amount of starter culture. The yogurts manufactured using the combined treatment of high pressure and heat presented enhanced textural characteristics.

The microstructure of heat-treated milk yogurt was composed of fewer interconnected chains of irregular shaped casein micelle, forming a network that enclosed the void spaces, while the microstructure of HHP treated yogurt exhibited more interconnected clusters of densely aggregated protein with reduced particle size, appearing more spherical in shape and exhibiting a smoother more regular surface and more uniform size distribution. The combined heat and HHP milk treatments led to compact yogurt gels with increasingly larger casein micelle clusters interspaced by void spaces, and exhibited a high degree of cross-linking. However, the exact mechanisms of combined effects on the unfolding and folding of secondary and territory structures and the energy states are not fully known and are worthy of thorough research.

There are relatively very few research studies demonstrating the efficacy of high pressure processing using a combination of heat and high pressure treatments. Before considering these technologies for commercialization, comparing these studies with commercial yogurts is extremely important. Also, effect of heat and then high pressure and a heat treatment with in high pressure chamber on the quality of yogurt would be interesting. The possibilities of studying and mathematically modelling the aggregation and re-

aggregation kinetics of casein micelles and casein sub-micelles can be a subject future research. Also, little is known about the implications of such changes during storage and shelf life studies are of utmost importance for the products developed by using these technologies.

Ultrasonification technique was used to rupture yogurt bacteria to release more β-*galactosidase*. The results showed that the probiotics grow better in sonicated culture yogurt than in unsonicated yogurt, indicating the availability of more nutrients for the probiotics due to more β-Gal availability. There is a clear trend that β-Gal activity increases due to sonification, improving the viability of probiotics. The β-Gal activity increased 4.73 times in sonicated culture yogurt compared to 3.28 times in unsonicated culture yogurt. The viability of probiotics increased by two log cycles in sonicated culture yogurt samples compared to just one-half log cycle in unsonicated culture yogurt. Also, ultrasonification reduced the post acidification in yogurt samples for both types of starter cultures. Water holding capacity did not show significant differences but showed a clear decreasing trend during storage. Sonicated culture yogurt samples showed lower syneresis compared to the control yogurt samples. Enumeration of yogurt and probiotic bacteria showed that sonification improved the viability of probiotics by two log cycles at the end of the 32 d storage.

The exact mechanism or the source that actually triggers the release of more β-gal is not fully understood and further research is necessary. Further areas of research possibilities are studying the biophysics and rupture kinetics of cell behavior to ultrasonification and

developing fundamental mathematical model. Ultrasonification is an energy consuming technique and scale-up for food processing especially for dairy products has seldom been done. Research should be especially focused on the process configuration and optimization to obtain high quality and cost effective food products. Equipment design improvements must be made to reduce the high energy losses of the currently available ultrasound equipment. Ultrasonification can be used as a possible homogenization technique for milk and the subsequent dairy applications.