5-2016

Effects of Thermosonication on Microbial Population Reduction and Solubillity Index in Skim Milk Powder

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EFFECTS OF THERMOSONICATION ON MICROBIAL POPULATION REDUCTION AND SOLUBILITY INDEX IN SKIM MILK POWDER

by

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

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

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Logan, Utah

2016

ABSTRACT

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and Solubility Index in Skim Milk Powder

by

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The effects of thermosonication (high intensity ultrasound coupled with thermal treatment), on the reduction of thermophilic spore-forming microorganisms and its effects on the solubility index in reconstituted skim milk powder (RSMP) were evaluated. Thermosonication was applied to RSMP at various solids concentrations, temperatures, and lengths of time based on commercial milk powder processing conditions. Microbial counts were determined prior to and after treatments to determine the log reduction of *Geobacillus stearothermophilus* vegetative cells and spores. Log reductions were recorded, and data were analyzed by response surface analysis. The log reductions induced by temperature and time without high intensity ultrasound (HIU) were compared to reductions observed with HIU. Thermosonication was also applied to RSMP to determine effects on solubility using a continuous flow cell system. Thermosonication yielded a significantly higher level of microbial destruction for both vegetative cells and

spores than heat treatment alone. For experiments involving vegetative cells, the interaction of treatment time and temperature proved to have the greatest influence on microbial inactivation. In comparison, the interaction of total solids content and length of HIU treatment demonstrated the greatest effect on the increased log reductions for spores. The solubility of RSMP treated with HIU did not significantly differ from the solubility of RSMP not treated with HIU. Further data showed the implementation of HIU, or thermosonication, during milk powder processing would be most effective before and after the evaporation stage when the total solids content of product is 9.2% and 50% at 75°C and 60°C, respectively. Based on preliminary data, it is assumed HIU applied for 10 s at these two locations would produce an additive effect, thereby reducing overall microbial counts by 5.76 log and 0.51 log for *G. stearothermophilus* vegetative cells and spores, respectively, in the product prior to entering the drying stage. All research findings and observations suggest HIU, or thermosonication, to be a successful method for reducing microbial populations during milk powder processing without sacrificing skim milk powder solubility.

(122 Pages)

PUBLIC ABSTRACT

Effects of Thermosonication on Microbial Population Reduction and Solubility Index in Skim Milk Powder

Nicola F. Beatty

Thermosonication has been researched as a means to improve shelf life, quality, and functional properties in dairy products. This study explored the effects of thermosonication on the inactivation of *Geobacillus stearothermophilus* in concentrated skim milk as a function of total solids content, temperature, and time and investigated changes in the solubility of the skim milk. Results showed thermosonication had an increased bactericidal effect on both vegetative cells and spores as compared to heat treatment alone without affecting solubility. A model was developed using response surface analysis showing that log reductions produced by thermosonication can be predicted based on a polynomial equation when certain conditions, such as treatment time, temperature, and total solids, are defined.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Marie Walsh for all of her help, support, and guidance that she has given me throughout the course of this project and during my entire time here at Utah State University. I would also like to thank the members of my committee, Dr. Donald McMahon and Dr. Silvana Martini, for their critique and valuable insight for multiple projects. In addition, thank you to the Dr. Niranjan R. Gandhi and Mrs. Josephine N. Gandhi Fellowship, the Office of Research and Graduate Studies, and the BUILD Dairy Program for funding my research and the research of many other students.

I'd like to thank my family, especially my parents, Korth and Joan Elliott, for teaching me the value of higher education and for encouraging me to never settle for anything less than my best. Most of all, I'd like to thank my husband, Nick Beatty, for loving and encouraging me every step of the way and for always being there even when we couldn't always be together.

Nicola F. Beatty

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LIST OF ABBREVIATIONS, NOTATIONS AND DEFINITIONS

cfu/ml, colony forming units per milliliter

CIP, cleaning in place

COP, clean out of place

Cp, specific heat capacity (J/g/°C)

D_{73}, D-value at 73°C

DHIA, Dairy Herd Improvement Association

dT/dt, change in temperature over time (°C/sec)

HAACP, hazard analysis critical control point

HIU, high intensity ultrasound

ISI, insolubility index

MPC-70, milk protein concentrate with 70% protein

NFDM, nonfat dry milk

PMO, Pasteurized Milk Ordinance 2013 revision

RMPC-70, reconstituted milk protein concentrate with 70% protein

RSMP, reconstituted skim milk powder

RSM, response surface methodology

SAS 9.4, Statistical Analysis System version 9.4

SI, solubility index

SMP, skim milk powder

spp., species

TS, total solids

TSA, tryptic soy agar

TSB, tryptic soy broth

WPC, whey protein concentrate

WPI, whey protein isolate

WPH, whey protein hydrolysate

w/v, weight-by-volume

w/w, weight-by-weight

CHAPTER 1
INTRODUCTION AND OBJECTIVES

The purpose of heat treating raw milk and milk products is to increase storage life by destroying microbial populations responsible for food borne-illnesses, which additionally results in a reduction of spoilage organisms. In the United States, the heat processing of milk and milk products is outlined in the Grade "A" Pasteurized Milk Ordinance (PMO) (2013 revision). A summary of these treatments and conditions are outlined in Table 1-1.

Generally, high temperature short time (HTST) and low temperature short time (LTLT) pasteurization conditions (Table 1-1) are used in the processing of fluid milks and milk powders. These conditions allow for the destruction of pathogenic and most spoilage-causing microorganisms without significantly affecting the physical and chemical composition of the final product (Walstra *et al.*1999). However, pasteurization is not always effective at producing the desired log reduction of mesophilic and thermophilic spore-forming bacteria, which are responsible for the spoilage and decreased quality in milk products (Cameron *et al*. 2009). Compared to pasteurization, ultra high temperature (UHT) and retort sterilization processing conditions (Table 1-1) destroy higher numbers of mesophilic and thermophilic spore-formers. However, higher heat treatments tend to produce sulfide-like cooked flavors, often described as burnt, scalded or caramel, that consumers find undesirable (Piyasena *et al.* 2003; Alvarez 2009; Bermúdez-Aguirre *et al.* 2009).

Bacillus (and related) spp. are of particular concern to the dairy industry, specifically in milk powder manufacturing, due to their ability to form spores, which are capable of surviving adverse conditions affected by pH, heat, moisture, and disinfectants (Scott *et al.* 2007; Lücking *et al.* 2013; Watterson *et al.* 2014). Once introduced to a more favorable environment during reconstitution of dry milk powder, the spores can germinate, grow, and begin metabolic processes, such as proteolysis and lipolysis, resulting in off-flavor development and spoilage (Scott *et al.* 2007; Lücking *et al*. 2013).

Skim milk powder (SMP) is a concentrated milk powder generally used as an ingredient in products termed "value-added foods", which consist of soups, sauces, confectionary, bakery, and meat products (Sharma *et al.* 2012). One of the main functional properties associated with SMP as an ingredient is its level of solubility, which can be influenced by milk heat treatment, type of spray drying, salt ion concentration, heat stabilizing agents added prior to powder manufacture, and bacterial contamination capable of inducing proteolysis and forming lactic acid (Sharma *et al.* 2012).

In recent years, the application of thermosonication, or high intensity ultrasound (HIU), has been explored as a means to increase the inactivation of vegetative and spore-forming bacterial populations when coupled with standard thermal processing conditions (Villamiel and Jong 2000; Awad *et al*. 2012; Herceg *et al*. 2012). Such treatments could potentially increase dairy product shelf life and quality without imparting undesirable cooked flavors that often occur in milk products treated at higher processing temperatures, such as UHT and retort sterilization (Bermúdez-Aguirre *et al*. 2009).

Cameron *et al*. (2009) found HIU was able to eliminate 100% of *Escherichia coli* and *Pseudomonas fluorescens* vegetative cells and 99% of *Listeria monocytogenes* vegetative cells after 10 min of application. This was observed in both raw and commercially pasteurized milk beverages. In addition, they observed no negative impacts on crude protein, casein content, fat content, or lactose content in milk as a result of HIU. Additional work (Bermúdez-Aguirre *et al*. 2009), determined HIU treatments to be effective in reducing microbial counts as a result of cell injury induced by cavitation and adverse environmental conditions produced by HIU treatment.

RATIONALE AND SIGNIFICANCE

While there has been previous research investigating HIU as a means to reduce microbial populations in the processing of fluid milks and beverages, little research has been conducted regarding the application of this technology in SMP and other concentrated milk products. Exploration of the effects of HIU on microbial reduction and functional properties of these types of dairy products would give valuable insight into the parameters necessary to achieve and predict microbial destruction rates when HIU is coupled with thermal processing conditions, such as HTST. In addition, it would provide evidence as to whether HIU would have any adverse effects on the solubility function of SMP.

This thesis focuses on the effects of HIU treatments as opposed to heat treatments without HIU on vegetative and spore-forming bacterial populations in reconstituted SMP at varying total solids content, temperatures, and length of HIU time. The effects of HIU

on changes in SMP solubility were additionally investigated due to its importance as a primary functional property of SMP.

HYPOTHESIS

Time, temperature, and total solids content each contribute toward the inactivation of thermophilic vegetative cells and spores in skim milk powder when HIU is applied. The bactericidal effect produced by HIU is greater and more significant than heat treatment alone without altering powder solubility.

OBJECTIVES

1. Investigate the effects of HIU on the reduction of *Geobacillus stearothermophilus* vegetative cells and spores in reconstituted skim milk powder using response surface methodology (RSM).
    a. Determine optimal HIU conditions (time, temperature, and total solids) and verify the predicted reductions to experimental microbial reductions.
    b. Evaluate and determine optimal location(s) in milk powder processing lines for implementation of HIU based on verification experiments.
    c. Compare microbial reduction in reconstituted skim milk powder to that of reconstituted milk protein concentrate (70% protein) when treated with HIU under milk powder processing conditions.
    d. Determine decimal reduction time (D-value) for HIU treatments.
2. Investigate the effects of HIU on the solubility of SMP using a continuous flow system.

TABLES AND FIGURES

Table 1-1 Thermal processing conditions for dairy products in the United States

Pasteurization Type	Conditions[a]	Products and Storage[a]	References
Low Temperature, Low Time (LTLT), vat or batch	145-155°F (63°C), 30 min	Milk, egg nog, frozen dessert mixes, viscous products; Must be refrigerated	PMO 2013
Continuous, High Temperature, Short Time (HTST)	161°F (72°C), 15 s	Milk, frozen dessert mixes, viscous products; Must be refrigerated	PMO 2013
Continuous, Higher Heat, Shorter Time (HHST)	191-212°F (89-100°C), 0.01-1 s	Milk; Must be refrigerated	PMO 2013
Continuous, Ultrapasteurization	≥280°F (138°C), 2 s	Milk and cream; Must be refrigerated, but extended shelf life	Lewis *et al.* 2009; PMO 2013
Aseptic, Ultra high temperature (UHT)	275-302°F (135-150°C), 4-15 s	Milk; Can be stored at room temperature	Lewis *et al.* 2009; PMO 2013
Sterilization	≥240°F (116°C), 20 min	Canned products; Can be stored at room temperature	PMO 2013

[a]Adapted from the Grade "A" Pasteurized Milk Ordinance (PMO), 2013 revision.

CHAPTER 2

LITERATURE REVIEW

INTRODUCTION

This literature review will first provide an overview concerning the formation of biofilms in dairy processing units since biofilms are the primary source of thermophilic bacteria vegetative cells and spores found in dairy products post-processing. This will include a description of how and why they form as well as characteristics contributing toward microbial growth, survival, and eventual contamination of product as it flows through the processing line. Next, this review will discuss the presence and types of thermophilic spore-forming bacteria in dairy products, specifically milk powders and concentrated milk products. Characteristics of *Bacillus* and *Bacillus* related thermophilic spore-formers will be discussed as well as how this organism is able to survive pasteurization and contribute toward product contamination and decreased quality. Thermosonication will then be discussed and previous research relating to its application and effects on microbial reduction in different food systems, including fluid milk and high protein milk powders. Lastly, solubility and the definition and determination of the solubility or insolubility index (SI, ISI) will be reviewed as it relates to milk powders, specifically skim milk powder (SMP). In addition, a brief overview of the effects of thermosonication on solubility will be reviewed from previous studies involving high protein milk powders.

BIOFILMS IN DAIRY PROCESSING

Biofilms are a common issue in all industrial dairy processing facilities (Simões *et al.* 2010). Their presence results in mechanical blockages, insufficient heat transfer, and corrosion of machinery, which translates to billions of dollars lost each year in revenue (Mittelman 1998; Houdt and Michiels 2010). In addition to losses correlated with the processing unit, biofilms are reservoirs for bacterial growth, specifically mesophilic and thermophilic spore-formers, the spores of which are capable of surviving heat treatment and cleaning. The spores living within these biofilms are responsible for contaminating and inducing product spoilage at an accelerated rate (Sharma and Anand 2002; Hill and Smythe 2012).

Biofilms are microcolonies of bacteria surrounded by an extracellular polysaccharide matrix growing together on a surface (Costerton *et al.* 1994; Burgess *et al.* 2009; Srey *et al*. 2013). Attachment to a surface and growth are a result of several factors, which include bacterial strain, surface material, pH, nutrient availability, and temperature (Srey *et al.* 2013). Dairy biofilms, or foulant, are mostly composed of bacteria, bacterial extracellular polymeric substances, milk proteins, and calcium phosphate (Mittelman 1998; Simões *et al.* 2010). Common microbes associated with food biofilms are *Pseudomonas aeruginosa*, *Pseudomonas fragi*, *Micrococcus* spp., *Bacillus subtilis*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Escherichia coli* 0157:H7, and *Campylobacter jejuni*, with *Bacillus* (and related) spp. (such as *Geobacillus*) being the most predominant genera in diary biofilms (Flint *et al.* 2001; Sharma and Anand 2002; Simões *et al.* 2010).

Biofilms contain both vegetative cells and spores (Burgess *et al*. 2009). Under ideal conditions, *Anoxybacillus flavithermus* and *Geobacillus* spp. (both *Bacillus* related spp.), thermophiles commonly present in milk powder processing facilities, can form biofilms within 8 h after introduction into the processing facility (Burgess *et al.* 2009). In their study, Burgess *et al.* (2009) found the inoculation of *A. flavithermus* vegetative cells into a continuous flow laboratory reactor resulted in spore formation at 55°C and 60°C. After 8 h, spore concentrations had reached 10-50% of the biofilm. No spore formation, however, was observed at 48°C. Average maximum cell density within the biofilms reached 6 $\log_{10}$ cells cm^{-2} after 8.5 h at 55°C (similar to a previous study by Flint *et al.* (2001) involving *G. stearothermophilus*), while spore counts continued to increase to 7 $\log_{10}$ spores cm^{-2} after 14.5 h at 55°C. They, therefore, concluded spore formation to be dependent upon temperature. Furthermore, they determined spores that survive pasteurization to be capable of germination in order to continue biofilm growth. Vegetative cells and spores from the biofilm can then contaminant product as individual cells or spores slough off during processing (Burgess *et al.* 2009).

Development of biofilms depends on the frequency and effectiveness of cleaning and sanitizing procedures. Proper cleaning in place (CIP) and cleaning out of place (COP) protocols are necessary components of Hazard Analysis Critical Control Point (HACCP) plans in order to minimize the development and growth of biofilms as much as possible (Sharma and Anand 2002). However, biofilms have enhanced resistance to antimicrobial agents compared to planktonic cells, making them difficult to remove (Mittelman 1998; Srey *et al.* 2013). In a study conducted by Sharma and Anand (2002), it

was found that biofilms continued to be present in certain segments of pasteurization lines in a commercial plant even after CIP and sanitation protocols were performed. These biofilms contained spoilage and pathogenic microorganisms that could potentially contaminate the final product.

THERMOPHILIC SPORES IN CONCENTRATED MILK PRODUCTS

Thermophilic spore-forming microbes are organisms capable of growing from 40-65°C and are often present during the manufacture of milk powder (Scott *et al.* 2007; Hill and Smythe 2012). Because of their tolerance to high temperatures, these organisms are capable of reproducing within the regeneration sections of plate heat exchangers and within the evaporators of dairy processing units, which operate at temperatures between 45°C and 75°C (Scott *et al*. 2007; Burgess *et al.* 2009). The most predominant thermophilic bacteria of concern in the dairy industry is the genus *Bacillus* and related spp. (Hill and Smythe 2012; Lücking *et al*. 2013).

One of the primary issues with thermophilic spore-forming microbes is their capability of producing acids, lipases, and proteases, causing spoilage and decreased quality in dairy products (Hill and Smythe 2012). Dairy products are generally stored below 37°C, but certain thermophilic spore-formers are still capable of germination at such temperatures (Burgess *et al.* 2010). *Bacillus* (and related) spp. commonly associated with the spoilage of a variety of products include: *G. stearothermophilus, B. subtilis, B. coagulans, B. sporothermodurans,* and *B. licheniformis* (Burgess *et al.* 2010; Hill and Smythe 2012). In a study analyzing milk powders from 18 different countries, 92% of the bacteria found consisted of *Bacillus* (and related) spp., specifically *G.*

stearothermophilus, *B. licheniformis*, and *A. flavithermus* (Rückert *et al.* 2004). *A. flavithermus* and *Geobacillus* spp. are among the most common thermophilic spore forming bacteria found in milk powder processing units, specifically near the plate heat exchanger and evaporator units (Flint *et al.* 2001; Ronimus *et al.* 2003; Burgess *et al.* 2009). A list of thermophilic bacilli and their characteristics commonly found in dairy processing facilities is outlined in Table 2-1.

Bacillus (and related) spp. are spoilage spore-formers commonly found in dehydrated foods, specifically concentrated milk products, such as skim milk powder (SMP) (Jiménez-Flores 1999). A study conducted in the San Joaquin Valley in 1997 and 1998, found that both mesophilic and thermophilic *Bacillus* spore-formers were present throughout all stages of milk powder manufacturing (Jiménez-Flores 1999). Although sources for contamination were suggested, there were many possible points of entry of spore-forming microorganisms into the final product.

In a more recent study by Buehner *et al.* (2015), nonfat dry milk samples were collected from 39 lots among 3 milk powder processing companies in the Midwest region of the United States. Mesophilic and thermophilic spores and bacteria counts were determined to be approximately 3.24 ± 0.09 and 3.23 ± 0.10 log cfu/g of powder, respectively, similar to observations from a previous study conducted at the University of Wisconsin-Madison by Ali *et al.* (2013). In that particular study, thermophilic spore counts measured from <1 to 4.1 log cfu/g of nonfat dry milk powder and SMP produced in the United States (Ali *et al.* 2013). The counts observed in both studies were measured in the final product, and no initial counts were taken in the milk prior to processing. The

similarity in counts suggest thermophilic bacteria and spores are capable of surviving milk powder thermal processing and entering the final product as viable microbes.

In a study conducted by Scott *et al.* (2007), spores were detected approximately 9 h following the initiation of 2 milk powder runs in a processing plant. At 18 h, spore collections showed an increase in counts by approximately 4 $\log_{10}$ cfu/ml in samples collected from the evaporator pass. This provided evidence of spore formation occurring inside the processing unit rather than through external contamination (Scott *et al.* 2007). Further sample collection showed fouling residues after CIP to be the source of thermophile (*Geobacillus* spp.) contamination within the plant and not from raw milk (Scott *et al.* 2007).

Spores produced during milk powder manufacturing are generally more heat-resistant and more tolerant of low water activity than those produced in a traditional laboratory setting (Hill and Smythe 2004; Burgess *et al.* 2010; Kotzekidou 2014). Production of highly heat resistant (80-100°C for 10-30 min; >106°C for 30 min) endospores by thermophilic bacilli (and related) are of concern for milk powder manufacture, such as SMP, since these products often become ingredients for high-heat treated concentrated dairy products and "value-added foods" (Burgess *et al.* 2010; Hill and Smythe 2012; Sharma *et al.* 2012). Certain thermophiles, such as *Geobacillus* and *B. sporothermodurans* spores are capable of surviving UHT and retort sterilization (Burgess *et al.* 2010; Hill and Smythe 2012) and therefore, begin lipolysis and proteolysis upon germination, causing increased rates of spoilage to products containing SMP as an ingredient.

SOLUBILITY AS A FUNCTIONAL PROPERTY OF SKIM MILK POWDER

Skim milk powder (SMP) is pasteurized non-fat dry milk (NFDM) containing ≤ 5% moisture and ≤ 1.5% milkfat (American Dairy Products Institute 2014). Unlike NFDM, which contains approximately 34% protein, SMP is standardized to a protein content ≥32% protein. Compared to other protein powders, such as whey protein isolate (WPI) and milk protein concentrate (MPC), SMP has a higher lactose content and lower protein content (Table 2-2). Solubility is one of the primary functional properties of SMP and is defined as the measure of the ability of milk powder particles or constituents to dissolve in solution (Sharma *et al.* 2012). These constituents generally consist of lactose, whey protein, salts, and casein (Fang *et al.* 2008). Powder particle (specifically protein) dissolution rate is influenced by the ability of hydrophilic amino acid residues to successfully form hydrogen bonds with water while weak interactions form among hydrophobic residues clustered among milk proteins (Schein 1990; Fang *et al.* 2008). For SMP and other dried milk products, complete solubility and dispersion of colloidal particles during rehydration is necessary in order for other functional properties, such as flowability, hygroscopicity, heat stability, emulsifying properties, water activity, stickiness, and caking, to be fully expressed (Mimouni *et al.* 2010).

Solubility Index

Because solubility is an essential function of SMP and milk powders in general, the solubility index (SI) or insolubility index (ISI) is used as a method for determining the solubility of milk powders. The SI is measured by reconstituting a certain amount of milk powder into a certain volume of water under specified conditions (Fang *et al.* 2008).

Following dispersion, the sample is centrifuged, and the sediment is then recovered and measured by volume in terms of milliliters to yield ISI, or amount that did not remain suspended in solution. The inverse of the obtained measurement is termed SI. A high SI or low ISI is desirable since that correlates to a high degree of solubility and the expression of other functional properties. Likewise, a low SI or high ISI indicates poor solubility and incomplete suspension of powder particles in solution. A standardized method does not exist for determining SI, which makes comparing results among studies relatively difficult (Fang *et al.* 2008). However, previous solubility studies have outlined multiple methods and techniques appropriate for measuring SI depending on milk powder lactose, fat, and protein content.

GEA NIRO (2010), a dairy technology processing division of GEA Group, cited the unfolding, or denaturation, of β-lactoglobulin to be the primary reason for a high ISI, or poor solubility. The unfolded β-lactoglobulin forms aggregates with casein, leading to a conformational change in the molecule to a more hydrophobic form that does not interact well with water (Baldwin 2010; GEA NIRO, 2010; Sharma *et al.* 2012). Factors contributing to this process include poor milk or powder quality as a result of bacterial contamination, increased viscosity and poor atomization due to drying temperatures or incomplete drying, low lactose content, and cross-linking of proteins to prevent proper hydrophobic and hydrophilic interactions in water (Fang *et al.* 2008; GEA NIRO 2010). Bacterial contamination is, in itself, a major concern for milk powder manufacturers due to poor shelf life of milk powders and of products utilizing milk powders as an ingredient. In addition, the presence of bacteria from foulant causes lactic acid

development and proteolysis (Table 2-3), which contribute to the denaturation of β-lactoglobulin and, therefore, high ISI or low SI (GEA NIRO 2010).

HIGH INTENSITY ULTRASOUND

Overview

High intensity ultrasound (HIU) refers to the presence of sound waves above the maximum limits of human hearing, greater than or equal to 20 kHz (Chandrapala *et al.* 2012). Different applications of ultrasound that have been explored or implemented in food processing include ultrasonic emulsification, lipid crystallization, filtration, viscosity modification, improvement of whey protein heat stability, improvement of meat tenderness, and inactivation of spoilage microbes (Chandrapala *et al.* 2012; Piyasena *et al.* 2003).

Application of HIU to Reduce Microbial Populations

The use of HIU to reduce spoilage microorganisms in foods has been researched since the late 1920's as a means of sterilization (Cameron *et al.* 2009). Recent technology within the last two decades has improved HIU methods, making it possible to achieve higher levels of microbial reduction in food systems, specifically fluid foods, not possible in earlier experiments (Cameron *et al.* 2009). HIU is of particular interest to the dairy industry due to its potential to improve the "tailored" functionality of foods as well as improve shelf life and quality (Knorr *et al.* 2004; Chandrapala *et al.* 2012).

In liquid media, HIU generates acoustic cavitation as a result of the development of localized regions of high and low pressure (Milly *et al.* 2007). Areas of low or negative pressure (expansion or rarefaction of the sound wave) induce formation of

vapor-filled bubbles while areas of high or positive pressure (compression of the sound wave) induce bubble growth (Gera and Doores 2011). Several cycles of compression and rarefaction generate large unstable bubbles, leading to implosion or eventual collapse of the bubble (Gera and Doores 2011). The implosion releases a series of shock waves while generating high-localized temperatures and the formation of free radicals, (Gera and Doores 2011). This series of bubble generation and implosion is referred to as hydrodynamic cavitation, or simply cavitation.

The collapse is thought to directly damage the microbial cell wall, making bacterial cells vulnerable to temperature and free radicals (Cameron *et al.* 2009). The effectiveness of HIU, however, is dependent on microbial strain, medium, cell size, temperature, and power input (Piyasena *et al.* 2003; Cameron *et al.* 2009). Milly *et al.* (2007) used the mechanisms of hydrodynamic cavitation at temperatures below that of conventional thermal processing to determine its lethal effect on common spoilage microorganisms in low- and high-acid foods. Vegetative cells and yeast were observed to be more susceptible to the effects of cavitation at low temperatures while spores proved more resistant (Milly *et al.* 2007).

The combination of temperature and HIU in fluid foods has shown improvements in microbial inactivity particularly with spores (Piyasena *et al*. 2003; Cameron *et al.* 2009). Garcia *et al.* (1989) determined HIU treatments to be more effective at increasing bactericidal effects on *B. subtilis* spores in milk when HIU (20 kHz; 150 W) was coupled with heat treatment at 100°C. Bermúdez-Aguirre *et al.* (2009) reported a 1-2 log decrease in the growth of mesophiles (spore and non-spore-formers) in pasteurized whole milk

during a 16 d storage period at 4°C following HIU treatments (63°C ± 0.5°C for 30 min) at amplitudes of 108 μm and120 μm, respectively. No visible signs of spoilage resulting from enzymatic or microbial origin were observed during the 16 d period. In addition, there were subtle decreases in protein, improved availability of butter fat content, and improvements in color, appearance, and homogenization without producing negative effects on lactose content (Bermúdez-Aguirre *et al.* 2009). However, research conducted by Gera and Doores (2011) has shown protein and lactose concentrations to have a protective effect on bacteria during HIU treatment, specifically in products containing concentrated amounts of protein and lactose.

HIU Effects on Solubility

In addition to microbial work, other research has explored the effects of HIU on the functional properties of milk powders, such as solubility. In a study conducted by Jambrak *et al.* (2008), it was determined that solubility improved in 10% w/w protein suspensions of WPI and whey protein hydrolysate (WPH) when treated with low- and high-intensity ultrasound for 15 and 30 min with a 20 kHz probe. The increased solubility was attributed to changes in protein conformation and structure. This change in protein conformation was likened to the mechanism by which inner hydrophilic areas of amino acids become exposed to water, ultimately allowing for enhanced protein solubility (Jambrak *et al.* 2008). They also assumed cavitation during sonication produces high local temperatures and pressures that lead to the formation of free radicals, corresponding to an increase in electrical conductivity leading to changes in protein solubility. The increase in conductivity translates to higher electrostatic forces, which

enables more protein-water interactions to occur and, therefore, increased protein solubility (Jambrak *et al.* 2008).

Whey protein concentrate (WPC) was also tested in the above study; however, minimal changes were observed in protein structure and solubility, which were attributed to a significantly higher lactose content in WPC (25%) than in WPI (1%) and WPH (1%) (Jambrak *et al*. 2008). They assumed the higher amount of lactose in WPC acted similarly to other disaccharides, which have been shown to exhibit a protective effect during pressurization treatments in earlier experiments (Dumay *et al.* 1994). Chandrapala *et al.* (2011) further showed this observation in WPC when exploring thermal and structural changes in proteins in WPC with low-intensity HIU.

Concerning high-protein milk powders, HIU has not been shown to produce a negative effect on solubility. However, the influence of HIU on solubility showed different results among different powders, specifically those with different lactose concentrations. Powders with lower concentrations in lactose displayed increases in solubility when treated with HIU, while powders with relatively higher amounts of lactose showed very little to no improvement in solubility. No adverse effects in solubility, however, were observed.

TABLES AND FIGURES

Table 2-1 Characteristics of thermophilic *Bacillus* (and related) spp. commonly found in dairy processing

Organism	Growth Range (°C)	Aerobic	pH Range	Spoilage in Dairy Products	Reference
Anoxybacillus flavithermus	30-72	No	6.0-9.0	Lactic acid production and off flavors	Lindsay and Flint 2009; Burgess *et al.* 2010
Geobacillus stearothermophilus	37-75	Yes	6.0-8.0	'Flat-sour' spoilage in canned evaporated milk	Burgess *et al.* 2010
Geobacillus thermoleovorans	35-70	Yes	5.2-8.0	Lactic acid and lipase production	DeFlaun *et al*. 2007 ; Burgess *et al.* 2010
Bacillus licheniformis	15-55	No	5.5-8.5	Production of slimy extracellular substance in cream	Burgess *et al.* 2010
Bacillus subtilis	5-55	Yes	5.5-8.5	Ropiness in pasteurized milk, UHT, and canned products	Burgess *et al.* 2010
Bacillus coagulans	15-61	No	4.0-10.5	Lactic acid production in UHT and canned milk products	Burgess *et al.* 2010
Bacillus pumilus	5-55	Yes	5.5-8.5	Off flavors and spoilage from lipases and proteases	Pirttijărvi *et al.* 1996; Burgess *et al.* 2010
Bacillus sporothermodurans	20-55	Yes	5.9	Contaminant, but no noticeable spoilage	Scheldeman *et al.* 2006; Burgess *et al.* 2010

Table 2-2 Composition of various milk powders commercially available in the United States

Composition (%)[a]	WPI[b]	WPC[c]	WPH[d]	MPC[e]	SMP[f]
Protein	95	60	94	70	34
Fat	1	6	1	2	1.25
Lactose	1	25	1	15-19	50-55
Ash	3	6	5	7-9	7.8-8.4
Moisture	5	3	5.5	5.0	4.0

[a]Composition (%) for WPI, WPC, and WPH from Jambrak *et al.* (2008).
[b]WPI – whey protein isolate (BiPRO®, Davisco Foods International, USA).
[c]WPC – whey protein concentrate ("Meggle" GmbH, Wasserburg, Germany, WPC-60).
[d]WPH – whey protein hydrolysate (BioZate 5®, Davisco Foods International, USA).
[e]MPC – milk protein concentrate (MilkPro™ 70, Grade A, Darigold, Inc., Seattle, WA, USA, MPC-70).
[f]SMP – skim milk powder (Extra Grade, Darigold, Inc., Seattle, WA, USA).

Table 2-3 *Bacillus* (and related) spp. commonly found in milk powder manufacturing[a]

Organism	Growth Range (°C)	Aerobic	Enzymatic Mechanisms Affecting Protein Solubility
Anoxybacillus flavithermus	30-72	No	Lactic acid production; proteolysis
Geobacillus stearothermophilus	37-75	Yes	Acid production; proteolysis
Bacillus licheniformis	15-55	No	Proteolysis; lipolysis
Bacillus subtilis	5-55	Yes	Proteolysis; lipolysis

[a]Buehner *et al*. (2015).

CHAPTER 3

USING THERMOSONICATION TO REDUCE THERMOPHILIC SPORE-FORMERS IN SKIM MILK POWDER

ABSTRACT

This study explored the influence of high intensity ultrasound (HIU) on the inactivation of *Geobacillus stearothermophilus* vegetative cells and spores in reconstituted skim milk powder (RSMP) using response surface methodology (RSM). The 3 variables being investigated were solids concentration (8-55%), temperature (45-75°C), and treatment time (5-30 s). Log_{10} reductions were determined from plate counts and analyzed using statistical analysis system software. Two models were generated to predict microbial inactivation, one for vegetative cells and one for spore reductions. Regression analysis showed treatment time and the influence of time and temperature together to be the primary variables contributing toward the inactivation of vegetative cells. For spores, solids concentration interacting with solids, and time interacting with time were determined to be the primary variables affecting microbial reduction. Optimization of vegetative cell reduction (4.8 log) was found to be at 19.75% total solids (TS), 45°C, and 30 s, while optimization of spore reduction (0.45 log) was found to be at 31.5% TS, 67.5°C, and 17.5 s. Additional experiments were performed using common milk powder processing conditions. Results showed the inactivation of vegetative cells and spores via HIU to be most effective at conditions before (9.2% TS, 75°C, and 10 s) and after (50% TS, 60°C, and 10 s) the evaporator, respectively, during milk powder processing and may, therefore, produce an additive effect in microbial reduction when the

2 treatments are combined, resulting in a 5.8 log reduction for vegetative cells and 0.51 log reduction for spores. The experimental reductions for both vegetative cells and spores fell within the predicted range of the model, confirming the accuracy of the model for this particular organism.

INTRODUCTION

The formation of biofilms is a constant concern for manufacturers of the milk powder industry due to their microbiological makeup of thermophilic spore-formers that survive pasteurization conditions (Simões *et al.* 2009). The ability of these organisms to form spores enables them to survive high processing temperatures and extreme pH conditions introduced by cleaning in place (CIP) and cleaning out of place (COP) (Scott *et al.* 2007; Burgess *et al.* 2009; Hill and Smythe 2012). As a result, surviving spores, specifically *Bacillus* (and related) spp., residing in biofilms contaminate product as it flows through the processing line. These spores can then become vegetative cells in the final product, leading to an accelerated decrease in quality over time (Burgess *et al.* 2010; Lücking *et al.* 2013; Watterson *et al.* 2014). High intensity ultrasound (HIU) has been explored as a means to reduce microbial populations in fluid milks and non-dairy beverages, but little work has been done in concentrated milk destined for powders, specifically skim milk powder (SMP) (Piyasena *et al.* 2003; Chandrapala *et al.* 2012). Previous research involving sonication of fluid milk has been successful at microbial reduction and has introduced the idea that many different factors play a role in the destruction of microorganisms. Factors considered to be contributing toward microbial reduction include bacterial strain (gram positive vs. gram negative), bacterial growth

phase, temperature, time, medium, solids concentration, and acoustic power (Piyasena *et al.* 2003; Milly *et al.* 2007; Cameron *et al.* 2009).

Herceg *et al.* (2012) investigated the influence of HIU on the reduction of *Staphylococcus aureus* and *Escherichia coli* in fluid milk containing 4% milk fat. Data analysis was performed using response surface methodology (RSM) in order to study the effect of 3 variables: HIU time (6, 9, and 12 min), temperature (20, 40, and 60°C), and amplitude (60, 90, and 120 µm). Ultrasound was observed to have a greater effect on *E. coli* (1.34-3.07 log reduction) than *S. aureus* (0.22-1.49 log reduction). They further observed that HIU applied at lower temperatures and lower amplitudes for less time was less effective in reducing *S. aureus* and *E. coli* counts than when applied at higher temperatures and amplitude for longer amounts of time. Overall, the results showed amplitude, treatment time, and treatment temperature to be the parameters significantly affecting the inactivation of both *S. aureus* and *E. coli* in fluid milk.

Compared to Herceg *et al.* (2012), Cameron *et al.* (2009) performed HIU treatments on *E. coli, Pseudomonas fluorescens,* and *Listeria monocytogenes* in fluid milk held at 24-26°C for 2.5-10 min at an amplitude of 124 µm. Observed log reductions ranged from 3.26-5.64, respectively, resulting in a 99-100% elimination for all organisms.

Previous work by Evelyn and Silva (2015) cited log reductions of less than 0.5 when exploring the microbial destruction of *Bacillus cereus* spores in reconstituted SMP when HIU was applied for 1.5 min at 70°C using a frequency of 24 kHz at an amplitude of 210 µm. In addition to SMP, Evelyn and Silva (2015) investigated the effects of

applying HIU (1.5 min, 70°C) to beef slurry, cheese slurry, and rice porridge inoculated with *B. cereus* spores. The observed log reductions in these experiments were greater than 3.2, suggesting that foods with higher solids concentration influence the effectiveness of HIU at higher temperatures. However, no explanation was offered as to why this effect was observed.

Recently, Ferrario *et al*. (2015) observed minimal to zero inactivation of *Alicyclobacillus acidoterrestris* spores in apple juice when treated with HIU for 30 min at 30°C and 44°C. In contrast, a 2.5 log reduction was shown in *Saccharomyces cerevisiae* when exposed to the same conditions, confirming a difference in microbes to be an important factor in the effectiveness of HIU. For both species, greater microbial inactivation was observed when combined with pulsed light, suggesting microbial inactivation of spores can be heightened when HIU is coupled with another inactivation technology (high pressure, pulsed light) traditionally used alone.

Other research has shown that HIU can promote increased microbial inactivation of mesophilic vegetative cells and spores in dairy and other fluid foods, but little research has been conducted investigating its application in concentrated milk with thermophilic bacteria. Since thermophilic bacteria form biofilms, reduction in these microbes may reduce biofilm formation as well as the prevalence of microbes in the final product following processing conditions. The objective of this study is to explore the influence and significance of 3 HIU variables (solids concentration, temperature, and treatment time) on the inactivation of thermophilic spore-formers in reconstituted SMP (RSMP) in order to develop a model capable of predicting levels of microbial inactivation under

specified conditions for each parameter. D values at 73°C were also calculated to compare the effectiveness of HIU to that of thermal processing without HIU.

MATERIALS AND METHODS

Experimental Design

The Box-Behnken response surface methodology (RSM) design from statistical analysis software (SAS 9.4) was used to create an experimental design consisting of 15 treatments (Table 3-1), taking into account 3 variables (time, temperature, and solids content). The parameters used for time, temperature, and solids content were generated based on the conditions commonly used in industrial milk powder processing facilities, which is depicted in Figure 3-1. Each HIU treatment was performed in duplicate for both vegetative cells and spores, and the data were analyzed using RSM in order to determine the significance of the variables contributing to microbial destruction.

The same number of treatments using the same conditions and parameters were then performed without HIU in order to compare bactericidal effects on vegetative cells and spores with and without HIU. Each treatment was performed in duplicate for both vegetative cells and spores, and the data were analyzed using a two-tail t-test.

Verification runs were performed in order to confirm the accuracy and precision of the predictive models generated by RSM for HIU treatments. Additional verification runs were performed to further test the accuracy of the models using the milk processing conditions shown in Figure 3-1. In addition to RSMP, reconstituted milk protein concentrate containing 70% protein (RMPC-70, MilkPro^{TM} 70, Grade A, Darigold, In.,

Seattle, WA, USA) to compare differences in log reductions between the two milk powders.

The microorganisms (*Bacillus subtilis* and *Geobacillus stearothermophilus*) selected for this study were chosen based on their ability to grow at high temperatures and form spores. *Bacillus subtilis* vegetative cells (not spores) were only used in HIU experiments while both *G. stearothermophilus* vegetative cells and spores were used for both experiments involving HIU and no HIU.

Growth and Preparation of *Bacillus subtilis*

Twenty-five milliliters of tryptic soy broth (TSB) were inoculated with 1 ml of *B. subtilis* stock culture (Western Dairy Center, Utah State University, Logan, UT, USA) in a sterile 250-ml Erlenmeyer flask covered with sterile foil and grown aerobically (shaker, 175 rpm) at room temperature (23°C) for 24 h. The $OD_{600\ nm}$ after 24 h was determined to be approximately 0.504 at a 1:4 dilution in TSB. Standard plate counts indicated a cell density of 10^8 cfu/ml.

A subculture was made by inoculating 25 ml TSB with 1 ml of freshly grown *B. subtilis* culture in a sterile 250-ml Erlenmeyer flask covered with sterile foil. The subculture was grown aerobically (shaker, 175 rpm) at room temperature (23°C) for 24 h, and the $OD_{600\ nm}$ measured to indicate 10^8 cfu/ml.

Freezer stocks were made from subcultures by inoculating 20 ml of 30% w/v glycerol TSB with 1 ml of subculture and stored in 2.0-ml cryo-vials at -20°C. Cultures for experiments were grown by inoculating 25 ml TSB with 1 ml of freezer stock and growing at room temperature (23°C) for 24 h in a shaker at 175 rpm.

Growth and Preparation of *Geobacillus stearothermophilus*

G. stearothermophilus spores were germinated using 0.1 ml of stock solution obtained from NAMSA *G. stearothermophilus* 10.0-ml spore suspension (2.4×10^6/0.1 ml, biological indicator for: STEAM, LOT: S90601) to inoculate 10 ml of sterile water. The sample was incubated for 10 min in an 80°C water bath. Twenty-five milliliters of TSB was inoculated with 1 ml of germinated bacteria in a sterile 250-ml Erlenmeyer flask covered with sterile foil and incubated at 55°C for 24 h in a shaker at 100 rpm. The OD_{600nm} was measured to be approximately 0.566 after 24 h (10^7 cfu/ml).

A subculture was grown by inoculating 25 ml of TSB with 0.1 ml of culture grown from germinated cells in a sterile 250-ml Erlenmeyer flask covered with sterile foil. Cells were grown aerobically at 55°C in a shaker at 100 rpm for 16-18 h (Kotzekidou 2014).

Freezer stocks were made by inoculating 20 ml of 30% w/v glycerol TSB with 2 ml of subculture and stored in 2.0-ml cyro-vials at -20°C. Cultures for experiments were grown by inoculating 25 ml TSB with 0.1 ml of freezer stock and incubated at 55°C in a shaker at 100 rpm for 16-18 h. For spore samples, *G. stearothermophilus* spores were obtained directly from NAMSA *G. stearothermophilus* 10-ml spore suspension indicated above, and no freezer stocks were made.

Preparation of SMP and MPC-70

Skim milk powder (Extra Grade Spray Process, Darigold, Inc., Seattle, WA, USA) was reconstituted to 8, 31.5, and 55% ± 0.5% w/v total solids (TS) (Table 3-1) as determined by parameters obtained from commercial milk powder processing facilities

(Figure 3-1). The powder was weighed and mixed with 60°C sterile water for 3 min using a high-speed blender, followed by a solids test performed using the oven drying method. The oven drying method was done by dispensing 3.5 ml of RSMP into a pre-weighed aluminum pan and leaving to dry in an oven at 80°C for 12 h. The pan with dried sample was then weighed again, and TS was determined using the equation: $TS = 100 \times (DP - P)/(WP - P)$, where TS is the solids concentration (%), DP is the weight (g) of the pan with sample after drying, P is the weight (g) of the aluminum pan by itself, and WP is the weight (g) of the pan with sample before drying.

The RSMP was then heat treated to 80°C for 20 min to destroy any existing bacteria that might cause potential contamination during experiments. The 8 and 31.5% TS RSMP was stored at 4°C for up to 1 week before a new batch was made. The 55% TS RSMP was remade prior to each experiment due to solidification at temperatures below 30°C. During treatments, RSMP was held at 60°C in 15-ml sterile tubes (8% and 31.5% TS) and 50-ml sterile tubes (55% TS) to ensure fluidity and easy pouring for experiments before use.

For verification experiments, SMP was reconstituted to 9.2%, 12.5%, and 50% ± 0.5% w/v TS, and MPC-70 was reconstituted to 9.2%, 12.5%, and 30% ± 0.5% w/v TS (Figure 3-1). Samples were reconstituted, heated treated, and tested for TS using the same methods described previously.

Experimental conditions

Treatments with HIU were performed in batch using a 10-ml double-walled glass cylinder (diameter: 2.8 cm outside, 1.7 cm inside; height: 6.3 cm outside, 5.3 cm inside)

containing 6 ml of sample. A water bath was used to control temperature fluctuations and to bring the RSMP and RMPC-70 up to the appropriate temperatures prior to inoculation. Experiments were performed using a 20 kHz Misonix Sonicator® 3000 (QSonica, LLC, Newtown, CT) coupled with a 0.32 cm (diameter) stainless steel tapered sonicator microtip (ID: 4418, QSonica, LLC, Newtown, CT) with an amplitude of 240 μm at a dial setting of 10. A complete layout of the HIU apparatus is shown in Figure 3-2. All materials were rinsed with 10% w/v bleach solution, followed by sterile water before and after each treatment to avoid cross-contamination.

Treatments not involving HIU (heat only) were done in a water bath using 15-ml sterile tubes containing 6 ml of sample. The RSMP was brought to temperature in the tubes, followed by inoculation of the microorganism.

Inoculation

For vegetative cells, 6 ml of reconstituted sample was brought to the specified treatment temperature in either the 10-ml glass cylinder (HIU treatments) or 15-ml sterile tube (non-HIU treatments) using the water bath (Table 3-1). Once brought to the appropriate temperature, the sample was inoculated with 1 ml (10^8 cfu/ml) of either *B. subtilis* (only used for HIU treatments) or *G. stearothermophilus* culture. After inoculation, 1 ml of sample was collected and placed on ice until ready to plate. The remaining sample was then treated with HIU or thermal processing. After treatment, the sample was poured into a 15-ml sterile tube and kept on ice until ready to plate. This entire procedure was performed each time for each experiment and its duplicate.

Dilutions were made in sterile water and plated at 0.1 ml on tryptic soy agar (TSA). For *B. subtilis*, TSA plates were incubated for 24 h at 37°C to determine counts and log reductions. For *G. stearothermophilus*, plates were incubated for 24-48 h in a humidified incubator at 55°C.

For spores, 6 ml of reconstituted sample was brought to the specified treatment temperature in either the 10-ml glass cylinder (HIU treatments) or 15-ml sterile tube (non-HIU treatments) using the water bath (Table 3-1). Once brought to the appropriate temperature, the sample was inoculated with 0.1 ml of *G. stearothermophilus* culture (10^6 spore/ml). Sample collections were performed in the same manner as sample collections done with vegetative cells.

Dilutions were made in sterile water and germinated at 80°C for 10 min. Germinated samples were plated at 0.1 ml on TSA and incubated for 24-48 h in a humidified incubator at 55°C to determine counts and log reductions.

Statistical Analysis and Calculations

Response surface methodology (RSM) was performed using SAS 9.4. For determination of the effect of HIU on microbial activity, microbial cell counts were entered into the program as $\log_{10}$ reductions, followed by analysis via linear regression (Bezerra *et al.* 2008; Ganesan *et al.* 2015). The data was analyzed using analysis of variation (ANOVA) with $P \leq 0.05$ to determine the significance of parameters (time, temperature, and solids content) affecting microbial reduction during HIU treatments (Bezerra *et al.* 2008; Herceg *et al.* 2012; Ganesan *et al.* 2015). Analysis comparing the

effect of HIU with that of thermal processing was done using a two-tail t-test. Significance was declared at $P \leq 0.05$.

D-values (the amount of time required to destroy 90% of the initial microbial population) were determined for *G. stearothermophilus* vegetative cells at 73°C with and without HIU in TSB. D-values (termed D_{73}) were determined from the negative reciprocal of the slope of the regression line ($\log_{10}$ cfu/ml versus treatment time) and calculated using the equation $D = t/(\log N_0 - \log N_f)$, where D = decimal reduction time, t = duration of treatment, N_0 = initial bacterial population, and N_f = surviving bacterial population after treatment (Mazzola *et al.* 2003).

Acoustic power delivered to the samples during HIU was calculated using $P = M \cdot Cp \cdot (dT/dt)$ where P is the acoustic power (W), M is the mass of the HIU sample (g), Cp is the specific heat capacity of medium at constant pressure (J/g/°C), and dT/dt is the increase in temperature (°C/s) during HIU (Jambrak *et al.* 2011; Ganesan *et al.* 2015). Increase in temperature during HIU was measured using a thermocouple (Traceable® Total-Range Thermometer Model: 23609-232, VWR International, LLC, Radnor, PA 19087) and plotted as a linear graph to determine precision among replicates. Specific heat capacity was determined using a differential scanning calorimeter (DSC, Auto Q20 2910, TA Instruments, USA; uses Refrigerated Cooling System 90 and Nitrogen) for 8%, 31.5%, and 55% TS RSMP in duplicate. A baseline was run from 25-80°C with a 5 min holding period at 25°C and 80°C and a ramp rate of 5°C/min. Sapphire was then run using the same conditions using an empty Tzero hermetic aluminum pan as a reference. After running Sapphire, 5-15 mg of RSMP sample (8%, 31.5%, 55% TS) was placed in a

Tzero hermetic aluminum pan for DSC use. The sample was heated to 80°C with a ramp rate of 5°C/min to evaluate the specific heat capacity at 45°C, 60°C, and 75°C. Each sample was run in duplicate. The average specific heat capacities determined from the DSC as well as the acoustic power calculations are located in Appendix E, Table E-1 and Figures E-1 to E-3.

RESULTS AND DISCUSSION

Bacillus subtilis Vegetative Cells

Table 3-2 shows the log reductions of *B. subtilis* when HIU was applied (refer to Appendix A, Table A-1 for raw data). The greatest log reduction (5.21) was observed in 8% RSMP held at 75°C while sonicated for 17.5 s. In comparison, the lowest log reduction was observed as 0.088 when 31.5% RSMP was sonicated for 5 s at 45°C. However, no further statistics determining the significance of solids content, time, or temperature on microbial reduction were performed using this particular organism due to its rapid inactivation at temperatures above 65°C, making it difficult to determine accurate log reductions and generate an accurate predictive model for treatment temperatures at 75°C. Therefore, *G. stearothermophilus* vegetative cells were used since they are capable of surviving temperatures near 110°C (Lücking *et al.* 2013).

Geobacillus stearothermophilus Vegetative Cells

In general, log reductions of *G. stearothermophilus* vegetative cells with HIU were significantly greater than log reductions from thermal processing treatments as shown in Table 3-3 (refer to Appendix A, Tables A-2 and A-3 for raw data and Appendix D for t-test statistical analysis tables). Log reductions with HIU ranged from 0.77 ± 0.29

to 5.0 ± 0.38 while heat treatments without HIU yielded less than 1.5 log reductions. The D_{73} value for *G. stearothermophilus* vegetative cells treated without HIU was 2.1 min while the D_{73} value for cells treated with HIU was 5.3 s as shown in Figure 3-3 (Appendix A, Tables A-4 and A-5).

Just from looking at Table 3-3, higher log reductions were observed in samples treated with HIU for longer amounts of time, regardless of solids content or temperature. For example, RSMP at 31.5% TS treated with HIU for 30 s at 45°C yielded more than 3 times the microbial destruction seen in RSMP with the same solids content treated with HIU for 5 s at 45°C, which was less than 31.5% TS RSMP treated with HIU for 17.5 s at a higher temperature. This trend is similar among samples with the same solids content throughout the entire table, implying that higher log reductions are achieved after longer treatment times.

Another interesting aspect shown in the data is the influence of solids content. RSMP samples with 8% TS treated with HIU for 17.5 s (45°C) and 5 s (60°C) resulted in lower log reductions than 55% TS RSMP treated under similar conditions. However, 8% TS RSMP treated with HIU at 60°C for 30 s and 75°C for 17.5 s yielded higher log reductions than 55% TS RSMP treated under the same conditions. Higher solids concentration may, therefore, contribute to a greater bactericidal effect at lower temperatures coupled with lower treatment times since it results in a higher amount of energy, or acoustic power (refer to Table 3-3), being transferred into the media (refer to Appendix E for acoustic power calculations and graphs). The increase in acoustic power translates to greater acoustic cavitation and more direct damage to the cell to result in

increased numbers in cell death. However, greater acoustic power generated within the system did not always directly correlate with a higher log reduction. As such, log reductions induced by HIU must be a result of a combination of many factors as described by Chandrapala *et al.* (2011).

In comparison to the data obtained in this experiment, log reductions from HIU were greater than those observed by Herceg *et al.* (2012) when HIU was applied to *S. aureus* and *E. coli* in fluid milk using 20 kHz power ultrasound for 6-12 min at amplitudes of 60-120 μm. However, microbial inactivation was similar to the results Cameron *et al.* (2009) observed for *E. coli, P. fluorescens,* and *L. monocytogenes* in fluid milk using 20 kHz power ultrasound for 6-10 min at an amplitude of 124 μm.

According to the RSM model, the log reduction of *G. stearothermophilus* vegetative cells achieved by HIU can be described with the polynomial equation:

$$Y1 = 1.760621 + 0.063776*S + 0.109613*T + 0.306508*TT – 0.131153*S*T – 0.20836*S*TT – 0.271353*T*TT – 0.176426*TT^2$$

where S is solids concentration (%), T is temperature (°C), and TT is treatment time (s). The coefficient of determination (R^2) for both the master and predictive models were 0.92 and 0.82, respectively. Analysis of Variance (ANOVA) (Table 3-4) determined solids concentration, temperature, and treatment time to be significant to the model as well as the interaction of solids-temperature, solids-treatment time, temperature-treatment time, and treatment time-treatment time (refer to Appendix B for complete SAS report). Numerical optimization results (Appendix B) based on the conditions defined in the experimental parameters predicted the largest log reduction (4.8) to occur when HIU is

applied to 19.75% RSMP at 45°C for 30 s. A maximum optimum, however, was not observed in this model using the defined experimental parameters (Figure 3-4), which fall outside of common conditions utilized in milk powder processing facilities. Therefore, this may be an option to explore in future work.

The response surface plots shown in Figure 3-4 describe the predicted log reductions of *G. stearothermophilus* vegetative cells in RSMP treated with HIU. Each of the plots (a-c) indicates a fairly linear association between each of the parameters (temperature, solids, and time) and log reduction. In Figure 3-4a, there is an increase in log reduction at 45°C and 55% TS; likewise, a similar increase is seen at 8% TS and 75°C. At high solids concentration and low temperature, the acoustic power is 39.10 W (Table 3-3), which is higher than any other experimental conditions tested. This may be due to the HIU power supply having to work harder to produce the same level of cavitation within a more viscous sample at 55% TS as compared to a less viscous sample at 8 and 31.5% TS at 45°C. The increase in cavitation may, therefore, lead to higher frequencies of bubble formation and larger bubble sizes, which would cause a greater impact when the bubble collapses. This would translate to a higher degree of damage to surrounding cells, resulting in increased microbial destruction. The acoustic power generated at 8% TS and 75°C (Table 3-3) was approximately half the acoustic power generated at 55% TS and 45°C, however, Figure 3-4a shows a higher log reduction at the lower solids concentration and higher temperature. Even though power delivered to the system is less at this point, the sharp increase in temperature must be more detrimental to the vegetative cells since it exceeds their typical growth range.

In Figure 3-4b, there is an observed increase in log reduction at a low solids concentration over an increase in treatment time when temperature is held constant. This effect supports the equation generated for the predictive model where treatment time is the most significant predictor. A longer treatment time allows for longer exposure to elevated temperatures and cavitation produced as a result of HIU, resulting in a greater bactericidal effect. The second most significant predictor in the model is the interaction between temperature and time, which can be seen in Figure 3-4c. The log reductions observed over a change of temperature and treatment time when solids concentration is held constant is different than the effect of temperature and time as separate predictors in Figures 3-4a and 3-4b. In Figure 3-4c, log reductions are highest at low temperatures and longer treatment times. However, there is an observed decline in log reduction at higher temperatures and longer treatment times, which is different when compared to Figures 3-4a and 3-4b where the highest log reductions are observed at high temperatures and long treatment times. The interaction between temperature and treatment time shows a fairly linear relationship with that of log reduction, but the relationship is not as linear as when the predictors are independent of each other.

The significance of treatment time and the effects of temperature and time together is similar to the model generated by Herceg *et al.* (2012). The heavy influence of time and temperature is due to the instability of microbes as they approach and begin to exceed maximum growth range, which is 75°C for *G. stearothermophilus* (Burgess *et al.* 2010). Acoustic cavitation and power generated from HIU increases the temperature of the media. Prolonged exposure to these elevated temperatures near or above maximum

growth range can be expected to cause damage to the cell membrane, leading to cell death and overall decrease in microbial population within the sample (Cameron *et al.* 2009).

Further runs were performed to verify the predictive model using parameters corresponding to high, medium, and low points of the model shown in Figure 3-4. Observed results fell within the predictive ranges for all experiments (Table 3-5), validating the model and the influence of time and temperature on the log reduction of *G. stearothermophilus* vegetative cells when HIU is applied (refer to Appendix A, Table A- for raw data).

Additional verification runs were performed using parameters for solids content, temperature, and time outlined in Figure 3-1 using both RSMP and RMPC-70. Table 3-6 compares the actual log reductions observed in RSMP and RMPC-70 with that of the predicted log reduction from the model (refer to Appendix A, Tables A-6 and A-7 for raw data). Log reductions observed in RSMP fell within the predicted ranges while results observed in RMPC-70 were both within and greater than the predicted ranges. Higher microbial inactivation (those outside the range) in RMPC-70 occurred at the highest and lowest solids content at 60°C and 75°C.

The increased log reductions observed in RMPC-70 as compared to RSMP may be due to lower lactose levels in MPC-70. This assumption is supported by Gera and Doores' (2011) research exploring the function of milk components towards bacterial inactivation during HIU. Their results showed the interaction of milk components, such as lactose, may aid in protecting the cell membrane by stabilizing the protein structure of

bacteria. When lactose or other disaccharides are present in the environment, the cell cytoplasm of the bacteria experiences an uptake of the surrounding sugars, thereby increasing sugar concentrations in the cytoplasm and stabilizing the bacteria's secondary protein structure (Kilimann *et al.* 2006; Gera and Doores 2011). Compared to MPC-70, SMP contains a higher concentration of lactose, which may provide the protective effect described by Gera and Doores (2011).

Log reductions in RSMP observed for the verification runs performed using common milk powder processing conditions suggest the most beneficial location for placement of HIU treatment would be before the evaporator in the processing line (Table 3-6). More research is needed to determine optimal conditions for maximum cell destruction for multiple microbial species on a pilot-scale production line.

Effect of Thermosonication on *Geobacillus stearothermophilus* Spores

Log reductions observed for *G. stearothermophilus* spores treated with HIU were generally greater than those observed for spores treated without HIU (Table 3-7), with approximately one third of HIU treatments being significantly greater than log reductions observed for treatments utilizing just heat (refer to Appendix A, Tables A-8 and A-9 for raw data). Compared to vegetative cells, the log reductions observed for spores treated with HIU were less, ranging from 0.06 ± 0.04 to 0.44 ± 0.13, similar to results obtained by Evelyn and Silva (2015) for *B. cereus* spores. However, log reductions observed in this study were greater than those observed for *A. acidoterrestris* spores but less than reductions seen in *S. cerevisiae* spores in the research conducted by Ferrario *et al.* (2015). The decrease in microbial inactivation in spores compared to vegetative cells was

expected due to increased resistance of spores in adverse environmental conditions (Burgess *et al*. 2010; Hill 2004; Kotzekidou 2014).

Referring to Table 3-7, an increase in treatment time did not directly correlate to higher log reductions in spores unlike vegetative cells. Instead, log reductions with HIU seemed to be more heavily influenced by solids than time. Specifically, HIU treatments on 55% TS RSMP at 60°C for 30 s resulted in log reductions twice the level observed in 8% TS RSMP treated under the same conditions. Conversely, HIU on 55% TS RSMP at 75°C for 17.5 s yielded a lower reduction than in the 8% TS RSMP treated with the same temperature and time. In both cases, the higher log reductions corresponded to higher levels of acoustic power and differences in solids concentration. However, this relationship between log reduction and acoustic power does not follow through with all of the experiments concerning spores, but solids concentration does prove to be a significant predictor when analyzed with RSM. Overall, log reductions were fairly similar among samples with 8% and 55% TS and increased in samples with 31.5% TS. The largest reductions were observed in 31.5% TS at the high and mid temperatures with shorter treatment times (Table 3-7) as opposed to longer treatment times.

As mentioned previously, D-values were not determined since the destruction of 90% of the initial spore population at 73°C with HIU would require a time frame outside feasible processing conditions. However, it would require less time than thermal processing without HIU, which has been determined in previous studies to be indefinite at 73°C and 4-5 min at 121°C (Kotzekidou 2014).

According to the RSM model, the log reduction of *G. stearothermophilus* spores achieved by HIU can be described with the polynomial equation:

$$Y1 = 0.658961 + 0.0015*S + 0.065579*T - 0.023711*TT - 0.160592*S^2 - 0.08469*T^2 - 0.065341*T*TT - 0.159631*TT^2$$

where S is solids concentration (%), T is temperature (°C), and TT is treatment time (sec). The R^2 for both the master and predictive models were 0.82 and 0.81, respectively. Analysis of Variance (ANOVA) (Table 3-8) determined solids concentration, temperature, and treatment time to be significant to the model as well as the interaction of solids-solids, temperature-temperature, temperature-treatment time, and treatment time-treatment time (refer to Appendix C for complete SAS report). The maximum log reduction predicted by numerical optimization of the model was determined to be 0.45 when SMP is reconstituted to 31.5% TS and sonicated at 67.5°C for 17.5 s (Appendix C).

The fit of the model was not as tight as the model for *G. stearothermophilus* vegetative cells; however, it was still able to provide specificity for predictive log reductions when time, temperature, and solids content are defined. The response surface plots in Figure 3-5 illustrated maximum optimizations for the predictive model, unlike the planar projections exhibited in the model for vegetative cells. The optimums observed in Figure 3-5 and interaction terms in the model equation – depicted as squared terms – (temperature-temperature, solids-solids, treatment time-treatment time) show this not to be representative of a linear regression model. Instead, the fit of the model is more similar to a quadratic regression, or parabola. As such, an increase or decrease in any of

the predictors does not necessarily translate to a corresponding change in the log reduction of spores.

In Figure 3-5a, lower log reductions are shown at high and low solids as well as at low temperatures and high solids. Under these conditions, cavitation generated by HIU may not be as efficient as under conditions of mid temperatures and mid solids concentration. Increasing the solids concentration while decreasing the temperature (and vice versa) may lead to smaller bubble formation or less frequent collapsing of bubbles, which would lessen the degree of damage to cells within the sample. Figure 3-5b also displays a similar trend seen in Figure 3-5a, but for treatment time and solids concentration. Unlike the *G. stearothermophilus* vegetative cells, an increase in time does not correspond to an increase in log reduction whether at low or high solids concentrations. Instead, the largest log reductions are observed at median treatment times and solids concentrations. In Figure 3-5c, increases in temperature with a slight increase in treatment time result in a maximum log reduction of spores; however, the level of reduction plateaus shortly before the highest experimental temperature of 75°C, which is fairly similar to the shape of the graph in Figure 3-5a. The plateaued effect observed in both Figures 3-5a and 3-5c show that temperature as a single predictor – after a certain point – does not heavily influence the degree of microbial inactivation. At this point, solids concentration and treatment time prove more influential in dictating which way the plateau falls along the plain of the plot, which supports the level of significance of these two predictors and their degree of interaction within the model. According to the ANOVA for the predictive model, the two most significant predictors are the interaction

of solids and solids, followed by the effects of treatment time and treatment time – described as S^2 and TT^2 in the model equation.

The influence of solids and time support the conclusions reached in previous studies concerning the effects of higher solids concentrations and increased time as being influential factors in increasing microbial inactivation via HIU (Cameron *et al.* 2009; Herceg *et al.* 2012; Evelyn and Silva 2015). The conclusions from this study, however, differ from the experimental data collected for vegetative cells in that the inactivation of spores is not as dependent upon HIU temperature as vegetative cells. Instead, spore destruction is more heavily dependent on solids concentration, which is not a variable that was shown to substantially affect vegetative cell inactivation. In the case of both vegetative cells and spores, however, the length of exposure to HIU has proven to be a common significant factor.

Experiments were performed to verify the RSM predictive model at high and low points illustrated in the response surface plots (Figure 3-5). All observed log reductions were within the predictive range (Table 3-5), validating the model under these conditions and parameters (refer to Appendix A, Table A-6 for raw data). Verification experiments were then run using common milk powder processing conditions shown in Figure 3-1 for both RSMP and RMPC-70. For all experiments, higher log reductions were observed in RMPC-70 than in RSMP (Table 3-6), similar to the experiments performed with vegetative cells, which may be attributed to the protective effect induced by disaccharides described by Gera and Doores (2011). The overall results from verification experiments suggest the placement of HIU treatment directly after the evaporator within the powder

processing unit would be most effective in achieving the highest log reduction of spores (Figure 3-1).

CONCLUSION

Thermosonication proved to be more effective than heat treatment alone in reducing the microbial population of *G. stearothermophilus*. For vegetative cells, D_{73} values improved when HIU was applied as compared to D_{73} values observed for heat treatment without HIU. Based on the observed log reductions, predictive models were generated for *G. stearothermophilus* vegetative cells and spores. These models were validated and used to determine effective locations for implementing HIU treatments during milk powder processing. Treatments applied directly before and after the evaporator would theoretically produce an additive effect that would result in higher levels of microbial inactivation for vegetative cells and spores, respectively. Further research is necessary, however, to determine optimum conditions on a pilot-scale model with indigenous thermophilic spore-formers found naturally in milk as it moves through the processing equipment.

TABLES AND FIGURES

Table 3-1 Box-Behnken statistical experimental design

RSMP Total Solids (%)	Treatment Temperature[a] (°C)	Treatment Time[b] (s)
8	45	17.5
8	60	5.0
8	60	30.0
8	75	17.5
31.5	45	5.0
31.5	45	30.0
31.5	60	17.5
31.5	60	17.5
31.5	60	17.5
31.5	75	5.0
31.5	75	30.0
55	45	17.5
55	60	5.0
55	60	30.0
55	75	17.5

[a]Temperature of RSMP when treatment (HIU or heat without HIU) is applied.
[b]Length of time treatment is applied for.

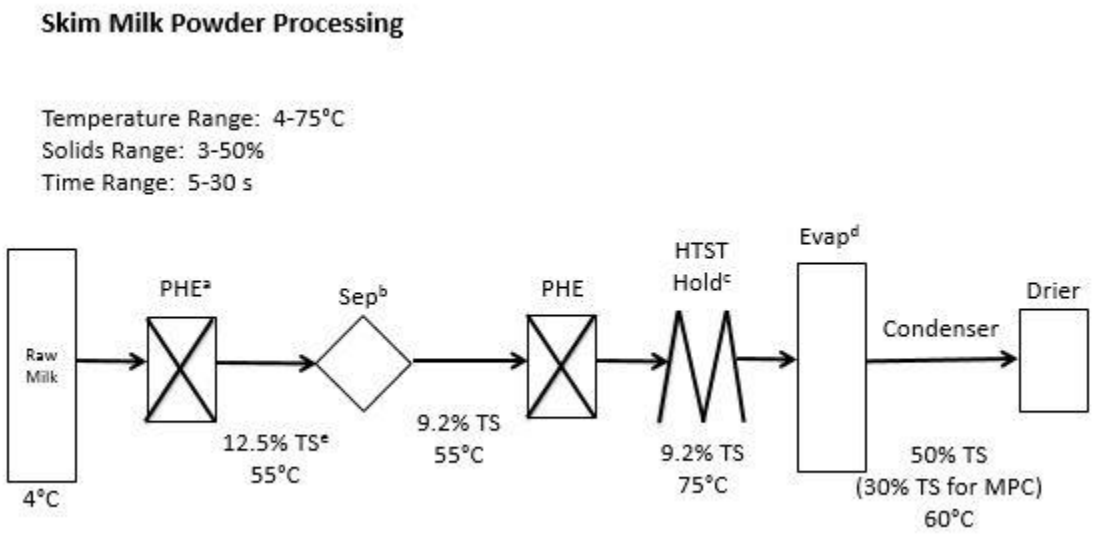


Figure 3-1 Diagram of SMP processing unit with specified temperature, time, and solids content parameters through each stage of processing. [a]PHE – plate heat exchanger, [b]Sep – fat separator, [c]HTST Hold– high temperature short time (where milk is held for specified amount of time until every particle is heated to a certain temperature), [d]Evap – evaporator, [e]TS – total solids.

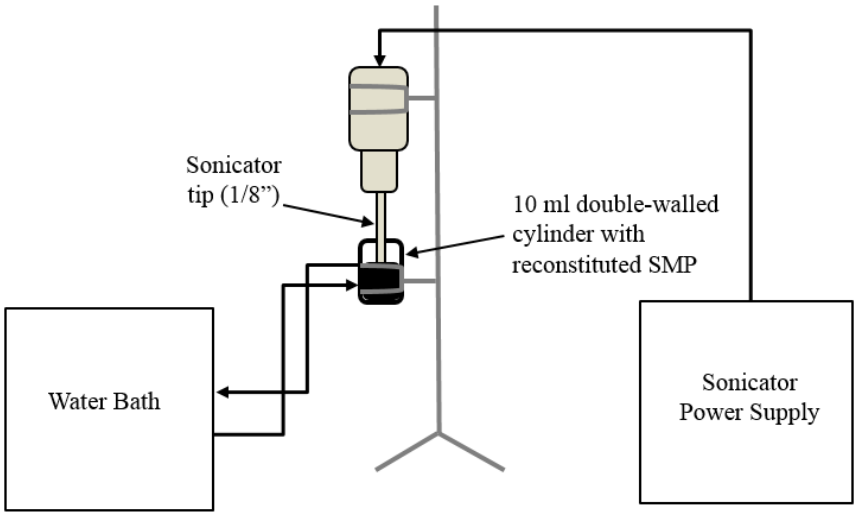


Figure 3-2 Apparatus for HIU treatments in a batch system.

Table 3-2 Average log reductions of *B. subtilis* vegetative cells in RSMP treated with HIU

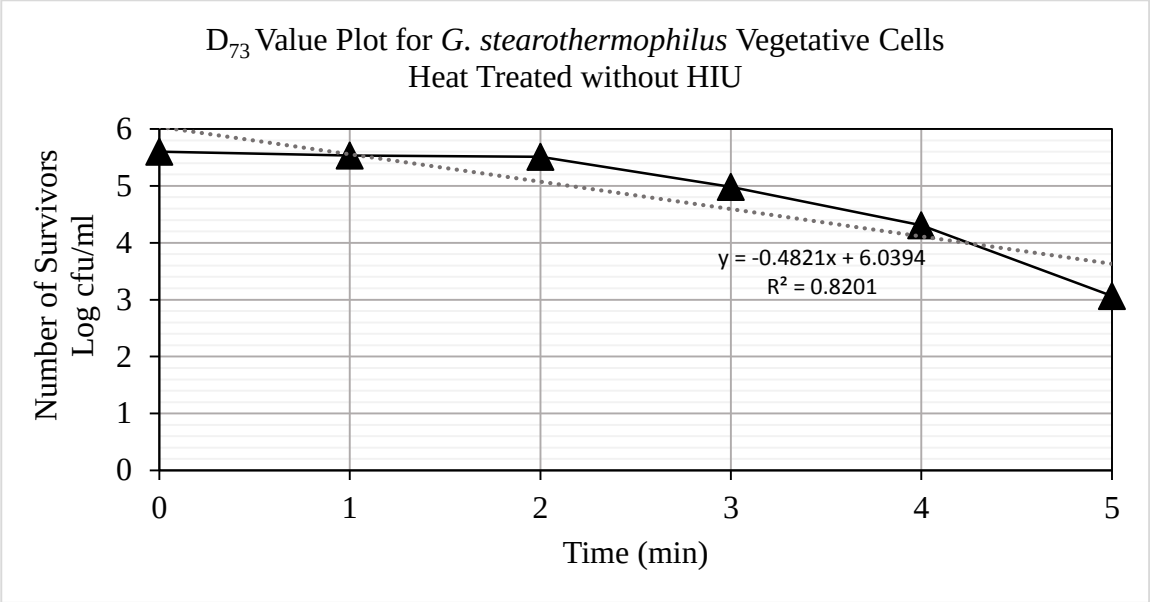
RSMP Total Solids (%)	Treatment Temperature (°C)	Treatment Time (s)	Log Reduction
8	45	17.5	3.8 ± 0.09
8	60	5	4.3 ± 0.55
8	60	30	4.7 ± 0.44
8	75	17.5	5.2 ± 0.55
31.5	45	5	0.088 ± 0.01
31.5	45	30	4.5 ± 0.05
31.5	60	17.5	3.9 ± 0.4[a]
31.5	75	5	3.8 ± 0.07
31.5	75	30	4.4 ± 0.12
55	45	17.5	4.1 ± 0.03
55	60	5	4.7 ± 0.39
55	60	30	4.4 ± 0.39
55	75	17.5	4.3 ± 0.21

[a]Midpoint of experimental design – performed in duplicate 3 times.

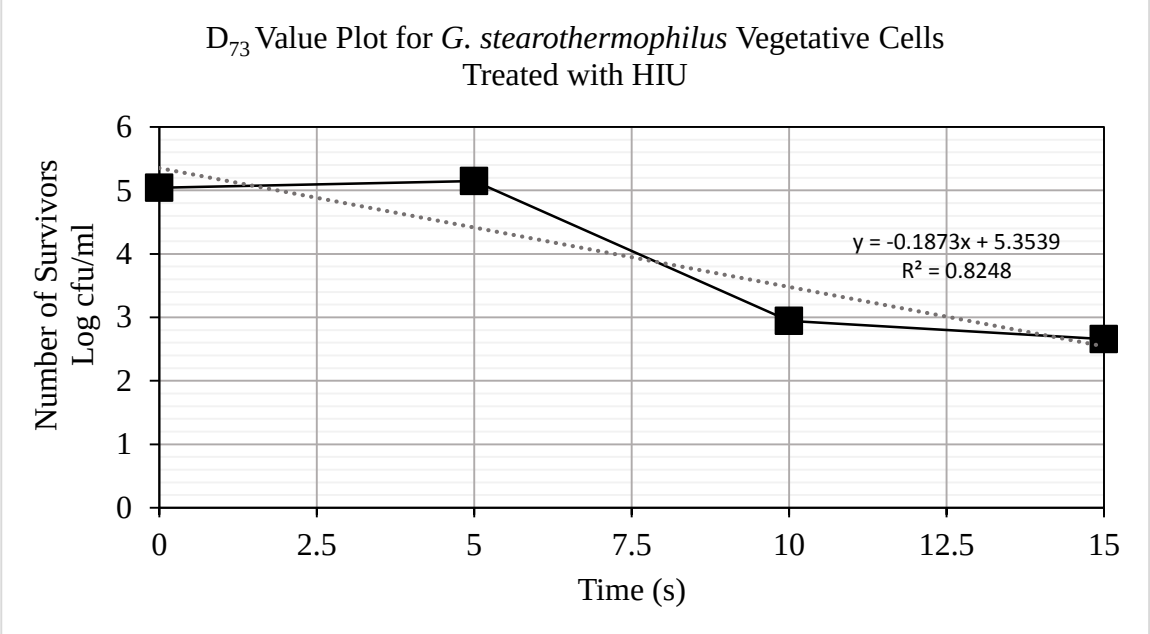
Table 3-3 Average log reductions of *G. stearothermophilus* vegetative cells observed in RSMP with and without HIU

RSMP Total Solids (%)	Treatment Temperature (°C)	Treatment Time (s)	Acoustic Power[a] (W)	Log Reduction with HIU	Log Reduction without HIU	*P*-value[b]
8	45	17.5	28.36	1.8 ± 0.53	0.27 ± 0.06	0.134
8	60	5	21.81	0.77 ± 0.29	0.23 ± 0.31	0.427
8	60	30	19.27	3.5 ± 0.29	0.10 ± 0.07	0.029
8	75	17.5	20.05	3.8 ± 0.11	1.0 ± 0.08	0.004
31.5	45	5	34.79	1.1 ± 0.03	0.24 ± 0.05	0.044
31.5	45	30	28.75	5.0 ± 0.38	0.18 ± 0.10	0.026
31.5	60	17.5	34.72	3.7 ± 0.35	0.27 ± 0.12	3.82E-6[c]
31.5	75	5	27.00	2.8 ± 0.05	0.80 ± 0.01	0.015
31.5	75	30	17.90	3.1 ± 0.01	0.94 ± 0.03	0.004
55	45	17.5	39.10	2.5 ± 0.08	0.09 ± 0.02	0.012
55	60	5	31.25	2.4 ± 0.10	0.16 ± 0.05	0.029
55	60	30	21.07	2.9 ± 0.23	0.51 ± 0.11	0.064
55	75	17.5	17.54	2.8 ± 0.05	1.4 ± 0.01	0.013

[a]Acoustic power was only calculated for treatments where HIU was applied.
[b]Significance declared at $P \leq 0.05$ to determine whether HIU as a whole (without taking into account different HIU conditions) rendered a significant difference in observed log reductions than without HIU.
[c]Midpoint of experimental design – performed in duplicate 3 times.



a



b

Figure 3-3 D-value plots for *G. stearothermophilus* vegetative cells treated with heat, no HIU (A) and with HIU (B). D-values were determined to be 2.1 min at 73°C without HIU and 5.3 s at 73°C with HIU.

Table 3-4 ANOVA analyzing the influence of solids content, temperature, and time on the log reduction of *G. stearothermophilus* vegetative cells in RSMP treated with HIU

ANOVA for Y1

	Master Model					Predictive Model				
Source	DF	SS	MS	F	Pr > F[a]	DF	SS	MS	F	Pr > F[a]
Solids	1	0.065079	0.065079	4.59273	0.0446	1	0.065079	0.065079	2.176802	0.1543
Temp	1	0.192239	0.192239	13.56667	0.0015	1	0.192239	0.192239	6.430154	0.0188
Time	1	1.503157	1.503157	106.0806	< .0001	1	1.503157	1.503157	50.27872	< .0001
Solids*Solids	1	0.367009	0.367009	25.90048	< .0001					
Solids*Temp	1	0.137609	0.137609	9.711349	0.0054	1	0.137600	0.137609	4.602858	0.0532
Solids*Time	1	0.347312	0.347312	24.51045	< .0001	1	0.347312	0.347312	11.61714	0.0025
Temp*Temp	1	0.017392	0.017392	1.227361	0.2811					
Temp*Time	1	0.589058	0.589058	41.57095	< .0001	1	0.589058	0.589058	19.70325	0.0002
Time*Time	1	0.283156	0.283156	19.98288	0.0002	1	0.232409	0.232409	7.773797	0.0107
Model	9	3.441187	0.382354	26.98345	< .0001	7	3.066864	0.438123	14.65467	< .0001
(Linear)	3	1.760475	0.586825	41.41335	< .0001					
(Quadratic)	3	0.606733	0.202244	14.27277	< .0001					
(Cross Product)	3	1.07379	0.357993	25.26425	< .0001					
Error	20	0.283399	0.01417			22	0.657723	0.029896		
(Lack of Fit)	3	0.154039	0.051346	6.74776	0.0034	5	0.528363	0.105673	13.88712	< .0001
(Pure Error)	17	0.12936	0.007609			17	0.12936	0.007609		
Total	29	3.724586				29	3.724586			

[a]Significance declared at $P \leq 0.05$.

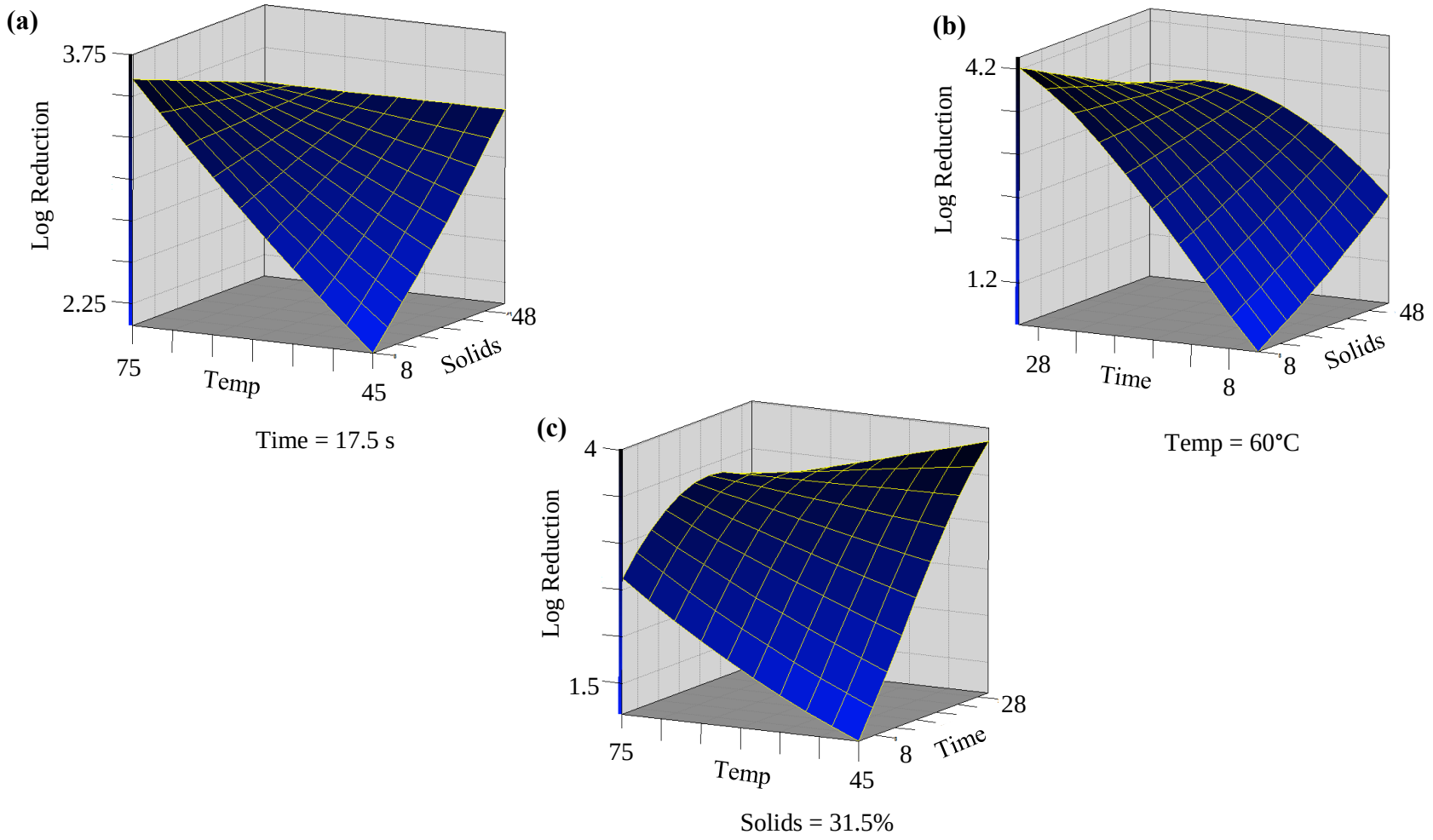


Figure 3-4 Response surface plots of the predicted log reduction (Y) for *G. stearothermophilus* vegetative cells in RSMP treated with HIU when temperature (°C), time (s), and solids content (%) are defined (a-c).

Table 3-5 Validation of RSM predictive models for *G. stearothermophilus* vegetative cells and spores in RSMP treated with HIU

Cell Type	RSMP Total Solids (%)	Treatment Temperature (°C)	Treatment Time (s)	Predicted Log Reduction from Model	Observed Log Reduction
Vegetative	10	45	10	0.899 (0.761, 1.036)	0.88 ± 0.02
Vegetative	30	45	10	1.489 (1.362, 1.616)	1.6 ± 0.40
Vegetative	55	45	10	2.436 (2.314, 2.558)	2.5 ± 0.25
Vegetative	55	72	20	3.155 (3.055, 3.256)	3.1 ± 0.11
Vegetative	55	72	30	2.286 (2.185, 2.387)	2.4 ± 0.02
Spores	8	60	10	0.206 (0.056,0.356)	0.24 ± 0.09
Spores	32	60	17	0.435 (0.349, 0.522)	0.43 ± 0.21
Spores	50	60	10	0.268 (0.162,0.374)	0.31 ± 0.05

Table 3-6 Predicted log reductions generated from RSM model vs observed log reductions of *G. stearothermophilus* vegetative cells and spores in RSMP and RMPC-70 under milk powder processing conditions

Cell Type	RSMP or RMPC-70 Total Solids (%)	Treatment Temperature (°C)	Treatment Time (s)	Predicted Log Reduction from Model	Observed Log Reduction in RSMP	Observed Log Reduction in RMPC-70
Vegetative	9.2	75	10	2.996 (2.901, 3.092)	3.0 ± 0.03	4.7 ± 0.06
Vegetative	9.2	55	10	1.444 (1.354, 1.535)	1.6 ± 0.60	1.3 ± 0.07
Vegetative	12.5	55	10	1.524 (1.436, 1.611)	1.6 ± 0.10	1.4 ± 0.11
Vegetative	50	60	10	2.762 (2.677, 2.847)	2.8 ± 0.08	---
Vegetative	30	60	10	2.253 (2.171, 2.336)	---	3.5 ± 0.16
Spores	9.2	75	10	0.240 (0.165, 0.315)	0.17 ± 0.12	0.28 ± 0.11
Spores	9.2	55	10	0.181 (0.114, 0.248)	0.21 ± 0.01	0.50 ± 0.06
Spores	12.5	55	10	0.216 (0.155, 0.278)	0.20 ± 0.04	0.38 ± 0.03
Spores	50	60	10	0.268 (0.211, 0.324)	0.31 ± 0.05	---
Spores	30	60	10	0.378 (0.325, 0.431)	---	0.61 ± 0.09

Table 3-7 Average log reductions of *G. stearothermophilus* spores in RSMP with and without HIU

RSMP Total Solids (%)	Treatment Temperature (°C)	Treatment Time (s)	Acoustic Power[a] (W)	Log Reduction with HIU	Log Reduction without HIU	*P*-value[b]
8	45	17.5	28.36	0.15 ± 0.02	0.23 ± 0.00	0.124
8	60	5	21.81	0.14 ± 0.01	0.04 ± 0.02	0.047
8	60	30	19.27	0.06 ± 0.04	0.11 ± 0.01	0.459
8	75	17.5	20.05	0.25 ± 0.05	0.34 ± 0.01	0.299
31.5	45	5	34.79	0.07 ± 0.02	0.30 ± 0.00	0.038
31.5	45	30	28.75	0.14 ± 0.01	0.08 ± 0.05	0.317
31.5	60	17.5	34.72	0.44 ± 0.13	0.05 ± 0.05	3.49E-4[c]
31.5	75	5	27.00	0.35 ± 0.02	0.04 ± 0.01	0.037
31.5	75	30	17.90	0.19 ± 0.02	0.10 ± 0.06	0.319
55	45	17.5	39.10	0.15 ± 0.02	0.38 ± 0.04	0.118
55	60	5	31.25	0.14 ± 0.00	0.10 ± 0.00	0.029
55	60	30	21.07	0.13 ± 0.01	0.16 ± 0.02	0.205
55	75	17.5	17.54	0.16 ± 0.06	0.08 ± 0.05	0.097

[a]Acoustic power was only calculated for treatments where HIU was applied.
[b]Significance declared at $P \leq 0.05$ to determine whether HIU as a whole (without taking into account different HIU conditions) rendered a significant difference in observed log reductions than without HIU.
[c]Midpoint of experimental design – performed in duplicate 3 times.

Table 3-8 ANOVA analyzing the influence of solids content, temperature, and time on the log reduction of *G. stearothermophilus* spores in RSMP treated with HIU

ANOVA for Y1

	Master Model					Predictive Model				
Source	DF	SS	MS	F	Pr > F[a]	DF	SS	MS	F	Pr > F[a]
Solids	1	0.000036	0.000036	0.006938	0.9344	1	0.000036	0.000036	0.005562	0.9412
Temp	1	0.068811	0.068811	13.25862	0.0016	1	0.068811	0.068811	10.63051	0.0034
Time	1	0.008996	0.008996	1.733276	0.2029	1	0.008996	0.008996	1.389708	0.2505
Solids*Solids	1	0.190448	0.190448	36.69594	< .0001	1	0.190448	0.190448	29.4221	< .0001
Solids*Temp	1	0.004092	0.004092	0.788474	0.3851					
Solids*Time	1	0.006832	0.006832	1.316394	0.2648					
Temp*Temp	1	0.052865	0.052965	10.20549	0.0046	1	0.052965	0.052965	8.182562	0.0088
Temp*Time	1	0.034156	0.034156	6.581278	0.0185					
Time*Time	1	0.188176	0.188176	36.25821	< .0001	1	0.188176	0.188176	29.07113	< .0001
Model	9	0.503034	0.055893	10.76955	< .0001	6	0.457954	0.076326	11.79149	< .0001
(Linear)	3	0.077842	0.025947	4.999612	0.0095					
(Quadratic)	3	0.380112	0.126704	24.41365	< .0001					
(Cross Product)	3	0.04508	0.015027	2.895382	0.0606					
Error	20	0.103798	0.00519			23	0.148878	0.006473		
(Lack of Fit)	3	0.033355	0.011118	2.683173	0.0795	6	0.078435	0.013072	3.154784	0.0288
(Pure Error)	17	0.070443	0.004144			17	0.070443	0.004144		
Total	29	0.606832				29	0.606832			

[a]Significance declared at $P \leq 0.05$.

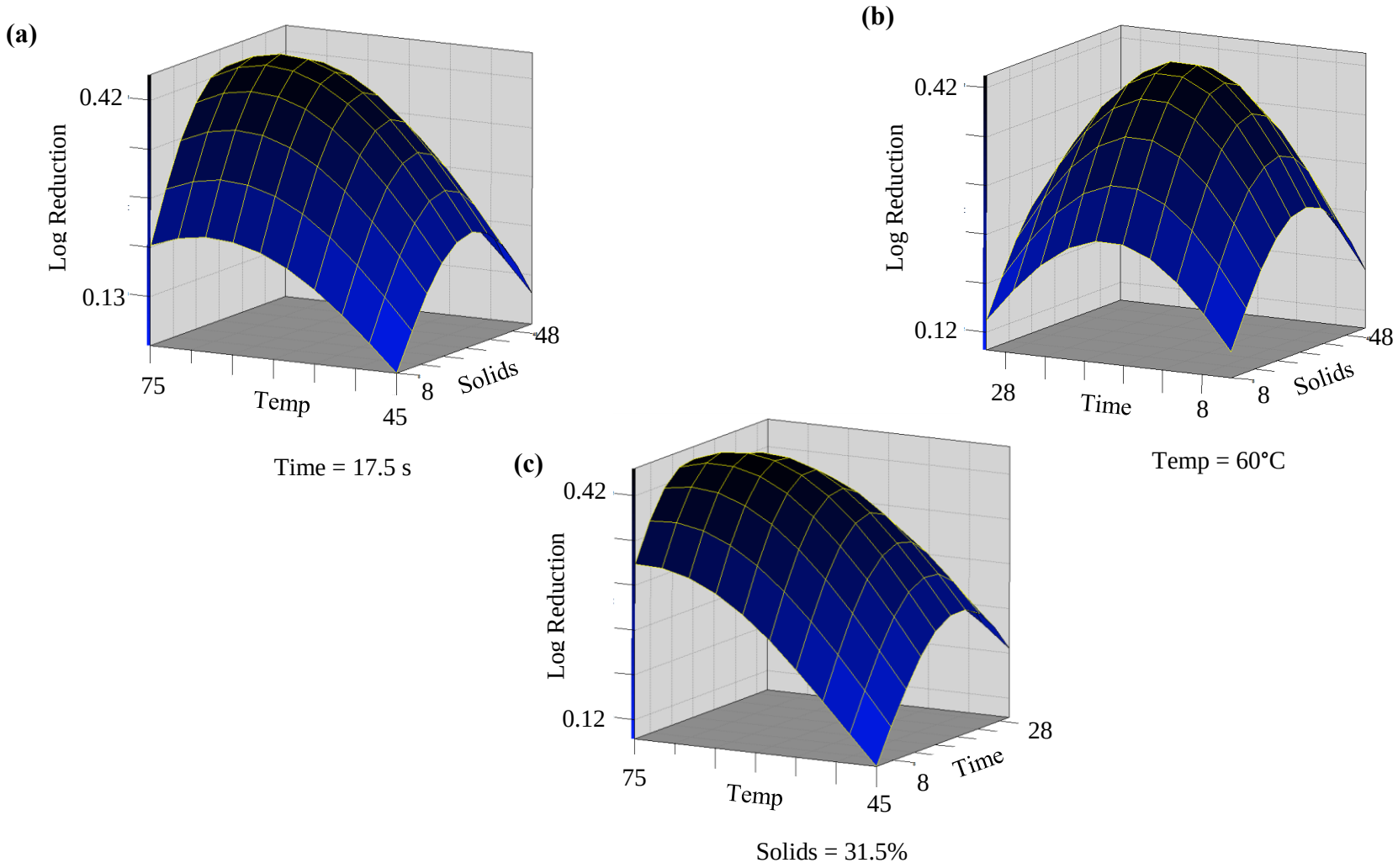


Figure 3-5 Response surface plots of the predicted log reduction (Y) for *G. stearothermophilus* spores in RSMP treated with HIU when temperature (°C), time (s), and solids (%) are defined (a-c).

CHAPTER 4

EFFECTS OF THERMOSONICATION ON SOLUBILITY IN SKIM MILK POWDER

ABSTRACT

Skim milk powder (SMP) was treated with high intensity ultrasound (HIU) to determine if HIU rendered a significant increase or decrease in solubility. SMP was reconstituted to 50% total solids (TS) and run through a continuous flow cell for HIU treatment at 60°C. Non-HIU and HIU samples were freeze-dried and reconstituted to a 2.5% weight-by-weight (w/w) solution followed by centrifugation and analysis of protein content to determine the solubility index (SI). Results indicated no significant change in solubility as a result of HIU.

INTRODUCTION

Skim milk powder (SMP) is nonfat dry milk (NFDM) that is produced within the United States, but not available for domestic sale. Unlike NFDM, which is produced and sold in the United States, SMP contains a minimum protein content of 32% rather than a minimum of 34% protein (American Dairy Products Institute 2014; 21CFR131.125 2015) Compared to other milk powders, such as whey protein concentrate (WPC), whey protein isolate (WPI), whey protein hydrolysate (WPH), and milk protein concentrate (MPC), SMP possesses a lower concentration of protein and a higher content of lactose as shown in Table 2-2 (Chapter 2). It is generally used as an ingredient for soups, sauces, milk

replacers, confectionary, bakery, and meat products labeled as “value-added foods” (Sharma *et al*. 2012).

As a result of its primary usage as a food ingredient, the degree to which milk powder particles dissolve in solution is an important characteristic dictating the quality of SMP. The dissolution of particles in solution is referred to as solubility and is often measured using the Solubility Index (SI) (Fang *et al.* 2008). For SMP, SI reflects the portion of proteins completely suspended in solution as compared to the total protein content. A high SI is indicative of a high ratio of soluble protein to insoluble protein, which is ideal for quality purposes. Solubility, however, can be influenced by a number of factors, specifically particle temperature during the drying stages of processing and bacterial contamination, which can lead to the denaturation of β-lactoglobulin and the formation of β-lactoglobulin-casein aggregates (Baldwin 2010; GEA NIRO 2010; Sharma *et al.* 2012). Bacterial contamination is of particular concern due to lactic acid production and proteolysis contributing toward the unfolding of β-lactoglobulin and overall decrease in solubility (GEA NIRO 2010).

The application of high intensity ultrasound (HIU) has proven to be a successful method for reducing microbial contaminants in dairy products, such as high protein milk powders. As a result, studies have explored the effects of HIU on the functional and rheological properties of milk powders (mostly high protein powders) to determine significant changes that could potentially change quality (Jambrak *et al.* 2008; Bermúdez-Aguirre *et al.* 2009; Chandrapala *et al.* 2011). The literature has shown HIU to have differing effects on milk powders depending on their composition and conditions of HIU

treatment (Jambrak *et al.* 2008; Jambrak *et al.* 2011). Yanjun *et al.* (2014) observed a significant increase from 35.78% to 88.30% in the solubility of MPC when treated with HIU for 5 min using a 20 kHz probe at 50% amplitude. This was different than the results obtained by Jambrak *et al.* (2008) where the solubility of WPI increased from 66.8% ± 1.8 to 85% ± 1.68 after 15 min of HIU and only slightly increased to 68% ± 1.23 after 30 min using a 20 kHz probe with an ultrasonic intensity of 43-48 W/cm^2. In the same study, Jambrak *et al.* (2008) observed a slight decrease in solubility in WPH from 72.1% ± 1.14 to 71% ± 1.34 after 15 min of HIU and an increase to 79% ± 1.36 after 30 min. In a later study, Jambrak *et al.* (2011) observed decreases in solubility of both WPI (31.9-41.9% decrease) and WPC (14.1-30.9% decrease) when samples were subjected to HIU for 5 and 10 min using a 30 kHz frequency probe with an ultrasonic intensity of 73-78 W/cm^2 at 100% amplitude. The difference in composition of WPI and WPH compared to MPC as well as the difference in the probe frequency may have influenced the effect HIU had on solubility when applied.

Because solubility is a major functional property of SMP, the expression of other functional properties, such as flowability, hygroscopicity, heat stability, emulsifying properties, water activity, stickiness, and caking, are dependent upon complete dissolution of powder particles during rehydration (Mimouni *et al.* 2010). As such, the effect of HIU on solubility is a necessary subject to explore in order to determine if the application of HIU to milk powder processing is a viable investment. The objective of this study, therefore, was to determine whether HIU had a significant impact on solubility

when applied to reconstituted SMP (RSMP) under conditions similar to that of a pilot-scale commercial milk powder processing unit.

MATERIALS AND METHODS

Experimental Design

The effects of HIU on solubility in RSMP was explored using a continuous flow cell Ultrasonic Processor UIP500hd (Heilscher Ultrasound Technology, Ringwood, NJ, USA) with a 2 cm in diameter stainless steel 20 kHz frequency probe. A volume of 3 L of 50% total solids (TS) RSMP (Extra Grade Spray Process, Darigold, Inc., Seattle, WA, USA) was run through the Ultrasonic Processor UIP500hd at 60°C while HIU was run continuously at a dial setting of 9, which is equivalent to 90% amplitude. Sample flowed through the cell at a flow rate of 1.818 L/min and residence time of 8.61 sec with a flow cell volume of 261 ml. Table F-2 (Appendix F) shows the residence times calculated for this particular flow cell based on different flow rates. These parameters were chosen in order to imitate the flow of milk through a commercial SMP powder processing unit as product flows from the evaporator and through the condenser to the drier (Chapter 3, Figure 3-1).

Experiments were conducted 3 times. Skim milk powder (Extra Grade Spray Process, Darigold, Inc., Seattle, WA, USA) was reconstituted to 50% ± 0.5% w/v TS. The powder was weighed and mixed with 60°C sterile water for 3 min at room temperature (23°C) using a high-speed blender, followed by a solids test performed using the oven drying method for each batch of RSMP. The oven drying method was done by dispensing 3.5 ml of RSMP into a pre-weighed aluminum pan and leaving to dry in an

oven at 80°C for 12 h. The pan with dried sample was then weighed again, and the solids concentration was determined using the equation: TS = 100 x (DP – P)/(WP – P), where TS is the solids concentration (%), DP is the weight (g) of the pan with sample after drying, P is the weight (g) of the aluminum pan by itself, and WP is the weight (g) of the pan with sample before drying. Approximately 200 ml of non-HIU sample and 200 ml of HIU sample were collected from each run for solubility assays to determine the effects of HIU on solubility.

Solubility Assay

Approximately 200 ml of non-HIU and HIU sample collected from each run were freeze-dried using a VirTis 5L Sentry™ freeze-dryer (The VirTis Company, LLC, Gardner, NY 12525) in separate 100 ml amounts for 48 h and then stored at -20°C. After freeze drying, all non-HIU samples and HIU samples were pooled separately, resulting in a non-HIU pool and HIU pool of freeze dried SMP. A third, or control pool was made using non-HIU, non-freeze dried SMP.

To determine solubility for HIU and non-HIU SMP, a combination of methods used in previous solubility studies was used since one specific standard method for determining SI does not exist (Morr *et al.* 1985; Moughal *et al.* 2000; Thomas *et al.* 2004; Jambrak *et al.* 2011). Freeze-dried SMP samples were first brought to room temperature (23°C) and then divided into 4 replicates per pool (12 replicates total). For each replicate, 25 g of sample was mixed with distilled water to achieve a 2.5% w/w solution. The solution was mixed with a high speed hand-held blender for 90 seconds at 23°C and then allowed to sit for 15 min. The mixture was then stirred with a spatula. Part

of the mixture was retained while the rest was poured into 50-ml centrifuge tubes. The tubes were centrifuged at 700 x g for 10 min followed by removal of the supernatant. Both the suspension before centrifugation and supernatant per replicate were sent to the Rocky Mountain Dairy Herd Improvement Association (DHIA, Wellsville, UT 84339) for protein analysis.

Determination of Solubility Index

The solubility index (SI) was determined to be the ratio of protein suspended in supernatant to protein in suspension prior to centrifugation. This ratio was determined by the equation $SI = 100 \times (P_{supernatant}/P_{retained})$, where SI is the solubility index, or the percentage of protein in suspension after treatment, $P_{supernatant}$ is the amount of protein in the supernatant, and $P_{retained}$ is the amount of protein in suspension before centrifugation.

The outlier from each data set was rejected based on a Q-test, where n=4 and $\alpha = 0.05$, leaving 3 replicates per sample pool. The SI for each replicate (9 total) was analyzed via an Analysis of Variance (ANOVA) with significance declared at $P \leq 0.05$ to determine significant differences in solubility of SMP as a result of HIU.

RESULTS AND DISCUSSION

No significant differences were detected between the SI of samples subjected to HIU versus samples not treated with HIU (Table 4-2). Replicates from all 3 pools were very similar (Figure 4-2) and had observed SI values greater than 97% (Appendix F).

The results obtained were similar to those observed by Jambrak *et al.* (2008) for WPC subjected to HIU under multiple conditions. In this study, WPC displayed no significant changes in solubility while WPI and WPH exhibited significant increases in

solubility after subjection to HIU treatments. WPC samples were thought to experience no significant changes in solubility due to its powder composition being different from that of WPI and HWP. Unlike WPI and WPH, WPC contains 25 times the amount of lactose (Chapter 2, Table 2-2), which is thought to display a protective effect similar to that of many other disaccharides, such as sucrose, during pasteurization (Dumay *et al.* 1994; Jambrak *et al.* 2008). Krešić *et al.* (2008) noted a similar observation of the effect of increased lactose when WPC exhibited a lower increase in solubility than that of WPI when HIU was applied. However, in this particular study, both WPI and WPC showed significant increases in solubility.

A follow-up study conducted by Jambrak *et al.* (2011) further observed that longer HIU times (5 and 10 min) showed significant decreases in WPC and WPI solubility. However, WPC continued to show smaller decreases in solubility than that of WPI as a result of increased lactose and fat content. In contrast, a more recent study in 2014 observed significant increases in solubility when MPC was treated with HIU for 5 min and longer (Yanjun *et al*. 2014).

The conclusions drawn from previous work suggest that increased lactose and fat content play a key role in the increase or decrease of solubility in milk powders when HIU is applied. Unlike other protein powders used in previous research, SMP has a higher lactose content (Table 4), which may attribute to its lack of significant change in water-solubility. However, Jambrak *et al.* (2011) observed significant changes in solubility for protein powders with medium lactose content when HIU was applied for an extended period of time, but at a slower rate than powders with lower lactose

concentration. Adversely, Yanjun *et al*. (2014) observed substantial increases in milk powder solubility sonicated for the same amount of time, but with milk powder containing higher concentrations of lactose. As such, it may be useful to explore the effects of longer HIU times on the solubility of SMP as well as the effect of HIU on other functional properties.

CONCLUSION

The application of HIU under conditions similar to milk powder processing parameters surrounding the evaporator stage of processing did not significantly change the solubility of SMP. These results were different than previous studies utilizing high protein powders, such as WPI and WPH. However, observations from this study were similar to research investigating the effects of HIU on the solubility of milk powders with lactose content greater than WPI and WPH, but less than that of SMP. In these studies, longer treatment times produced a decrease or increase in solubility depending on lactose concentration. Future research exploring whether longer HIU treatments cause a significant positive or negative impact on solubility of SMP and whether there is a significant improvement or decline in other functional properties would be useful in further validating the potential for HIU as a beneficial addition to milk powder processing.

TABLES AND FIGURES

Table 4-1 ANOVA determining significance of HIU on SMP Solubility Index

Analysis of Variance					
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F[a]
Model	2	1.47858811	0.73929406	4.26	0.0706
Error	6	1.04164596	0.17360766		
Corrected Total	8	2.52023407			

[a]Significance declared at $P \leq 0.05$.

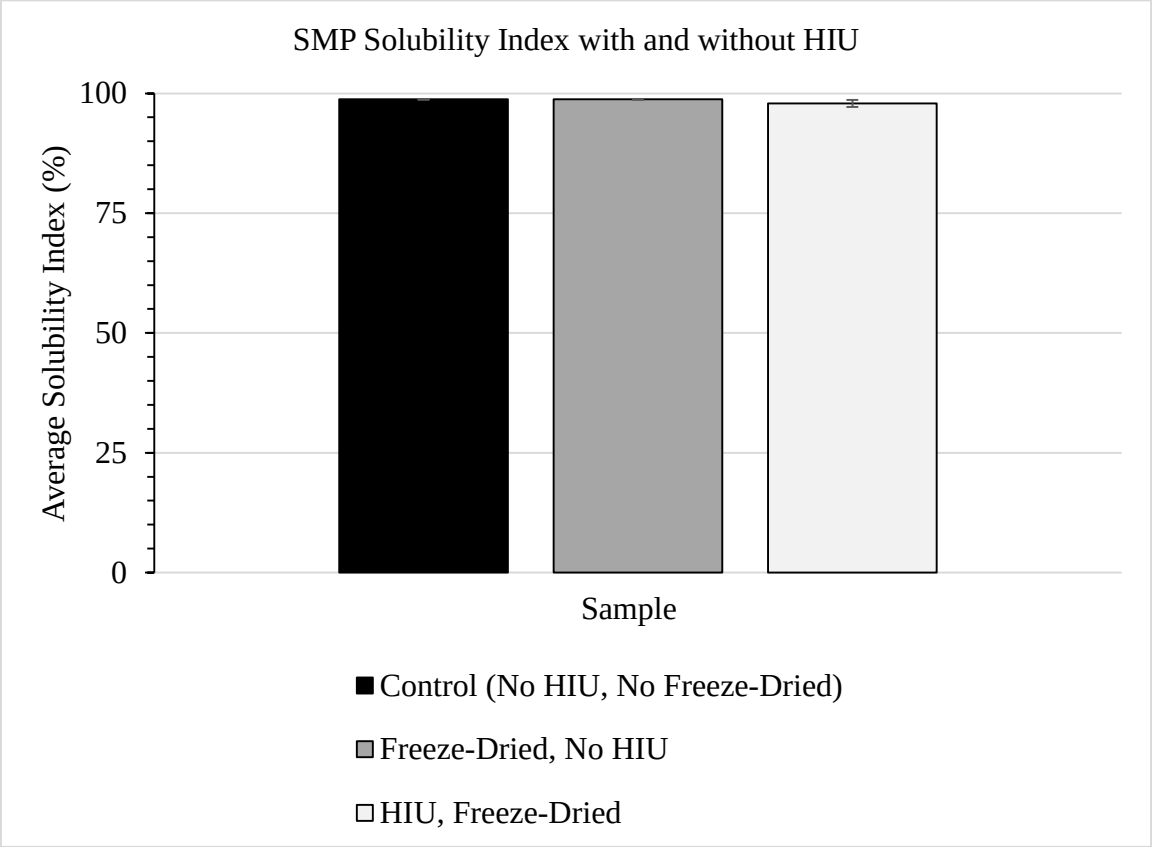


Figure 4-1 Comparison of SMP Solubility Index (%) determined from protein analysis among sample replicates from non-HIU and HIU sample pools.

CHAPTER 5
SUMMARY AND FUTURE WORK

Pasteurization treatments are effective methods in eliminating pathogens and reducing spoilage organisms in dairy products, but these methods lack the capability of destroying thermophilic spore-formers surviving in biofilms within dairy processing units near the plate heat exchangers and evaporators of milk powder processing plants (Walstra *et al*. 1999; Cameron *et al*. 2009; Watterson *et al*. 2014). *Bacillus* (and related) spp., in particular, have been found to be the most predominant contaminants in milk powders, leading to accelerated decrease in quality and loss of functional properties over time (Lücking *et al*. 2013; Buehner *et al*. 2015). Previous research has explored the application of high intensity ultrasound (HIU) in conjunction with traditional pasteurizing conditions as a means to reduce microbial populations, thereby prolonging the shelf life and quality of foods (Chandrapala *et al.* 2012). Specifically, HIU has been investigated to determine its effects on the reduction of vegetative microorganisms and rheological properties of fluid milks and high protein powders (Bermúdez-Aguirre *et al.* 2009; Chandrapala *et al.* 2012). However, little research has been conducted exploring the reduction of thermophilic spore-formers in whole milk or skim milk powder (SMP).

Based on previous research performed on fluid milk and high protein powders, this study provided information and observations on the effects of HIU on the microbial reduction of *G. stearothermophilus*, a thermophilic spore-former often found in dairy processing biofilms and milk powders, and the effects of HIU on the solubility of SMP.

Conditions and parameters used for experiments were based on solids concentration, temperature, and residence times used in commercial milk powder processing facilities.

The application of HIU proved to be significantly more effective in reducing *G. stearothermophilus* vegetative cells than heat treatment alone. Ultrasound yielded a D_{73} value equal to 5.3 s while heat treatment without HIU resulted in a D_{73} value equal to 2.1 min. Response surface analysis (RSM) identified the primary factors contributing toward bactericidal effects as being time, followed by the interaction of temperature and time together, suggesting longer HIU times and higher temperatures improve microbial destruction. Previous research conducted by Cameron *et al.* (2009) and Herceg *et al.* (2012) supported this conclusion of the influence of temperature and time interacting together depending on the strain and growth range of the bacterium.

Thermosonication significantly improved the bactericidal effect of *G. stearothermophilus* spores as compared to thermal processing alone in reconstituted SMP (RSMP); however, it was not as significant of an increase as in experiments involving vegetative cells. This was to be expected due to the increased resistance of spores (Scott *et al*. 2007). The influence of solids concentration and length of HIU were determined to be the primary factors contributing toward spore reduction, as previously observed by Evelyn and Silva (2015). However, the conditions necessary to achieve adequate microbial destruction is dependent upon the strain and type of bacteria as observed by Ferrario *et al*. (2015).

Based on the log reductions observed, RSM generated polynomial equations for models capable of predicting the microbial destruction of both vegetative cells and spores

when time, temperature, and solids content are defined during HIU. Additional experiments validated the accuracy of each of the models and provided information as to where HIU treatments would be most effective in the manufacturing of milk powder. Primary locations were determined to be before and after the evaporator units in order to induce an additive effect from 2 HIU treatments leading to increased destruction of bacterial cells and spores before entering the drying stage.

Additional research is needed to further explore the effects of HIU on the reduction of other thermophilic spore-formers commonly found in SMP and other milk powders to determine if the bactericidal effects are similar or different from those observed with *G. stearothermophilus* for this particular model. *Anoxybacillus flavithermus* would be a particular organism of interest due to its being one of the most common microbes prevalent in milk powders or concentrated milk products (Lindsay and Flint 2009). A more extensive study of *B. subtilis* spores may also be useful to determine the significance of solids content, temperature, and time during HIU treatment on the rate of inactivation compared to that of traditional heat processing, which was not further explored in this study.

Future work performing pilot-scale HIU treatments simulating the processing conditions of commercial milk powder manufacturing before and after the evaporation stage would also be ideal to determine if a large-scale application would render the same degree of microbial destruction observed in a small-scale apparatus for indigenous microorganisms present in product.

Thermosonication applied to RSMP was not observed to significantly change the level of solubility. This was thought to occur as a result of high lactose content, providing a "protective barrier" effect not commonly observed in high protein powders treated with HIU (Jambrak *et al.* 2008). Because solubility is a major functional property of SMP, it is correlated with the expression of additional functional properties important toward its powder quality (Sharma *et al.* 2012). Since HIU did not produce a significant effect upon the level of solubility, it can be assumed that the rest of the functional properties pertaining to SMP will not be affected by HIU treatment either.

In conjunction with further research exploring large-scale HIU effects on microbial reduction, investigation of the effects of HIU under similar conditions on solubility and other functional properties of milk powders, specifically SMP, should be addressed. Longer or shorter treatment times and changes in acoustic power generated by HIU may or may not influence significant changes in functional properties and components, such as lactose, protein, fat, and vitamin and mineral content. Furthermore, additional research should be conducted exploring the effect of HIU on the solubility of SMP throughout different stages of milk powder processing when temperature and solids concentrations vary in order to determine whether solubility ultimately remains unaffected.

The observations and results from this study suggest HIU to be a viable technique as a means to reduce mesophilic and thermophilic spore-formers in the processing of SMP without significantly altering solubility in order to produce a higher quality powder. This study also provided confirmation in the accuracy of predictive models generated

through statistical polynomial equations for determining the expected log reduction of *G. stearothermophilus* when time, temperature, and solids concentration are defined. The validation of these models enabled the determination of 2 effective locations (before and after the evaporator) for HIU treatment during milk powder processing. However, this conclusion and correctness of the predictive model may be dependent upon microbial strain and type, as observed in previous studies. As such, further research is needed to explore the impact of HIU on the destruction of indigenous bacteria in milk powders and other dairy products on a large scale in order to test the validity of the model equation and to determine how a large-scale application of HIU ultimately affects functional properties in addition to nutritional and structural components within the final product.

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APPENDICES

APPENDIX A

RAW DATA FOR CHAPTER 3

Table A-1 Log reduction of *B. subtilis* vegetative cells in RSMP treated with HIU

RSMP Total Solids (%)	Treatment Temperature (°C)	Treatment Time (s)	Bacterial Counts before HIU (cfu/ml)	Bacterial counts after HIU (cfu/ml)	Log Reduction
8	45	17.5	7.6E+6	1.28E+3	3.774
8	45	17.5	8.8E+6	1.08E+3	3.911
8	60	5	5.0E+6	1.0E+2	4.699
8	60	5	2.0E+6	1.2E+2	3.921
8	60	30	1.01E+6	1.0E+1	5.004
8	60	30	1.20E+6	5.0E+1	4.380
8	75	17.5	2.0E+6	3.0E+1	4.824
8	75	17.5	4.0E+6	1.0E+1	5.602
31.5	45	5	3.6E+7	2.9E+7	0.094
31.5	45	5	3.5E+7	2.9E+7	0.084
31.5	45	30	1.95E+7	5.2E+2	4.574
31.5	45	30	1.89E+7	5.9E+2	4.506
31.5	60	17.5	6.2E+7	2.8E+3	4.345
31.5	60	17.5	4.8E+7	2.5E+3	4.283
31.5	60	17.5	2.10E+7	8.7E+3	3.383
31.5	60	17.5	2.18E+7	6.3E+3	3.539
31.5	60	17.5	2.9E+7	4.1E+3	3.850
31.5	60	17.5	3.6E+7	2.7E+3	4.125
31.5	75	5	1.32E+6	1.7E+2	3.890
31.5	75	5	1.10E+6	1.8E+2	3.786
31.5	75	30	5.7E+6	1.7E+2	4.525
31.5	75	30	3.8E+6	1.7E+2	4.349
55	45	17.5	6.0E+6	5.0E+2	4.079
55	45	17.5	6.6E+6	6.0E+2	4.041
55	60	5	8.7E+6	1.0E+2	4.940
55	60	5	7.4E+6	3.0E+2	4.392
55	60	30	8.7E+6	2.0E+2	4.638
55	60	30	7.4E+6	6.0E+2	4.091
55	75	17.5	3.9E+6	3.0E+2	4.114
55	75	17.5	5.1E+6	2.0E+2	4.407

Table A-2 Log reduction of *G. stearothermophilus* vegetative cells in RSMP treated with HIU

RSMP Total Solids (%)	Treatment Temperature (°C)	Treatment Time (s)	Bacterial Count before HIU (cfu/ml)	Bacterial Counts after HIU (cfu/ml)	Log Reduction
8	75	17.5	1.69E+6	2.1E+2	3.906
8	75	17.5	1.08E+6	1.9E+2	3.755
31.5	75	5	3.6E+5	6.8E+2	2.724
31.5	75	5	3.6E+5	5.7E+2	2.800
31.5	45	30	8.0E+7	4.9E+2	5.213
31.5	45	30	4.0E+7	8.3E+2	4.683
8	60	5	1.13E+6	3.1E+5	0.562
8	60	5	2.5E+6	2.7E+5	0.967
8	60	30	1.22E+6	2.7E+2	3.655
8	60	30	1.03E+6	5.8E+2	3.249
31.5	45	5	1.28E+6	1.07E+5	1.078
31.5	45	5	1.29E+6	1.18E+5	1.039
31.5	60	17.5	2.43E+7	3.8E+3	3.806
31.5	60	17.5	2.43E+7	1.73E+3	4.148
31.5	60	17.5	1.01E+7	1.15E+3	3.944
31.5	60	17.5	1.01E+7	2.6E+3	3.589
31.5	75	30	8.5E+5	6.3E+2	3.130
31.5	60	17.5	1.50E+7	8.6E+3	3.242
31.5	60	17.5	1.1E+7	5.1E+3	3.334
31.5	75	30	3.6E+6	2.58E+3	3.145
8	45	17.5	1.55E+6	5.4E+4	1.458
8	45	17.5	3.6E+6	2.23E+4	2.208
55	45	17.5	3.6E+6	9.1E+3	2.597
55	45	17.5	3.6E+6	1.18E+4	2.484
55	60	5	3.6E+6	1.19E+4	2.481
55	60	5	3.6E+6	1.62E+4	2.347
55	60	30	3.6E+6	6.2E+3	2.764
55	60	30	3.6E+6	2.9E+3	3.094
55	75	17.5	3.6E+6	5.0E+3	2.857
55	75	17.5	3.6E+6	5.9E+3	2.785

Table A-3 Log reduction of *G. stearothermophilus* vegetative cells in RSMP heat treated without HIU

RSMP Total Solids (%)	Treatment Temperature (°C)	Treatment Time (s)	Bacterial Count before Heat Treatment (cfu/ml)	Bacterial Count after Heat Treatment (cfu/ml)	Log Reduction
8	45	17.5	1.12E+6	6.6E+5	0.23
8	45	17.5	1.59E+6	7.7E+5	0.315
8	60	5.0	6.5E+5	2.3E+5	0.451
8	60	5.0	5.2E+5	5.1E+5	8.43E-3
8	60	30.0	6.5E+5	4.6E+5	0.15
8	60	30.0	5.2E+5	4.6E+5	0.0532
8	75	17.5	2.05E+6	1.8E+5	1.056
8	75	17.5	2.05E+6	2.33E+5	0.944
31.5	45	5.0	4.5E+5	2.8E+5	0.206
31.5	45	5.0	3.2E+5	3.0E+5	0.28
31.5	45	30.0	4.5E+5	2.5E+5	0.255
31.5	45	30.0	4.1E+5	3.2E+5	0.108
31.5	60	17.5	4.8E+5	1.95E+5	0.391
31.5	60	17.5	5.4E+5	3.6E+5	0.176
31.5	60	17.5	3.1E+5	1.95E+5	0.201
31.5	60	17.5	5.4E+5	2.05E+5	0.421
31.5	60	17.5	3.8E+5	1.95E+5	0.29
31.5	60	17.5	5.4E+5	4.0E+5	0.13
31.5	75	5.0	1.45E+6	2.25E+5	0.809
31.5	75	5.0	1.45E+6	2.34E+5	0.792
31.5	75	30.0	2.05E+6	2.5E+5	0.914
31.5	75	30.0	2.05E+6	2.26E+5	0.958
55	45	17.5	3.6E+6	2.85E+6	0.101
55	45	17.5	3.6E+6	3.01E+6	0.078
55	60	5	1.36E+5	1.02E+5	0.125
55	60	5	2.14E+5	1.36E+5	0.197
55	60	30	1.36E+5	3.5E+4	0.589
55	60	30	1.36E+5	5.0E+4	0.435
55	75	17.5	1.36E+5	4.8E+3	1.452
55	75	17.5	1.36E+5	3.6E+3	1.435

Table A-4 Determination of D-value for *G. stearothermophilus* vegetative cells heat treated without HIU.

Media	Treatment Temperature (°C)	Treatment Time (min)	Bacterial Count before Heat Treatment (cfu/ml)	Bacterial Count after Heat Treatment (cfu/ml)	Log Reduction[b]
TSB[a]	73	1	2.0E+5	1.4E+5	0.155
TSB	73	1	6.0E+5	5.4E+5	0.046
TSB	73	2	2.0E+5	1.0E+5	0.301
TSB	73	2	6.0E+5	5.5E+5	0.038
TSB	73	3	2.0E+5	4.2E+4	0.678
TSB	73	3	6.0E+5	1.51E+5	0.599
TSB	73	4	2.0E+5	2.01E+4	0.998
TSB	73	4	6.0E+5	2.06E+4	1.464
TSB	73	5	2.0E+5	1.03E+3	2.288
TSB	73	5	6.0E+5	1.30E+3	2.664

[a]TSB – tryptic soy broth
[b]D_{73} - value = 2.1 min

Table A-5 Determination of D-value for *G. stearothermophilus* vegetative cells treated with HIU

Media	Temp (°C)	Time (sec)	Bacterial Count before HIU (cfu/ml)	Bacterial Count after HIU (cfu/ml)	Log Reduction[b]
TSB[a]	73	5	1.11E+6	1.06E+5	1.020
TSB	73	5	1.11E+6	1.76E+5	0.800
TSB	73	10	1.11E+6	6.8E+2	3.213
TSB	73	10	1.11E+6	1.08E+3	3.012
TSB	73	15	1.11E+6	7.8E+2	3.153
TSB	73	15	1.11E+6	1.3E+2	3.931

[a]TSB – tryptic soy broth
[b] D_{73}-value = 5.3 s

Table A-6 Verification runs for log reduction of *G. stearothermophilus* vegetative cells and spores treated with HIU

Cell Type	RSMP Total Solids (%)	Treatment Temperature (°C)	Treatment Time (s)	Bacterial Count before HIU (cfu/ml)	Bacterial Count after HIU (cfu/ml)	Log Reduction
Vegetative	TSB[a]	45	10	1.45E+6	7.5E+5	0.286
Vegetative	TSB	45	10	1.45E+6	3.6E+5	0.605
Vegetative	10	45	10	1.45E+6	1.99E+5	0.863
Vegetative	10	45	10	1.45E+6	1.84E+5	0.897
Vegetative	30	45	10	1.45E+6	7.7E+4	1.275
Vegetative	30	45	10	1.45E+6	2.08E+4	1.843
Vegetative	55	45	10	1.45E+6	2.7E+3	2.335
Vegetative	55	45	10	1.45E+6	3.0E+3	2.684
Vegetative	55	72	20	6.7E+5	4.0E+2	3.224
Vegetative	55	72	20	5.9E+5	5.0E+2	3.072
Vegetative	55	72	30	6.7E+5	2.9E+3	2.364
Vegetative	55	72	30	5.9E+5	2.7E+3	2.339
Vegetative	9.2	75	10	1.11E+6	1.26E+3	2.945
Vegetative	9.2	75	10	1.11E+6	1.15E+3	2.984
Vegetative	9.2	55	10	1.11E+6	7.7E+4	1.16
Vegetative	9.2	55	10	1.11E+6	1.09E+4	2.008
Vegetative	12.5	55	10	1.11E+6	3.60E+4	1.489
Vegetative	12.5	55	10	1.11E+6	2.62E+4	1.627
Vegetative	50	60	10	7.0E+5	1.3E+3	2.731
Vegetative	50	60	10	7.0E+5	1.0E+3	2.845
Spores	8	60	10	7.4E+4	3.7E+4	0.301
Spores	8	60	10	1.96E+5	1.32E+5	0.172
Spores	32	60	17	1.3E+5	6.8E+4	0.281
Spores	32	60	17	1.18E+5	3.1E+4	0.581
Spores	50	60	10	1.41E+5	7.6E+4	0.268
Spores	50	60	10	1.23E+5	5.6E+4	0.342
Spores	9.2	75	10	1.04E+5	5.8E+4	0.254
Spores	9.2	75	10	1.13E+5	9.4E+4	0.080
Spores	9.2	55	10	1.61E+5	9.7E+4	0.220
Spores	9.2	55	10	1.65E+5	1.03E+5	0.205
Spores	12.5	55	10	1.88E+5	1.10E+5	0.233
Spores	12.5	55	10	1.99E+5	1.33E+5	0.175

[a]TSB – tryptic soy broth

Table A-7 Verification runs for log reduction of *G. stearothermophilus* vegetative cells and spores in RMPC-70 treated with HIU under milk powder processing conditions.

Cell Type	RMPC-70 Total Solids (%)	Treatment Temperature (°C)	Treatment Time (s)	Bacterial Count before HIU (cfu/ml)	Bacterial Count after HIU (cfu/ml)	Log Reduction
Vegetative	9.2	75	10	7.6E+6	1.4E+2	4.735
Vegetative	9.2	75	10	7.6E+6	1.7E+2	4.65
Vegetative	9.2	55	10	1.06E+7	5.1E+5	1.318
Vegetative	9.2	55	10	1.06E+7	6.3E+5	1.226
Vegetative	12.5	55	10	1.06E+7	3.8E+5	1.446
Vegetative	12.5	55	10	1.06E+7	5.4E+5	1.293
Vegetative	30	60	10	5.0E+6	1.3E+3	3.585
Vegetative	30	60	10	5.0E+6	2.2E+3	3.357
Spores	9.2	75	10	5.7E+4	2.5E+4	0.207
Spores	9.2	75	10	6.6E+4	4.1E+4	0.358
Spores	9.2	55	10	8.4E+4	2.9E+4	0.462
Spores	9.2	55	10	6.3E+4	1.8E+4	0.544
Spores	12.5	55	10	1.03E+5	4.1E+4	0.4
Spores	12.5	55	10	1.59E+5	6.9E+4	0.363
Spores	30	60	10	1.45E+5	3.1E+4	0.67
Spores	30	60	10	2.01E+5	5.8E+4	0.54

Table A-8 Log reduction of *G. stearothermophilus* spores in RSMP treated with HIU

RSMP Total Solids (%)	Treatment Temperature (°C)	Treatment Time (s)	Bacterial Count before HIU (cfu/ml)	Bacterial Count after HIU (cfu/ml)	Log Reduction
8	45	17.5	1.49E+5	1.02E+5	0.165
8	45	17.5	1.44E+5	1.06E+5	0.133
8	60	5	1.71E+5	1.22E+5	0.147
8	60	5	1.71E+5	1.25E+5	0.136
8	60	30	1.20E+5	1.11E+5	0.0339
8	60	30	1.25E+5	1.01E+5	0.0926
8	75	17.5	1.63E+5	1.01E+5	0.208
8	75	17.5	1.95E+5	1.02E+5	0.281
31.5	45	5	1.13E+5	1.00E+5	0.0531
31.5	45	5	7.4E+4	6.1E+4	0.0839
31.5	45	30	7.8E+4	5.6E+4	0.144
31.5	45	30	1.17E+5	8.7E+4	0.129
31.5	60	17.5	1.53E+5	5.9E+4	0.414
31.5	60	17.5	1.32E+5	6.0E+4	0.342
31.5	60	17.5	1.53E+5	4.0E+4	0.583
31.5	60	17.5	1.32E+5	4.1E+4	0.508
31.5	60	17.5	1.53E+5	4.3E+4	0.551
31.5	60	17.5	1.32E+5	7.3E+4	0.257
31.5	75	5	9.0E+4	3.9E+4	0.363
31.5	75	5	6.7E+4	3.1E+4	0.335
31.5	75	30	1.73E+5	1.08E+5	0.204
31.5	75	30	1.73E+5	1.14E+5	0.181
55	45	17.5	1.61E+5	1.20E+5	0.128
55	45	17.5	2.32E+5	1.6E+5	0.161
55	60	5	1.25E+5	9.0E+4	0.143
55	60	5	1.84E+5	1.34E+5	0.138
55	60	30	1.86E+5	1.39E+5	0.126
55	60	30	1.55E+5	1.14E+5	0.133
55	75	17.5	1.65E+5	1.03E+5	0.205
55	75	17.5	1.84E+5	1.41E+5	0.116

Table A-9 Log reduction of *G. stearothermophilus* spores in RSMP heat treated without HIU

RSMP Total Solids (%)	Treatment Temperature (°C)	Treatment Time (s)	Bacterial Count before Heat Treatment (cfu/ml)	Bacterial Count after Heat Treatment (cfu/ml)	Log Reduction
8	45	17.5	7.5E+4	4.4E+4	0.232
8	45	17.5	8.2E+4	4.8E+4	0.233
8	60	5	5.7E+4	5.1E+4	0.0483
8	60	5	4.1E+4	3.9E+4	0.0217
8	60	30	1.22E+5	9.3E+4	0.118
8	60	30	7.9E+4	9.9E+4	0.098
8	75	17.5	9.1E+4	4.1E+4	0.346
8	75	17.5	9.1E+4	4.3E+4	0.326
31.5	45	5	2.6E+4	1.31E+4	0.298
31.5	45	5	3.0E+4	1.5E+4	0.301
31.5	45	30	5.7E+4	3.6E+4	0.12
31.5	45	30	6.7E+4	6.0E+4	0.0479
31.5	60	17.5	6.8E+4	6.7E+4	0.00643
31.5	60	17.5	6.2E+4	6.1E+4	0.00706
31.5	60	17.5	1.24E+5	9.0E+4	0.0895
31.5	60	17.5	1.02E+5	8.3E+4	0.139
31.5	60	17.5	4.6E+4	4.3E+4	0.0293
31.5	60	17.5	6.8E+4	6.2E+4	0.0401
31.5	75	5	1.16E+4	1.08E+4	0.0310
31.5	75	5	1.6E+4	1.46E+4	0.0398
31.5	75	30	6.9E+4	6.1E+4	0.0535
31.5	75	30	3.7E+4	2.7E+4	0.137
55	45	17.5	2.3E+4	9.0E+3	0.407
55	45	17.5	7.2E+4	3.2E+4	0.352
55	60	5	1.01E+5	8.1E+4	0.0958
55	60	5	9.5E+4	7.6E+4	0.0969
55	60	30	7.9E+4	5.6E+4	0.149
55	60	30	7.1E+4	4.7E+4	0.179
55	75	17.5	4.3E+4	3.3E+4	0.115
55	75	17.5	4.6E+4	4.1E+4	0.05

APPENDIX B

SAS REPORT – VEGETATIVE CELLS IN CHAPTER 3

DESIGN DETAILS

Design type:	Response Surface
Design description:	Box-Behnken
Number of factors:	3
Number of runs:	30
Customization:	
Point replication:	Yes

FACTORS

Factors and Levels:

Factor	Low	Center	High
SOLIDS	8	31.5	55
TEMP	45	60	75
TIME	5	17.5	30

RESPONSE

Response
Y1

DESIGN POINTS (Coded)

RUN	SOLIDS	TEMP	TIME	Y1
1	-1	-1	0	1.458
2	-1	-1	0	2.208
3	-1	1	0	3.906
4	-1	1	0	3.755
5	1	-1	0	2.597
6	1	-1	0	2.484
7	1	1	0	2.857
8	1	1	0	2.785
9	0	-1	-1	1.078
10	0	-1	-1	1.039
11	0	-1	1	5.213
12	0	-1	1	4.683
13	0	1	-1	2.724
14	0	1	-1	2.800
15	0	1	1	3.130
16	0	1	1	3.145
17	-1	0	-1	0.562
18	-1	0	-1	0.967
19	1	0	-1	2.481
20	1	0	-1	2.347
21	-1	0	1	3.655
22	-1	0	1	3.249
23	1	0	1	2.764
24	1	0	1	3.094
25	0	0	0	3.806
26	0	0	0	4.148
27	0	0	0	3.944
28	0	0	0	3.589
29	0	0	0	3.242
30	0	0	0	3.334

DESIGN POINTS (Uncoded)

RUN	SOLIDS	TEMP	TIME	Y1
1	8.0	45	17.5	1.458
2	8.0	45	17.5	2.208
3	8.0	75	17.5	3.906
4	8.0	75	17.5	3.755
5	55.0	45	17.5	2.597
6	55.0	45	17.5	2.484
7	55.0	75	17.5	2.857

8	55.0	75	17.5	2.785
9	31.5	45	5.0	1.078
10	31.5	45	5.0	1.039
11	31.5	45	30.0	5.213
12	31.5	45	30.0	4.683
13	31.5	75	5.0	2.724
14	31.5	75	5.0	2.800
15	31.5	75	30.0	3.130
16	31.5	75	30.0	3.145
17	8.0	60	5.0	0.562
18	8.0	60	5.0	0.967
19	55.0	60	5.0	2.481
20	55.0	60	5.0	2.347
21	8.0	60	30.0	3.655
22	8.0	60	30.0	3.249
23	55.0	60	30.0	2.764
24	55.0	60	30.0	3.094
25	31.5	60	17.5	3.806
26	31.5	60	17.5	4.148
27	31.5	60	17.5	3.944
28	31.5	60	17.5	3.589
29	31.5	60	17.5	3.242
30	31.5	60	17.5	3.334

FIT DETAILS:

Y1 Check Assumptions Analysis

Response Transformation
- Optimal power from Box-Cox plot: Y1**0.6
- Power recommended by ADX: Y1**0.6
- Power applied for response transformation: SQRT(Y1)
- Response Scaling Shift: 0

Outlier Observations
- Run numbers deleted from analysis: None

Influential Observations
- Run numbers deleted from analysis: None

ANOVA for Y1

	Master Model					Predictive Model				
Source	DF	SS	MS	F	Pr > F	DF	SS	MS	F	Pr > F
SOLIDS	1	0.065079	0.065079	4.59273	0.0446	1	0.065079	0.065079	2.176802	0.1543
TEMP	1	0.192239	0.192239	13.56667	0.0015	1	0.192239	0.192239	6.430154	0.0188
TIME	1	1.503157	1.503157	106.0806	<.0001	1	1.503157	1.503157	50.27872	<.0001
SOLIDS*SOLIDS	1	0.367009	0.367009	25.90048	<.0001					
SOLIDS*TEMP	1	0.137609	0.137609	9.711349	0.0054	1	0.137609	0.137609	4.602858	0.0432
SOLIDS*TIME	1	0.347312	0.347312	24.51045	<.0001	1	0.347312	0.347312	11.61714	0.0025
TEMP*TEMP	1	0.017392	0.017392	1.227361	0.2811					
TEMP*TIME	1	0.589058	0.589058	41.57095	<.0001	1	0.589058	0.589058	19.70325	0.0002
TIME*TIME	1	0.283156	0.283156	19.98288	0.0002	1	0.232409	0.232409	7.773797	0.0107
Model	9	3.441187	0.382354	26.98345	<.0001	7	3.066864	0.438123	14.65467	<.0001
(Linear)	3	1.760475	0.586825	41.41335	<.0001					
(Quadratic)	3	0.606733	0.202244	14.27277	<.0001					
(Cross Product)	3	1.073979	0.357993	25.26425	<.0001					
Error	20	0.283399	0.01417			22	0.657723	0.029896		
(Lack of fit)	3	0.154039	0.051346	6.74776	0.0034	5	0.528363	0.105673	13.88712	<.0001
(Pure Error)	17	0.12936	0.007609			17	0.12936	0.007609		
Total	29	3.724586				29	3.724586			

Fit Statistics for Y1

	Master Model	Predictive Model
Mean	1.666527	1.666527
R-square	92.39%	82.34%
Adj. R-square	88.97%	76.72%
RMSE	0.119038	0.172906
CV	7.142852	10.37523

Canonical Analysis: Stationary point for Y1

Stationary point:	Critical value is a Saddle Point
Predicted response at stationary point:	3.95294
Standard error of predicted value:	0.004012

Canonical Analysis: Critical value for Y1

Factor Name	Coded	Uncoded
SOLIDS	-0.25039	25.6157
TEMP	1.16617	77.4925
TIME	0.10785	18.8481

Canonical Analysis: Eigenvectors for Y1

Eigenvalues	SOLIDS	TEMP	TIME
0.03225	-0.01495	0.86231	-0.50616
-0.12383	-0.77856	0.30759	0.54701
-0.37570	0.62739	0.40226	0.66676

Ridge Analysis for Y1

Radius	Predicted Response	Standard Error	Dependent variable	Type of ridge	SOLIDS	TEMP	TIME
0.0	1.91574	0.048597	Y1	MINIMUM	0.00000	0.00000	0.00000
0.1	1.87940	0.048446	Y1	MINIMUM	-0.02483	-0.03557	-0.09010
0.2	1.83647	0.048012	Y1	MINIMUM	-0.05891	-0.07438	-0.17606
0.3	1.78665	0.047343	Y1	MINIMUM	-0.09967	-0.11478	-0.25864
0.4	1.72977	0.046524	Y1	MINIMUM	-0.14524	-0.15591	-0.33852
0.5	1.66570	0.045677	Y1	MINIMUM	-0.19428	-0.19738	-0.41629
0.6	1.59437	0.044971	Y1	MINIMUM	-0.24590	-0.23895	-0.49238
0.7	1.51570	0.044617	Y1	MINIMUM	-0.29945	-0.28054	-0.56713
0.8	1.42967	0.044867	Y1	MINIMUM	-0.35447	-0.32208	-0.64079
0.9	1.33624	0.045981	Y1	MINIMUM	-0.41066	-0.36355	-0.71358
1.0	1.23538	0.048192	Y1	MINIMUM	-0.46776	-0.40494	-0.78563
0.0	1.91574	0.048597	Y1	MAXIMUM	0.00000	0.00000	0.00000
0.1	1.94592	0.048447	Y1	MAXIMUM	0.01266	0.02909	0.09483
0.2	1.97059	0.048016	Y1	MAXIMUM	0.01110	0.04593	0.19434
0.3	1.99065	0.047372	Y1	MAXIMUM	-0.00345	0.04201	0.29702
0.4	2.00727	0.046631	Y1	MAXIMUM	-0.02490	0.00954	0.39911
0.5	2.02169	0.045941	Y1	MAXIMUM	-0.04553	-0.05072	0.49533
0.6	2.03497	0.045460	Y1	MAXIMUM	-0.06148	-0.12903	0.58273
0.7	2.04783	0.045370	Y1	MAXIMUM	-0.07288	-0.21587	0.66188
0.8	2.06068	0.045892	Y1	MAXIMUM	-0.08095	-0.30602	0.73471
0.9	2.07374	0.047267	Y1	MAXIMUM	-0.08676	-0.39721	0.80293
1.0	2.08715	0.049715	Y1	MAXIMUM	-0.09101	-0.48850	0.86780

Alias Structure for Y1

Master Model	Predictive Model
No effects aliased.	No effects aliased.

Predictive Model for Y1

Coded Levels(-1,1):

Y1 = 1.760621 + 0.063776*SOLIDS + 0.109613*TEMP + 0.306508*TIME
- 0.131153*SOLIDS*TEMP - 0.20836*SOLIDS*TIME - 0.271353*TEMP*TIME
- 0.176426*TIME*TIME

Uncoded Levels:

Y1 = -2.15201 + 0.037451*SOLIDS + 0.044354*TEMP + 0.173216*TIME
- 0.000372*SOLIDS*TEMP - 0.000709*SOLIDS*TIME - 0.001447*TEMP*TIME
- 0.001129*TIME*TIME

Effect Estimates for Y1

	Master Model				Predictive Model			
Term	Estimate	Std Err	t	Pr > |t|	Estimate	Std Err	t	Pr > |t|
SOLIDS	0.0637763	0.029759	2.143066	0.0446	0.0637763	0.043227	1.475399	0.1543
TEMP	0.1096127	0.029759	3.683296	0.0015	0.1096127	0.043227	2.535775	0.0188
TIME	0.3065083	0.029759	10.29955	<.0001	0.3065083	0.043227	7.090749	<.0001
SOLIDS*SOLIDS	-0.222933	0.043805	-5.08925	<.0001				
SOLIDS*TEMP	-0.131153	0.042086	-3.1163	0.0054	-0.131153	0.061132	-2.14543	0.0432
SOLIDS*TIME	-0.20836	0.042086	-4.9508	<.0001	-0.20836	0.061132	-3.40839	0.0025
TEMP*TEMP	-0.04853	0.043805	-1.10786	0.2811				

TEMP*TIME	-0.271353	0.042086	-6.44755	<.0001	-0.271353	0.061132	-4.43883	0.0002
TIME*TIME	-0.195816	0.043805	-4.47022	0.0002	-0.176426	0.063277	-2.78815	0.0107

OPTIMIZATION

Factors:

Factor	Setting
SOLIDS	31.5
TEMP	60
TIME	17.5

Response(s):

Response	Est. Value
Y1	3.099788 [3.095359,3.104217]

Desirability:

Overall
83.99%

Y1
D(Y1) = 0 when Y1 < 1.5
D(Y1) = 0.5 when Y1 = 2.25
D(Y1) = 1 when Y1 > 3.5

Function power:
Lower half: 1
Upper half: 1

Response Calculator

SOLIDS	TEMP	TIME	Y1
20	72	20	3.609 (0.004)[3.25,3.967]
50	72	20	3.218 (0.006)[2.964,3.472]
55	72	20	3.155 (0.008)[2.948,3.363]
15	72	20	3.676 (0.006)[3.496,3.856]
25	72	20	3.542 (0.004)[3.382,3.703]
30	72	20	3.476 (0.003)[3.33,3.623]
10	45	10	0.899 (0.011)[0.761,1.036]
30	45	10	1.489 (0.005)[1.362,1.616]
55	45	10	2.436 (0.012)[2.314,2.558]

Numerical Optimization Results

SOLIDS	TEMP	TIME	Y1
19.75	45	30	4.7922627787
19.75	52.5	30	4.5724807092
31.5	45	30	4.4551923084
19.75	75	23.75	4.4042285178
19.75	75	17.5	4.4037869502
19.75	60	30	4.3578574368
31.5	75	17.5	4.3359460668
19.75	67.5	23.75	4.3146785936
31.5	52.5	30	4.2433728586
19.75	60	23.75	4.2260484407
31.5	75	23.75	4.2078992971
19.75	67.5	30	4.1483929614
19.75	52.5	23.75	4.138338059
31.5	67.5	23.75	4.1203784467
19.75	45	23.75	4.0515474486
31.5	60	30	4.0367122059
31.5	60	23.75	4.0337773676
19.75	67.5	17.5	4.0193935004
31.5	75	11.25	4.0020501906
31.5	67.5	17.5	3.9545927791
31.5	52.5	23.75	3.9480960598
19.75	75	30	3.9440872831
19.75	75	11.25	3.9428337576
31.5	45	23.75	3.8633345232
31.5	67.5	30	3.8352103503

APPENDIX C

SAS REPORT – SPORES IN CHAPTER 3

DESIGN DETAILS

Design type:	Response Surface
Design description:	Box-Behnken
Number of factors:	3
Number of runs:	30
Customization:	
Point replication:	Yes

FACTORS

Factors and Levels:

Factor	Low	Center	High
SOLIDS	8	31.5	55
TEMP	45	60	75
TIME	5	17.5	30

RESPONSE

Response
Y1

DESIGN POINTS (Coded)

RUN	SOLIDS	TEMP	TIME	Y1
1	-1	-1	0	0.1650
2	-1	-1	0	0.1330
3	-1	1	0	0.2080
4	-1	1	0	0.2810
5	1	-1	0	0.1280
6	1	-1	0	0.1610
7	1	1	0	0.2050
8	1	1	0	0.1160
9	0	-1	-1	0.0531
10	0	-1	-1	0.0839
11	0	-1	1	0.1440
12	0	-1	1	0.1290
13	0	1	-1	0.3630
14	0	1	-1	0.3350
15	0	1	1	0.1810
16	0	1	1	0.2040
17	-1	0	-1	0.1360
18	-1	0	-1	0.1470
19	1	0	-1	0.1430
20	1	0	-1	0.1380
21	-1	0	1	0.0339
22	-1	0	1	0.0926
23	1	0	1	0.1260
24	1	0	1	0.1330
25	0	0	0	0.4140
26	0	0	0	0.3420
27	0	0	0	0.5830
28	0	0	0	0.5080
29	0	0	0	0.5510
30	0	0	0	0.2570

DESIGN POINTS (Uncoded)

RUN	SOLIDS	TEMP	TIME	Y1
1	8.0	45	17.5	0.1650
2	8.0	45	17.5	0.1330
3	8.0	75	17.5	0.2080
4	8.0	75	17.5	0.2810
5	55.0	45	17.5	0.1280
6	55.0	45	17.5	0.1610
7	55.0	75	17.5	0.2050
8	55.0	75	17.5	0.1160

9	31.5	45	5.0	0.0531
10	31.5	45	5.0	0.0839
11	31.5	45	30.0	0.1440
12	31.5	45	30.0	0.1290
13	31.5	75	5.0	0.3630
14	31.5	75	5.0	0.3350
15	31.5	75	30.0	0.1810
16	31.5	75	30.0	0.2040
17	8.0	60	5.0	0.1360
18	8.0	60	5.0	0.1470
19	55.0	60	5.0	0.1430
20	55.0	60	5.0	0.1380
21	8.0	60	30.0	0.0339
22	8.0	60	30.0	0.0926
23	55.0	60	30.0	0.1260
24	55.0	60	30.0	0.1330
25	31.5	60	17.5	0.4140
26	31.5	60	17.5	0.3420
27	31.5	60	17.5	0.5830
28	31.5	60	17.5	0.5080
29	31.5	60	17.5	0.5510
30	31.5	60	17.5	0.2570

FIT DETAILS:

Y1 Check Assumptions Analysis

Response Transformation
- Optimal power from Box-Cox plot: Y1**0.4
- Power recommended by ADX: Y1**0.4
- Power applied for response transformation: SQRT(Y1)
- Response Scaling Shift: 0

Outlier Observations
- Run numbers deleted from analysis: None

Influential Observations
- Run numbers deleted from analysis: None

ANOVA for Y1

	Master Model					Predictive Model				
Source	DF	SS	MS	F	Pr > F	DF	SS	MS	F	Pr > F
SOLIDS	1	0.000036	0.000036	0.006938	0.9344	1	0.000036	0.000036	0.006905	0.9345
TEMP	1	0.068811	0.068811	13.25862	0.0016	1	0.068811	0.068811	13.19572	0.0015
TIME	1	0.008996	0.008996	1.733276	0.2029	1	0.008996	0.008996	1.725053	0.2026
SOLIDS*SOLIDS	1	0.190448	0.190448	36.69594	<.0001	1	0.190448	0.190448	36.52185	<.0001
SOLIDS*TEMP	1	0.004092	0.004092	0.788474	0.3851					
SOLIDS*TIME	1	0.006832	0.006832	1.316394	0.2648					
TEMP*TEMP	1	0.052965	0.052965	10.20549	0.0046	1	0.052965	0.052965	10.15707	0.0043
TEMP*TIME	1	0.034156	0.034156	6.581278	0.0185	1	0.034156	0.034156	6.550055	0.0179
TIME*TIME	1	0.188176	0.188176	36.25821	<.0001	1	0.188176	0.188176	36.08619	<.0001
Model	9	0.503034	0.055893	10.76955	<.0001	7	0.49211	0.070301	13.4816	<.0001
(Linear)	3	0.077842	0.025947	4.999612	0.0095					
(Quadratic)	3	0.380112	0.126704	24.41365	<.0001					
(Cross Product)	3	0.04508	0.015027	2.895382	0.0606					
Error	20	0.103798	0.00519			22	0.114722	0.005215		
(Lack of fit)	3	0.033355	0.011118	2.683173	0.0795	5	0.044279	0.008856	2.137163	0.1103
(Pure Error)	17	0.070443	0.004144			17	0.070443	0.004144		
Total	29	0.606832				29	0.606832			

Fit Statistics for Y1

	Master Model	Predictive Model
Mean	0.443007	0.443007
R-square	82.90%	81.09%
Adj. R-square	75.20%	75.08%
RMSE	0.072041	0.072212
CV	16.26177	16.30048

Canonical Analysis: Stationary point for Y1

Stationary point:	Critical value is a Maximum
Predicted response at stationary point:	0.457023
Standard error of predicted value:	0.000771

Canonical Analysis: Critical value for Y1

Factor Name	Coded	Uncoded
SOLIDS	-0.04335	30.4812
TEMP	0.45942	66.8912
TIME	-0.17226	15.3467

Canonical Analysis: Eigenvectors for Y1

Eigenvalues	SOLIDS	TEMP	TIME
-0.06970	-0.17206	0.91658	-0.36094
-0.15712	0.82543	0.33413	0.45499
-0.17810	-0.53764	0.21965	0.81407

Ridge Analysis for Y1

Radius	Predicted Response	Standard Error	Dependent variable	Type of ridge	SOLIDS	TEMP	TIME
0.0	0.44250	0.030337	Y1	MINIMUM	0.00000	0.00000	0.00000
0.1	0.43559	0.030243	Y1	MINIMUM	0.00100	-0.09316	0.03633
0.2	0.42690	0.029973	Y1	MINIMUM	-0.01379	-0.18642	0.07111
0.3	0.41628	0.029558	Y1	MINIMUM	-0.07680	-0.27015	0.10546
0.4	0.40317	0.029027	Y1	MINIMUM	-0.19966	-0.31152	0.15195
0.5	0.38695	0.028455	Y1	MINIMUM	-0.32098	-0.32103	0.20955
0.6	0.36738	0.027976	Y1	MINIMUM	-0.42991	-0.31973	0.27009
0.7	0.34441	0.027749	Y1	MINIMUM	-0.53065	-0.31413	0.33125
0.8	0.31801	0.027953	Y1	MINIMUM	-0.62618	-0.30640	0.39245
0.9	0.28818	0.028770	Y1	MINIMUM	-0.71820	-0.29744	0.45357
1.0	0.25490	0.030352	Y1	MINIMUM	-0.80775	-0.28770	0.51456
0.0	0.44250	0.030337	Y1	MAXIMUM	0.00000	0.00000	0.00000
0.1	0.44766	0.030243	Y1	MAXIMUM	-0.00702	0.09257	-0.03716
0.2	0.45108	0.029973	Y1	MAXIMUM	-0.01690	0.18472	-0.07478
0.3	0.45277	0.029561	Y1	MAXIMUM	-0.02835	0.27659	-0.11266
0.4	0.45273	0.029071	Y1	MAXIMUM	-0.04075	0.36827	-0.15072
0.5	0.45096	0.028593	Y1	MAXIMUM	-0.05378	0.45981	-0.18889
0.6	0.44745	0.028253	Y1	MAXIMUM	-0.06723	0.55126	-0.22714
0.7	0.44222	0.028209	Y1	MAXIMUM	-0.08099	0.64263	-0.26545
0.8	0.43526	0.028639	Y1	MAXIMUM	-0.09497	0.73395	-0.30381
0.9	0.42657	0.029722	Y1	MAXIMUM	-0.10913	0.82522	-0.34220
1.0	0.41615	0.031599	Y1	MAXIMUM	-0.12343	0.91646	-0.38062

Alias Structure for Y1

Master Model	Predictive Model
No effects aliased.	No effects aliased.

Predictive Model for Y1

Coded Levels(-1,1):

$$Y1 = 0.658961 + 0.0015*SOLIDS + 0.065579*TEMP - 0.023711*TIME - 0.160592*SOLIDS*SOLIDS - 0.08469*TEMP*TEMP - 0.065341*TEMP*TIME - 0.159631*TIME*TIME$$

Uncoded Levels:

$$Y1 = -1.89454 + 0.018384*SOLIDS + 0.055638*TEMP + 0.05477*TIME - 0.000291*SOLIDS*SOLIDS - 0.000376*TEMP*TEMP - 0.000348*TEMP*TIME - 0.001022*TIME*TIME$$

Effect Estimates for Y1

	Master Model				Predictive Model			
Term	Estimate	Std Err	t	Pr > |t|	Estimate	Std Err	t	Pr > |t|
SOLIDS	0.0015001	0.01801	0.083292	0.9344	0.0015001	0.018053	0.083094	0.9345
TEMP	0.0655795	0.01801	3.641239	0.0016	0.0655795	0.018053	3.632592	0.0015
TIME	-0.023711	0.01801	-1.31654	0.2029	-0.023711	0.018053	-1.31341	0.2026

SOLIDS*SOLIDS	-0.160592	0.02651	-6.05772	<.0001	-0.160592	0.026573	-6.04333	<.0001
SOLIDS*TEMP	-0.022617	0.02547	-0.88796	0.3851				
SOLIDS*TIME	0.0292231	0.02547	1.147342	0.2648				
TEMP*TEMP	-0.08469	0.02651	-3.1946	0.0046	-0.08469	0.026573	-3.18702	0.0043
TEMP*TIME	-0.065341	0.02547	-2.5654	0.0185	-0.065341	0.025531	-2.55931	0.0179
TIME*TIME	-0.159631	0.02651	-6.02148	<.0001	-0.159631	0.026573	-6.00718	<.0001

OPTIMIZATION

Factors:

Factor	Setting
SOLIDS	31.5
TEMP	60
TIME	17.5

Response(s):

Response	Est. Value
Y1	0.43423 [0.432427,0.436032]

Desirability:

Overall
78.08%

Y1
D(Y1) = 0 when Y1 < 0.2
D(Y1) = 0.5 when Y1 = 0.35
D(Y1) = 1 when Y1 > 0.5

Function power:
Lower half: 1
Upper half: 1

Response Calculator

SOLIDS	TEMP	TIME	Y1
8	60	10	0.206 (97E-5)[0.056,0.356]
50	60	10	0.268 (72E-5)[0.162,0.374]
32	60	17	0.435 (87E-5)[0.349,0.522]
9.2	75	10	0.24 (0.001)[0.165,0.315]
9.2	55	10	0.181 (87E-5)[0.114,0.248]
12.5	55	10	0.216 (72E-5)[0.155,0.278]
50	60	10	0.268 (72E-5)[0.211,0.324]
30	60	10	0.378 (76E-5)[0.325,0.431]

Numerical Optimization Results

SOLIDS	TEMP	TIME	Y1
31.5	67.5	17.5	0.4496754223
31.5	60	17.5	0.434229808
31.5	67.5	11.25	0.4340985363
31.5	75	11.25	0.4153405778
31.5	75	17.5	0.4094089581
43.25	67.5	17.5	0.3983887932
31.5	60	11.25	0.3980460545
19.75	67.5	17.5	0.396497374
43.25	60	17.5	0.3838585717
43.25	67.5	11.25	0.3837351491
19.75	60	17.5	0.3820020067
19.75	67.5	11.25	0.3818788829
31.5	60	23.75	0.3686891151
43.25	75	11.25	0.3661112466
31.5	52.5	17.5	0.3660237109
19.75	75	11.25	0.3642981603
31.5	67.5	23.75	0.3629817322
43.25	75	17.5	0.3605435451
19.75	75	17.5	0.3587443152
43.25	60	11.25	0.3498852201
19.75	60	11.25	0.3481128175
31.5	52.5	23.75	0.3244110117
31.5	75	5	0.3240706824
43.25	60	23.75	0.3223966225
31.5	67.5	5	0.3218622155

APPENDIX D

T-TEST STATISTICS FOR CHAPTER 3

Tables D-1a to D-1m: Paired two sample for means t-test determining significance of average log reductions of *G. stearothermophilus* vegetative cells in RSMP treated with HIU vs without HIU. Significance declared at $P \leq 0.05$.

Table D-1a Total Solids = 8%, Temp = 45°C, Time = 17.5 se

	Variable 1	*Variable 2*
Mean	1.833	0.2725
Variance	0.28125	0.0036125
Observations	2	2
Pearson Correlation	1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	4.693233083	
P(T<=t) one-tail	0.06682385	
t Critical one-tail	6.313751515	
P(T<=t) two-tail	0.133647701	
t Critical two-tail	12.70620474	

Table D-1b Total Solids = 8%, Temp = 60°C, Time = 5 s

	Variable 1	*Variable 2*
Mean	0.7645	0.229715
Variance	0.0820125	0.097934102
Observations	2	2
Pearson Correlation	-1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	1.261925269	
P(T<=t) one-tail	0.213303952	
t Critical one-tail	6.313751515	
P(T<=t) two-tail	0.426607903	
t Critical two-tail	12.70620474	

Table D-1c Total Solids = 8%, Temp = 60°C, Time = 30 s

	Variable 1	*Variable 2*
Mean	3.452	0.1016
Variance	0.082418	0.00468512
Observations	2	2
Pearson Correlation	1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	21.67141009	
P(T<=t) one-tail	0.014677598	
t Critical one-tail	6.313751515	
P(T<=t) two-tail	0.029355197	
t Critical two-tail	12.70620474	

Table D-1d Total Solids = 8%, Temp = 75°C, Time = 17.5 s

	Variable 1	*Variable 2*
Mean	3.8305	1
Variance	0.0114005	0.006272
Observations	2	2
Pearson Correlation	1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	145.1538462	
P(T<=t) one-tail	0.002192879	
t Critical one-tail	6.313751515	
P(T<=t) two-tail	0.004385758	
t Critical two-tail	12.70620474	

Table D-1e Total Solids = 31.5%, Temp = 45°C, Time = 5 s

	Variable 1	*Variable 2*
Mean	1.0585	0.243
Variance	0.0007605	0.002738
Observations	2	2
Pearson Correlation	-1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	14.4336283	
P(T<=t) one-tail	0.02201817	
t Critical one-tail	6.31375151	
P(T<=t) two-tail	0.04403633	
t Critical two-tail	12.7062047	

Table D-1f Total Solids = 31.5%, Temp = 45°C, Time = 30 s

	Variable 1	*Variable 2*
Mean	4.948	0.1815
Variance	0.14045	0.0108045
Observations	2	2
Pearson Correlation	1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	24.89033943	
P(T<=t) one-tail	0.012781617	
t Critical one-tail	6.313751515	
P(T<=t) two-tail	0.025563234	
t Critical two-tail	12.70620474	

Table D-1g Total Solids = 31.5%, Temp = 60°C, Time = 17.5 s

	Variable 1	*Variable 2*
Mean	3.677166667	0.26816667
Variance	0.124877767	0.01420377
Observations	6	6
Pearson Correlation	-0.099982439	
Hypothesized Mean Difference	0	
Df	5	
t Stat	21.74212085	
P(T<=t) one-tail	1.90973E-06	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	3.81945E-06	
t Critical two-tail	2.570581836	

Table D-1h Total Solids = 31.5%, Temp = 75°C, Time = 5 s

	Variable 1	*Variable 2*
Mean	2.762	0.8005
Variance	0.002888	0.0001445
Observations	2	2
Pearson Correlation	-1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	42.1827957	
P(T<=t) one-tail	0.00754455	
t Critical one-tail	6.31375151	
P(T<=t) two-tail	0.0150891	
t Critical two-tail	12.7062047	

Table D-1i Total Solids = 31.5%, Temp = 75°C, Time = 30 s

	Variable 1	*Variable 2*
Mean	3.1375	0.936
Variance	0.0001125	0.000968
Observations	2	2
Pearson Correlation	1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	151.8275862	
P(T<=t) one-tail	0.002096492	
t Critical one-tail	6.313751515	
P(T<=t) two-tail	0.004192984	
t Critical two-tail	12.70620474	

Table D-1j Total Solids = 55%, Temp = 45°C, Time = 17.5 s

	Variable 1	*Variable 2*
Mean	2.5405	0.08935
Variance	0.0063845	0.000271445
Observations	2	2
Pearson Correlation	1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	54.6521739	
P(T<=t) one-tail	0.00582364	
t Critical one-tail	6.31375151	
P(T<=t) two-tail	0.01164727	
t Critical two-tail	12.7062047	

Table D-1k Total Solids = 55%, Temp = 60°C, Time = 5 s

	Variable 1	*Variable 2*
Mean	2.414	0.161
Variance	0.008978	0.002592
Observations	2	2
Pearson Correlation	-1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	21.87378641	
P(T<=t) one-tail	0.014541991	
t Critical one-tail	6.313751515	
P(T<=t) two-tail	0.029083982	
t Critical two-tail	12.70620474	

Table D-1l Total Solids = 55%, Temp = 60°C, Time = 30 s

	Variable 1	*Variable 2*
Mean	2.929	0.512
Variance	0.05445	0.011858
Observations	2	2
Pearson Correlation	-1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	9.987603306	
P(T<=t) one-tail	0.031764635	
t Critical one-tail	6.313751515	
P(T<=t) two-tail	0.063529269	
t Critical two-tail	12.70620474	

Table D-1m Total Solids = 55%, Temp = 75°C, Time = 17.5 s

	Variable 1	*Variable 2*
Mean	2.821	1.4435
Variance	0.002592	0.0001445
Observations	2	2
Pearson Correlation	1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	50.09090909	
P(T<=t) one-tail	0.0063538	
t Critical one-tail	6.313751515	
P(T<=t) two-tail	0.0127076	
t Critical two-tail	12.70620474	

Tables D-2a to D-2m: Paired two sample for means t-test determining significance of average log reductions of *G. stearothermophilus* spores in RSMP treated with HIU vs without HIU. Significance declared at $P \leq 0.05$.

Table D-2a Total Solids = 8%, Temp = 45°C, Time = 17.5 s

	Variable 1	*Variable 2*
Mean	0.149	0.2325
Variance	0.000512	5E-07
Observations	2	2
Pearson Correlation	-1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	-5.06060606	
P(T<=t) one-tail	0.06209953	
t Critical one-tail	6.31375151	
P(T<=t) two-tail	0.12419905	
t Critical two-tail	12.7062047	

Table D-2b Total Solids = 8%, Temp = 60°C, Time = 5 s

	Variable 1	*Variable 2*
Mean	0.1415	0.035
Variance	6.05E-05	0.00035378
Observations	2	2
Pearson Correlation	1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	13.65384615	
P(T<=t) one-tail	0.023271287	
t Critical one-tail	6.313751515	
P(T<=t) two-tail	0.046542574	
t Critical two-tail	12.70620474	

Table D-2c Total Solids = 8%, Temp = 60°C, Time = 30 s

	Variable 1	*Variable 2*
Mean	0.06325	0.108
Variance	0.001722845	0.0002
Observations	2	2
Pearson Correlation	-1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	-1.137229987	
P(T<=t) one-tail	0.229589572	
t Critical one-tail	6.313751515	
P(T<=t) two-tail	0.459179144	
t Critical two-tail	12.70620474	

Table D-2d Total Solids = 8%, Temp = 75°C, Time = 17.5 s

	Variable 1	*Variable 2*
Mean	0.2445	0.336
Variance	0.0026645	0.0002
Observations	2	2
Pearson Correlation	-1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	-1.967741935	
P(T<=t) one-tail	0.149664045	
t Critical one-tail	6.313751515	
P(T<=t) two-tail	0.29932809	
t Critical two-tail	12.70620474	

Table D-2e Total Solids = 31.5%, Temp = 45°C, Time = 5 s

	Variable 1	*Variable 2*
Mean	0.0685	0.2995
Variance	0.00047432	4.5E-06
Observations	2	2
Pearson Correlation	1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	-16.618705	
P(T<=t) one-tail	0.01913064	
t Critical one-tail	6.31375151	
P(T<=t) two-tail	0.03826129	
t Critical two-tail	12.7062047	

Table D-2f Total Solids= 31.5%, Temp = 45°C, Time = 30 s

	Variable 1	*Variable 2*
Mean	0.1365	0.08395
Variance	0.0001125	0.002599205
Observations	2	2
Pearson Correlation	1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	1.840630473	
P(T<=t) one-tail	0.158416022	
t Critical one-tail	6.313751515	
P(T<=t) two-tail	0.316832044	
t Critical two-tail	12.70620474	

Table D-2g Total Solids = 31.5%, Temp = 60°C, Time = 17.5 s

	Variable 1	*Variable 2*
Mean	0.4425	0.05189833
Variance	0.0162251	0.00274566
Observations	6	6
Pearson Correlation	0.495228663	
Hypothesized Mean Difference	0	
Df	5	
t Stat	8.605990865	
P(T<=t) one-tail	0.000174747	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.000349493	
t Critical two-tail	2.570581836	

Table D-2h Total Solids = 31.5%, Temp = 75°C, Time = 5 s

	Variable 1	*Variable 2*
Mean	0.349	0.0354
Variance	0.000392	3.872E-05
Observations	2	2
Pearson Correlation	-1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	17.04347826	
P(T<=t) one-tail	0.018654958	
t Critical one-tail	6.313751515	
P(T<=t) two-tail	0.037309916	
t Critical two-tail	12.70620474	

Table D-2i Total Solids = 31.55%, Temp = 75°C, Time = 30 s

	Variable 1	*Variable 2*
Mean	0.1925	0.09525
Variance	0.0002645	0.003486125
Observations	2	2
Pearson Correlation	-1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	1.82629108	
P(T<=t) one-tail	0.15946253	
t Critical one-tail	6.31375151	
P(T<=t) two-tail	0.31892505	
t Critical two-tail	12.7062047	

Table D-2j Total Solids = 55%, Temp = 45°C, Time = 17.5 s

	Variable 1	*Variable 2*
Mean	0.1445	0.3795
Variance	0.0005445	0.0015125
Observations	2	2
Pearson Correlation	-1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	-5.34090909	
P(T<=t) one-tail	0.0589163	
t Critical one-tail	6.31375151	
P(T<=t) two-tail	0.1178326	
t Critical two-tail	12.7062047	

Table D-2k Total Solids = 55%, Temp = 60°C, Time = 5 s

	Variable 1	*Variable 2*
Mean	0.1405	0.09745
Variance	1.25E-05	6.05E-07
Observations	2	2
Pearson Correlation	1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	22.07692308	
P(T<=t) one-tail	0.014408369	
t Critical one-tail	6.313751515	
P(T<=t) two-tail	0.028816738	
t Critical two-tail	12.70620474	

Table D-2l Total Solids = 55%, Temp = 60°C, Time = 30 s

	Variable 1	*Variable 2*
Mean	0.1295	0.164
Variance	0.0000245	0.00045
Observations	2	2
Pearson Correlation	1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	-3	
P(T<=t) one-tail	0.102416382	
t Critical one-tail	6.313751515	
P(T<=t) two-tail	0.204832765	
t Critical two-tail	12.70620474	

Table D-2m Total Solids = 55%, Temp = 75°C, Time = 17.5 s

	Variable 1	*Variable 2*
Mean	0.1605	0.0825
Variance	0.0039605	0.0021125
Observations	2	2
Pearson Correlation	1	
Hypothesized Mean Difference	0	
Df	1	
t Stat	6.5	
P(T<=t) one-tail	0.04858979	
t Critical one-tail	6.313751515	
P(T<=t) two-tail	0.097179581	
t Critical two-tail	12.70620474	

APPENDIX E

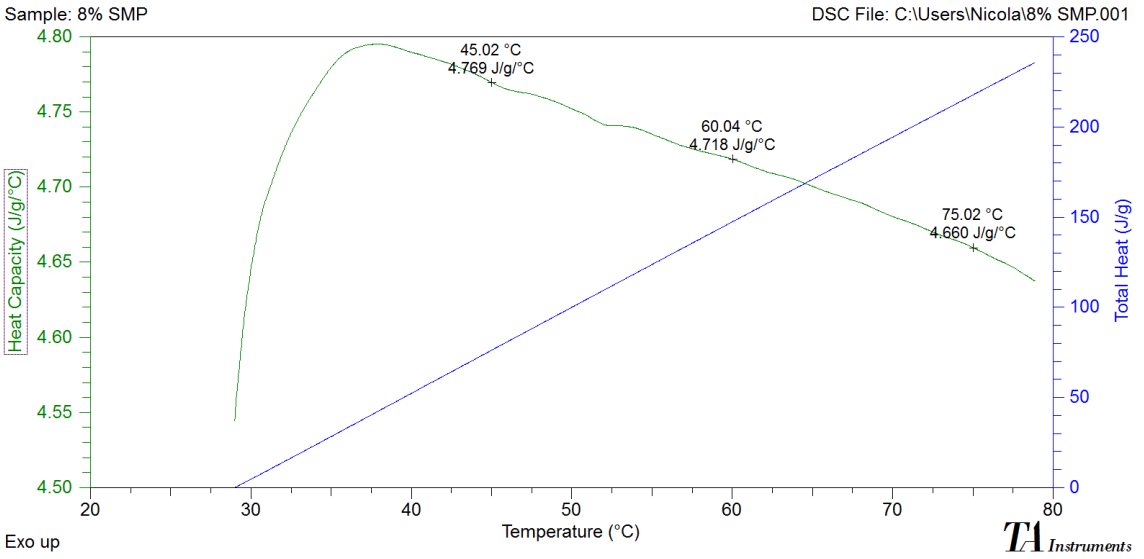
ACOUSTIC POWER CALCULATIONS FOR CHAPTER 3

Table E-1 Acoustic power calculations in RSMP treated with HIU

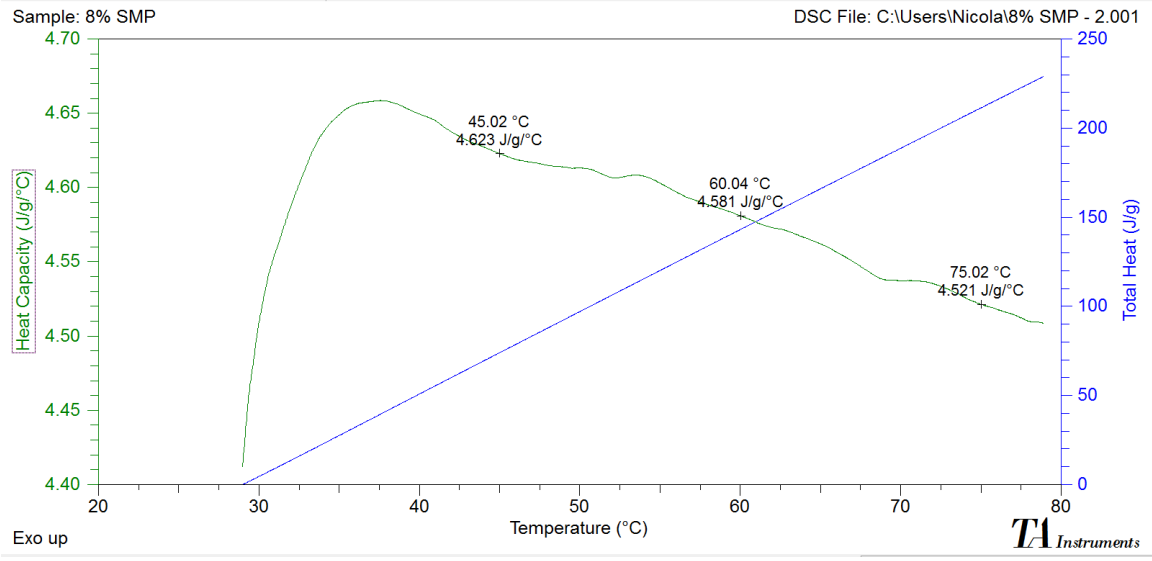
RSMP Total Solids (%)	Treatment Temperature (°C)	Treatment Time (s)	Average Sample Mass (g)	Cp (J/g/°C)[a]	Average dT/dt (°C/s)[b]	Power (W)
8	45	17.5	6.42435	4.696	0.94	28.359
8	60	5	6.42435	4.6495	0.73	21.805
8	60	30	6.42435	4.6495	0.645	19.266
8	75	17.5	6.42435	4.5905	0.68	20.054
31.5	45	5	6.98565	4.185	1.19	34.790
31.5	45	30	6.98565	4.185	0.983	28.748
31.5	60	17.5	6.98565	4.172	1.289	37.554
31.5	60	17.5	6.98565	4.172	1.089	31.725
31.5	60	17.5	6.98565	4.172	1.197	34.890
31.5	75	5	6.98565	4.156	0.93	27.000
31.5	75	30	6.98565	4.156	0.617	17.903
55	45	17.5	6.66535	3.399	1.726	39.097
55	60	5	6.66535	3.399	1.38	31.265
55	60	30	6.66535	3.399	0.93	21.070
55	75	17.5	6.66535	3.399	0.774	17.542

[a]Cp – specific heat capacity of sample at constant pressure. Measured in J/g/°C.
[b]dT/dt – change in temperature during HIU. Measured as °C/s.

Figure E-1 to E-3: Differential scanning colorimetry (DSC) of RSMP to determine specific heat capacity (Cp) at 45°C, 60°C, and 75°C. TS = Total Solids.

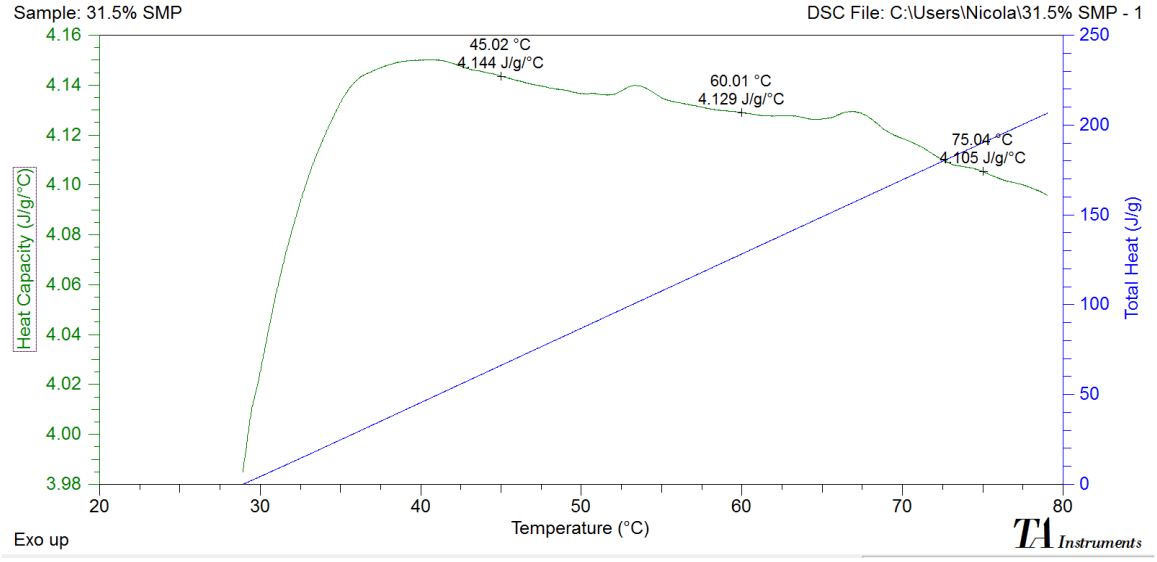


(a)

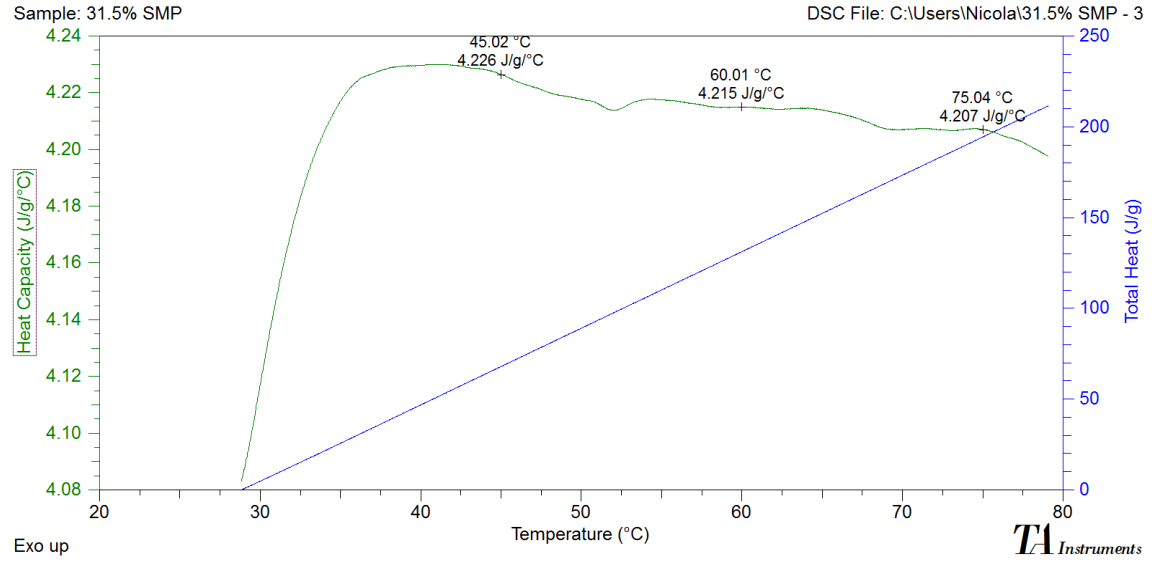


(b)

Figure E-1 8% TS RSMP. Figures (a) and (b) are replicates of each other.

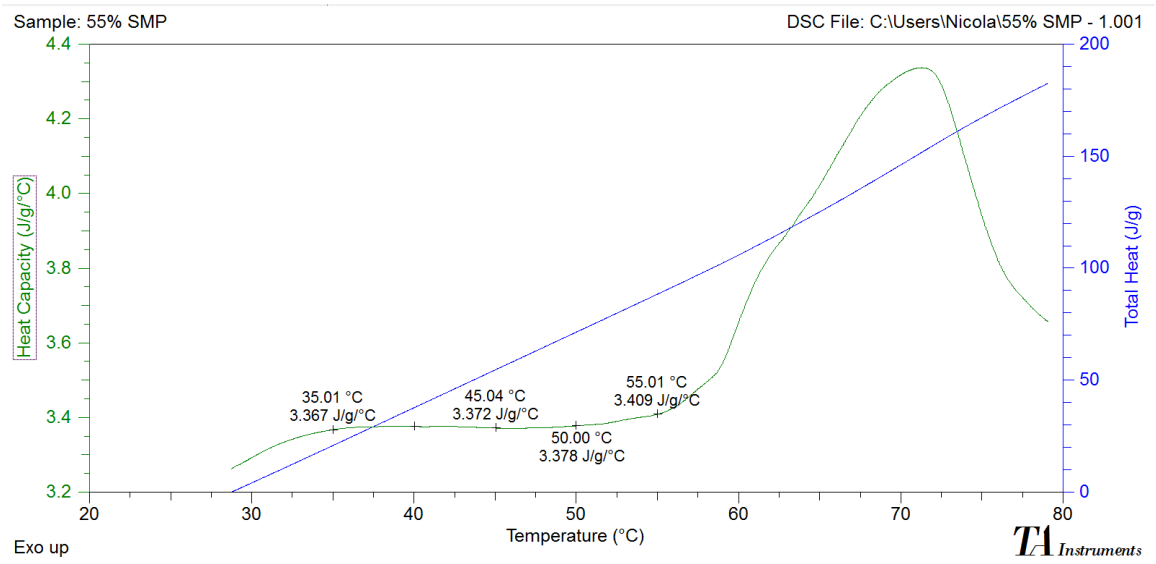


(a)

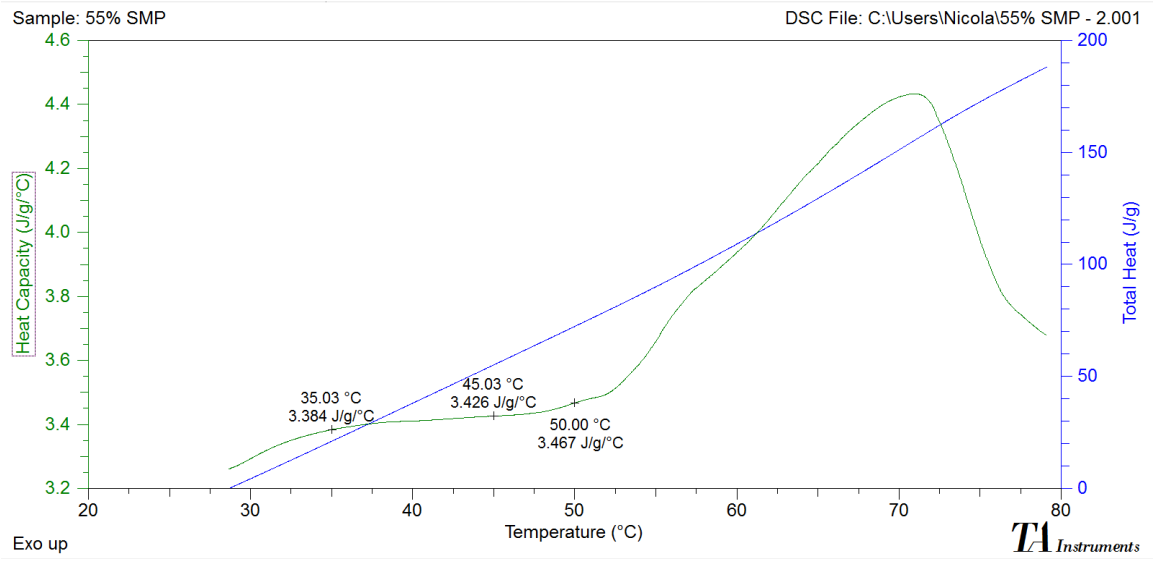


(b)

Figure E-2 31.5% TS RSMP. Figures (a) and (b) are replicates of each other.



(a)



(b)

Figure E-3 55% TS RSMP. Figures (a) and (b) are replicates of each other.

APPENDIX F

DATA AND STATISTICS FOR CHAPTER 4

Table F-1 Dairy Herd Improvement Association hot sheet

Sample[a]	Fat (%)	Protein (%)	Lactose (%)	SNF (%)[b]	SCC[c]
C-1A[d]	0.17	0.81	1.35	2.42	5
C-1B	0.15	0.80	1.36	2.42	0
C-2A	0.16	0.80	1.35	2.40	11
C-2B	0.16	0.79	1.35	2.40	0
C-3A	0.14	0.79	1.35	2.40	14
C-3B	0.14	0.79	.35	2.40	0
C-4A	0.14	0.79	1.35	2.40	10
C-4B	0.14	0.78	1.35	2.40	0
FC-1A[e]	0.14	0.79	1.36	2.42	8
FC-1B	0.14	0.7	1.36	2.41	0
FC-2A	0.15	0.80	1.37	2.43	8
FC-2B	0.15	0.79	1.38	2.43	0
FC-3A	0.14	0.79	1.37	2.43	9
FC-3B	0.14	0.78	1.38	2.42	0
FC-4A	0.14	0.79	1.36	2.41	8
FC-4B	0.13	077	1.36	2.40	0
S-1A[f]	0.13	0.78	1.36	2.40	7
S-2A	0.14	0.79	1.36	2.41	6
S-1A	0.14	0.77	1.36	2.39	0
S-2B	0.14	0.77	1.36	2.40	0
S-3A	0.14	0.80	1.37	2.43	11
S-3B	0.14	0.77	1.37	2.41	0
S-4A	0.14	0.79	1.37	2.42	6
S-4B	0.14	0.77	1.37	2.40	0

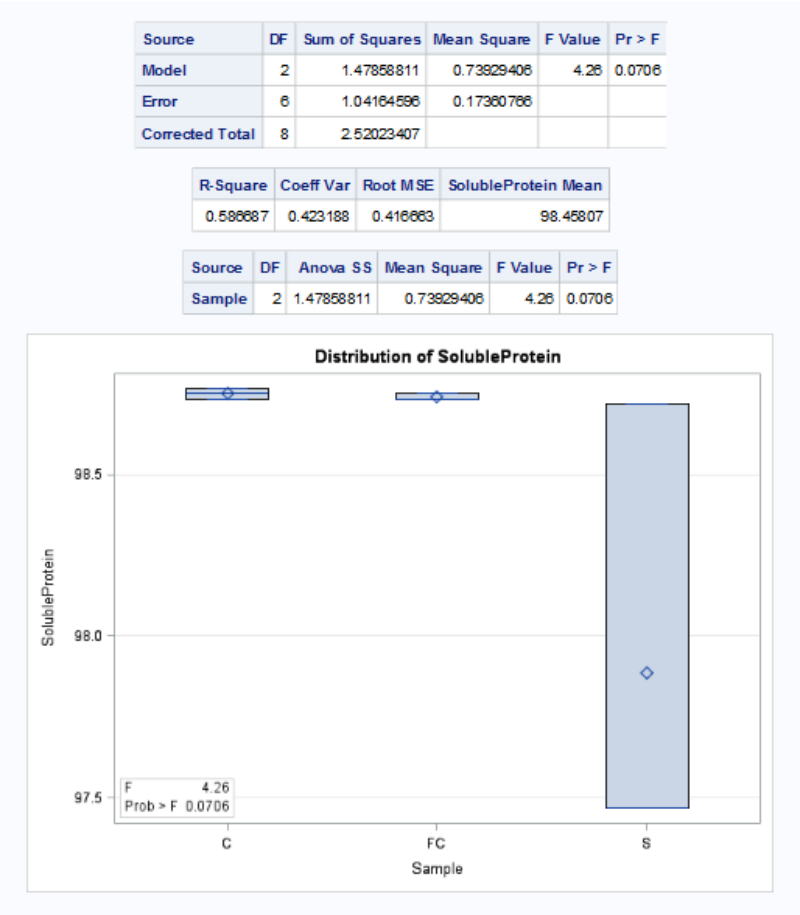
[a]SMP (2.5% w/w) samples before centrifugation and supernatant after from solubility assay (A = before centrifugation, B = supernatant).
[b]SNF - solids-not-fat
[c]SCC – somatic cell count
[d]C – no HIU, not freeze-dried, samples
[e]FC – no HIU, freeze-dried samples
[f]S – HIU, freeze-dried samples

Table F-2 Residence times calculated for Ultrasonic Processor UIP500 hd continuous flow system.

Setting[a]	Time Needed to Fill 1 Liter (s)	L/min	Residence Time (s)
10	72	0.833	18.8
15	47	1.277	12.27
20	33	1.818	8.61
25	28	2.143	7.31

[a]Dial setting on the pump.

Figure F-1 SAS report for one-way ANOVA.

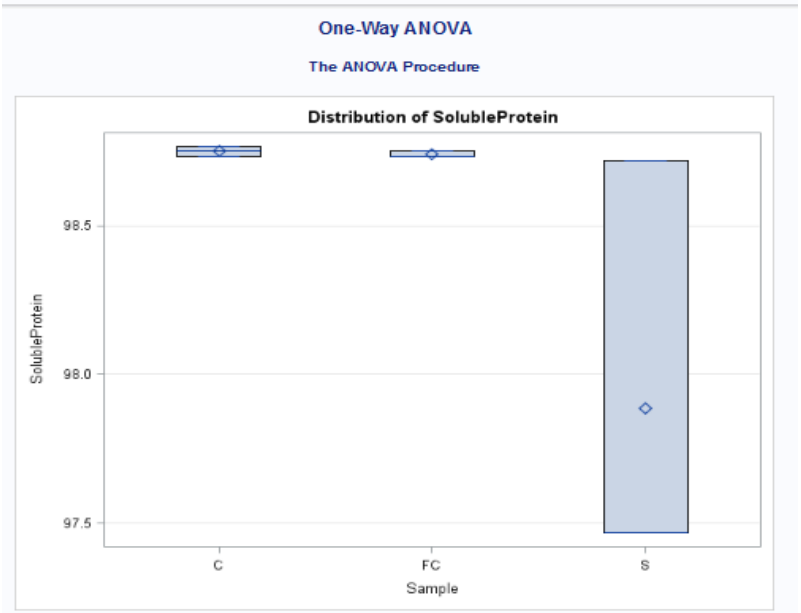


Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	1.47858811	0.73929406	4.26	0.0706
Error	6	1.04164596	0.17360766		
Corrected Total	8	2.52023407			

R-Square	Coeff Var	Root MSE	SolubleProtein Mean
0.586687	0.423188	0.416663	98.45807

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Sample	2	1.47858811	0.73929406	4.26	0.0706

Figure F-1 continued



One-Way ANOVA

The ANOVA Procedure

Student-Newman-Keuls Test for SolubleProtein

Note: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.173608

Number of Means	2	3
Critical Range	0.832437	1.0438315

Means with the same letter are not significantly different.

SNK Grouping	Mean	N	Sample
A	98.7499	3	C
A			
A	98.7395	3	FC
A			
A	97.8849	3	S

CURRICULUM VITAE

NICOLA F. BEATTY

EDUCATION

Utah State University, 2014 – 2016
Master of Food Science, 3.95 GPA

The University of Idaho, 2010 – 2014
Bachelor of Animal and Veterinary Sciences, Microbiology Minor
Magna cum laude

RESEARCH EXPERIENCE

Food Science Intern, January 2016 – Present
Mathew McDonald, J.R. Simplot Company

- Collaboration with plant sciences division working on Fresh Cut Project
- Managed recovery project on Innate™ Fresh Cut dices and fries
- Assisted in sensory panel and marketing analysis

Graduate Research Assistant, 2014 - 2016
Marie K. Walsh, Utah State University

- Investigated effects of sonication on reconstituted skim milk powder microbial and functional properties
- Performed microbiological assays and statistical analysis using response surface analysis
- Presented findings in a written thesis and oral presentations

Mathematical Biology Intern, 2013 - 2014
Mark A. McGuire, University of Idaho

- Explored nutrition relating to microbiome diversity during human and animal lactation
- Performed DNA extractions, PicoGreen® Assay, PCR, and data analysis via RStudio
- Kept lab records, wrote protocols, and performed diagnostics

Research Assistant, 2010 – 2011
Matt Doumit, University of Idaho

- Performed enzyme treatments on cattle biceps to improve tenderness
- Collected and analyzed pH, color, temperature, and sheer force data
- Assisted in conducting a taste panel

OTHER WORK EXPERIENCE

Teaching Assistant, 2013
Genetics of Livestock Improvement, University of Idaho

- Graded exams and assignments; conducted review sessions

Dental Assistant, 2009 - 2013
Korth B. Elliott, D.D.S.

- Assisted in and prepped for examinations and surgery (i.e. extractions, endodontics, orthodontics)
- Provided customer service and basic patient care
- Balanced financial statements and processed insurance claims

Farm/Orchard Worker, 2004 - 2012
Plaza Fruit Ranch

- Picked and handled fruit for local sales and Tree Top, Inc.
- Irrigated, performed maintenance work, and operated farm equipment and machinery

ACHIEVEMENTS AND AWARDS

- 2015 Idaho Milk Processors Association Product Development Grand Champion team
- Dr. Niranjan R. Gandhi and Mrs. Josephine N. Gandhi Fellowship recipient
- BUILD Dairy graduate student member – Western Dairy Center
- 2015-2016 Utah State University Food Science Club IFT Event Coordinator
- University of Idaho Dairy Club Member of the Year Scholarship recipient
- Farm Bureau officer; state and national discussion meet participant, 2011-2014
- University of Idaho College of Agriculture and Life Sciences Ambassador, 2011-2013
- Research Presentation/Poster Finalist
    - 3rd place – 2015 Utah Food and Candy Expo/IFT Supplier's Night
    - 3rd place – 2014 University of Idaho Innovation Showcase
    - Finalist – 2014 IBEST Genomics Symposium
    - 1st place – 2014 ADSSA Western Division

VOLUNTEER EXPERIENCE

- 2015 IFT Annual Meeting student monitor
- Relay for Life
- University of Idaho Lionel Hampton Jazz Festival

RESEARCH POSTERS AND PRESENTATIONS

- J. E. Williams, J. M. Carrothers, K. A. Lackey, N. F. Beatty, M. A. York, S. L. Brooker, M. A. McGuire and M. K. McGuire. Effects of time postpartum and maternal diet on the human milk microbiome. 2016 ISRHML Conference, South Africa.
- N. F. Beatty and M. K. Walsh. Effects of thermosonication on the reduction of *Geobacillus stearothermophilus* cells in skim milk powder. 2015 Utah Food and Candy Expo/IFT Supplier's Night.
- E. D. Benda, N. F. Beatty, J. E. Williams, M. L. Settles, J. P. McNamara and M. A. McGuire. Bacterial communities in rumen fluid from lactating Holstein cows from Washington dairies. 2015 ADSA-ASAS Joint Annual Meeting.
- J. E. Williams, J. M. Carrothers, K. A. Lackey, M. A. York, S. L. Brooker, E. D. Benda, N. F. Beatty, K. M. Steinkamp, H. K. Peterson, M. A. McGuire and M. K. McGuire. Relationships among the microbial communities in human milk, maternal feces, and infant feces during breastfeeding. 2015 FASEB conference.
- J. E. Williams, W. I. Loucks, E. D. Benda, N. F. Beatty, K. M. Steinkamp, M. E. Doumit and M. A. McGuire. Bacterial communities in the gastrointestinal tract of preruminant dairy calves. 2015 ADSA-ASAS Joint Annual Meeting.
- E. D. Benda, N. F. Beatty, J. E. Williams, M. L. Settles, J. P. McNamara and M. A. McGuire. Bacterial diversity in rumen fluid of dairy cattle in Washington. 2014 University of Idaho Innovation Showcase, 2014 IBEST Genomics Symposium.