

**Sonic boom or bust?**  
**Application of high-power ultrasound**  
**for fluid milk processing**

by

**Lily Claire Benner**

A thesis submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of  
MASTER OF SCIENCE

Major: Food Science and Technology

Program of Study Committee:  
Stephanie Clark, Major Professor  
Angela Shaw  
David Grewell

Iowa State University

Ames, Iowa

2015

Copyright © Lily Claire Benner, 2015. All rights reserved

## TABLE OF CONTENTS

LIST OF TABLES	iii
ACKNOWLEDGEMENTS	iv
ABSTRACT	v
CHAPTER 1. INTRODUCTION	1
CHAPTER 2. LITERATURE REVIEW	5
Milk as a Bacterial Environment	5
Conventional Thermal Processing	6
Revenge of the Spores	8
Using Ultrasound Energy to Control Bacterial Growth	10
Ultrasound's Effects on Milk Macronutrients	12
Microbial Destruction via Ultrasound	15
Ultrasound's Effects on Milk Quality and Sensory	16
References	19
CHAPTER 3. EFFECT OF COLD SONICATION AND THERMOSONICATION ON THE TOTAL AEROBIC COUNT OF MILK SUPPLEMENTED WITH <i>PAENIBACILLUS AMYLOLYTICUS</i>	24
Abstract	24
Introduction	25
Materials and Methods	27
Results and Discussion	30
Conclusion	37
References	37
Tables	40
CHAPTER 4. DESCRIPTIVE SENSORY EVALUATION OF THERMOSONICATED MILK	44
Abstract	44
Introduction	45
Materials and Methods	47
Results and Discussion	49
Conclusion	53
References	53
Tables	55
CHAPTER 5. GENERAL CONCLUSIONS	56

**LIST OF TABLES**

Table 3.1	Sonication settings, mean temperatures, and energy density values for thermosonicated and cold sonicated skim milk	40
Table 3.2	Least squares mean log TAC for storage days 1-22 $\pm$ standard error from linear mixed model	41
Table 3.3	Estimated mean log TAC for storage days 29-50	42
Table 3.4	Treatments that differ significantly from pasteurized control (PC) in mixed linear model for storage days 1-22	42
Table 3.5	One-way ANOVA analysis of mean log difference from control and standard error for storage days 1-22	43
Table 4.1	Terms and anchors for the aroma attributes of thermosonicated skim milk	53
Table 4.2	Mean panelist rating (on 15-cm line scale) of cooked, lacks freshness, and rubbery aroma attributes of skim milk subjected to thermosonication or pasteurization	53

## ACKNOWLEDGMENTS

Biggest thanks to Dr. Stephanie Clark for being a wonderful mentor. I would never have finished this journey without your support and understanding. Thanks also to my other committee members, Dr. David Grewell and Dr. Angela Shaw, for your guidance on this project.

Apologies to family and friends who had to hear me moan about my bacterial woes over the past 2 years. I appreciated the sympathy and tough love, depending on the day.

Shout-out to my sensory panelists who sniffed some pretty nasty and/or confusing things in the name of science: Carmen Au, Justin Banach, Danielle Franchetti, Angelika Merz, Molly Paterson, Katie Ranard, Abigail Sparks, and Vaishnavi Manimanna Sankarlal. Y'all are rockstars.

Finally, to whomever is reading this, have a wonderful day, you wonderful person.

## ABSTRACT

Ultrasonication has potential application in the dairy industry for extending milk shelf life by killing bacteria and spores that survive pasteurization. However, ultrasound treatment may result in off-aromas or undesirable milk quality consequences, which increase as treatment time and intensity increase. The primary objective of this research was to determine whether short treatments of acoustic energy, in conjunction with pasteurization, increase refrigerated shelf life while producing no adverse aroma effect. Shelf life was determined by performing total aerobic counts (TAC) on skim milk that had been inoculated with *Paenibacillus amylolyticus*, a spore-forming, thermotolerant and psychrophilic milk contamination bacterium. Pasteurized control milk was plated against thermosonicated (TS) milk and cold sonicated (CS) milk. Both treatments were pasteurized; however TS milk was sonicated after pasteurization while CS milk was sonicated before pasteurization. CS delivered significantly more acoustic energy to milk compared to TS under the same amplitude and treatment duration. TAC for almost all TS and CS treatments were higher than TAC for pasteurized control through 50 d refrigerated storage. Aroma quality of two TS treatment intensities (20 J/mL and 80 J/mL) and pasteurized controls were also evaluated by a trained descriptive sensory panel. No differences in cooked or lacks freshness aroma attributes were noted. The 80 J/mL TS sample had significantly higher rubbery aroma on day 1 after treatment compared to 20 J/mL sample but the aroma dissipated by day 8 or 22. Under the conditions evaluated in the present study, neither TS nor CS were more effective at reducing bacteria in milk than standard pasteurization conditions. Neither TS nor CS extended the shelf life of milk beyond standard pasteurization.

## CHAPTER 1: GENERAL INTRODUCTION

The fluid milk industry is currently at a crossroads. Consumption of milk as a beverage has been steadily decreasing over the past 30 years (Stewart et al., 2013). This could be due to a variety of reasons: more non-milk beverage options available in stores, the convenience of beverages not requiring refrigeration, misconceptions about milk's nutritional value, lactose intolerance and milk allergies, or simply changing taste preferences (Bus and Worsley, 2003). White milk is not as sweet as a soda, and its viscosity and protein content make it less refreshing than flavored waters or juice drinks. People on diets, particularly women, consider milk to be too high in fat and calories (Bus and Worsley, 2003). It is prone to spoilage, which can result in unpleasant sensory experiences. Shelf life can be extended through ultra-pasteurization or ultra-high-temperature (UHT) processing and aseptic packaging, but these processes can result in extremely cooked flavors that some consumers find unacceptable (Christensen and Reineccius, 1992). In the meantime, a vocal minority of consumers demand the right to drink raw milk. Although strictly forbidden across state lines since 1987 (Weisbecker, 2007), it is a legal retail product in 10 US states, with an additional 20 allowing farm-direct sales for human or pet consumption (FTCLDF, 2013). Proponents claim that milk tastes better raw, and that there are many health benefits associated with it, including probiotic lactic acid bacteria (LAB), increased vitamin content, native digestive enzymes, and immune factors (Loss et al., 2011; RAWMI, 2015). Proponents swear by raw milk's abilities to cure allergies, asthma, and lactose intolerance, among other ailments. Between 1% and 3% of Americans currently consume raw milk (Pediatrics, 2014). These consumers consider any processing, including homogenization and standard high-temperature, short-time pasteurization, to be too much. The dairy industry

might recapture some raw milk devotees, while also providing a safe and wholesome product, if they consider the idea of minimal processing.

Milk has been recognized as a source of harmful bacteria for over 100 years. In 1938, milk accounted for 25% of foodborne illnesses in the United States. By the start of the 21<sup>st</sup> century, that percentage had decreased to 1% (MacDonald et al., 2011). What changed? Pasteurization. Simply put, pasteurization is the heat treatment of milk to kill all pathogenic bacteria and induce a 5-log decrease in total aerobic bacteria count (FDA, 2011). This eliminates risk of illness or even death from bacteria such as *Campylobacter jejuni*, *Escherichia coli*, *Salmonella* spp., and *Listeria monocytogenes*, which commonly contaminate milk (Quigley et al., 2013). High-temperature, short-time (HTST) pasteurization generally gives fluid milk a refrigerated shelf life of 21-28 days. Ultra-pasteurization can make milk last 50 days, while UHT milk can be stored at room temperature for 6 months if packed aseptically (FDA, 2011).

Despite these important technological advances in milk processing, which have saved consumers' lives and improved quality, milk is still not most people's beverage of choice. The dairy industry is seeking ways to improve sales, and alternative processing techniques are a popular strategy to explore for this purpose. The idea is that a flashy new method can improve some aspect of milk taste, shelf life, or other functionality. FairLife, from Coca-Cola, is one recent development in the dairy industry to do just that. Filtration and ultra-pasteurization are used to create a high-protein, lactose-free product (Fairlife, 2015). The end result is something between regular milk and a protein shake with a cooked flavor indicative of the heat treatment that gives it its long shelf life. Although this kind of product may not win over raw milk activists, it worked for consumers seeking long shelf life and high protein. If it worked for

ultrafiltration, who's to say it won't work for another novel processing technique such as ultrasound?

High-power sound waves have been studied for their application in food processing for over 50 years. While low-power, high-frequency sound waves are used for imaging and diagnostic purposes, high-power, low-frequency acoustic energy is capable of inducing chemical and physical changes in a medium. High-power ultrasound can work in conjunction with heat (thermosonation, TS) or pressure (manosonation, MS) or both (manothermosonation, MTS) to alter the properties or functionality of foods. Early research has shown the ability of high-power ultrasound to kill bacteria, inactivate enzymes, and improve the cheese- or yogurt-making process (Martini et al., 2012; Reiner et al., 2009; Shanmugam et al., 2012; Villamiel and DeJong, 2000).

Despite the body of research that has demonstrated the beneficial effects of high-power ultrasound, there are many unanswered questions about its effects on fluid milk quality. Some studies have suggested that even short periods of ultrasound (US) treatment result in undesirable sensory attributes (Chouliara et al., 2010; Marchesini et al., 2015). It is possible that the difference between US's positive and negative effects is simply due to amplitude, duration, or temperature differences during processing. More research needs to be done to determine the minimal amount of US needed to induce desired changes to milk without damaging it.

This thesis focuses on the use of short high-power US treatment times ( $\leq 60$  s) to improve the microbial shelf life of skim milk without sacrificing sensory quality. The review of literature in the following chapter introduces milk as a complex environment for microorganisms, outlines the major bacteria present in milk, and discusses US as a method for their inactivation.



Ultrasound is defined and explored, and its effects on various milk components are examined. Finally, the methods and results of the two US studies are presented.

The objective of this research project was to determine the feasibility of integrating high-power US with standard HTST pasteurization in order to improve shelf life while maintaining sensory quality of milk. The following hypotheses were tested:

1. Thermosonicated skim milk will have a lower total aerobic bacteria count than pasteurized control over 50 days of refrigerated storage.
2. Thermosonicated skim milk will have a similar or superior aroma quality to pasteurized control after 21 days of refrigerated storage as determined by a trained sensory panel.

## CHAPTER 2: LITERATURE REVIEW

### Milk as a Bacterial Environment

There is a reason that young humans and mammals thrive on milk. It is a delicious source of nutrition, full of macronutrients and micronutrients alike. Raw whole cow's milk averages 3.4% fat, 4.9% lactose, 3.3% protein, and less than 1% ash (non-combustible materials, including minerals). Calcium is the most well-known micronutrient found in milk, and it is also a good source of phosphorus (Jenness, 1988).

All of these components make milk a rich, pH-neutral environment for bacteria as well. Milk is sterile when it is inside the udder, but as soon as it passes through the teat, it is exposed to millions of bacteria in the environment (Quigley et al., 2013). *Aerococcus*, *Acinetobacter*, *Corynebacterium*, *Staphylococcus*, *Streptococcus*, and *Bifidobacterium* are some of the common teat bacteria genera (Braem et al., 2012). Lactic acid bacteria (LAB) thrive in milk because, as their name suggests, they are able to metabolize milk sugar (lactose) into lactic acid through glycolysis and fermentation to produce energy for themselves as well as by-products such as lactic acid and compounds such as diacetyl and citrate. LAB are mesophiles, meaning that they thrive at temperatures between 30-40°C.

In most cases, LAB are not harmful to human health and are economically important due to their use in production of cultured dairy products. *Lactobacillus*, *Streptococcus*, *Lactococcus*, and *Leuconostoc* species have applications in the cheese, yogurt, and sour cream industries, as well as for non-dairy products such as sauerkraut (Quigley et al., 2013). Dairy fermentations used to rely on these naturally-occurring LAB to out-compete pathogens and spoilage microbes. However, modern practice employs the more reliable method of inoculating pasteurized milk with known cultures. This is a wise decision because of how easily milk can become

contaminated by pathogens. *Campylobacter jejuni* is the most common harmful bacteria found in milk, and it can cause unpleasant gastrointestinal symptoms. More serious are bacteria such as *Escherichia coli* 0157:H7 and *Listeria monocytogenes*, which are known to cause death or severe complications in immunocompromised individuals, particularly the very young, the elderly, or pregnant women. Between 1998 and 2009, over 1,800 people became sick from drinking raw milk contaminated from these types of bacteria, and 2 people died (Pediatrics, 2014).

Less problematic to health, but as economically devastating, are the spoilage bacteria. Present in soil, feed, equipment, or on dairy workers' hands, these bacteria contribute to quality defects that can render milk unfit for commercial sale. LAB are considered spoilage microorganisms when they are present in a product not intended for fermentation, since their acid production is undesirable in fresh milk (Quigley et al., 2013).

*Pseudomonas* are another characteristic milk spoilage bacteria. These Gram negative rods, typically from the species *fluorescens* and *fragi*, can grow under refrigeration conditions and produce a fruity off-flavor in addition to protein degradation (Ternstrom et al., 1993).

Finally, some Gram positive sporeforming bacteria are common in milk. *Clostridia* are the most well-known genus of sporeformers, and several species, such as *Clostridium sporogenes* and *C. tyrobutyricum* can cause cheese defects (Quigley et al., 2013). *Bacillus* and *Paenibacillus* are two other groups of sporeformers that have recently been targeted for their role in milk spoilage, and will be discussed at length later in this review.

### **Conventional Thermal Processing**

Pasteurization has been law for all milk sold across state lines (and within many states) in the U.S. since 1987 (Weisbecker, 2007). Pathogenic and spoilage bacteria such as those described in the previous section are killed by proper pasteurization. There are three main types

of thermal treatments employed for the modern retail milk market. High-temperature, short-time (HTST) pasteurization is the most common. The legal definition of this process involves heating every particle of milk to 72°C and holding thereat for at least 15 s, although most dairy processing plants exceed this time and temperature. HTST milk has an expected refrigerated shelf life of 21-28 days. A higher treatment temperature can also be used, resulting in the process of ultra-pasteurization. The milk must be heated to 125 to 138°C for 2 to 4 s, giving it a refrigerated shelf life of up to (but generally shorter than) 56 days. Finally, ultra-high-temperature (UHT) processing is an option more popular in countries where refrigeration is not as readily available. Heating the milk to 135°C for at least 2 s and packaging aseptically renders the product commercially sterile. It has a room-temperature shelf life of 6 to 9 months (FDA PMO, 2011).

HTST milk, in order to be deemed Grade 'A' in the U.S., must contain < 20,000 CFU/mL (4.3 log CFU/mL) bacteria, < 10 CFU/mL coliforms, and < 350 milliunits/L phosphatase, which is the indicator enzyme for a successful pasteurization. Law also requires rapid cooling of the milk to <4°C. This applies to raw milk storage in a farm's bulk tank after milking as well as finished packages of pasteurized milk (FDA 2011). If improperly cooled, milk spends too much time in the mesophilic temperature range. Spoilage microorganisms could proliferate, especially in raw bulk tank milk. Although these bacteria will likely be killed during proper pasteurization, damage to the milk quality may already have been done.

*Pseudomonas*, as previously mentioned, are a common contaminant found in raw milk. *P. fluorescens* and *P. fragi*, in particular, pose a problem for the dairy industry (Ternstrom et al., 1993). *Pseudomonas* contribute to spoilage via the production of proteolytic and lipolytic enzymes (Hayes et al., 2002; Ternstrom et al., 1993). These bacteria produce strong off-aromas

which can vary from fruity to barny and rotten depending on the species (Hayes et al., 2002). Since pasteurization kills vegetative *Pseudomonas* cells, any found in retail-ready milk are an indication of post-pasteurization contamination (PPC) (Ranieri and Boor, 2009).

### **Revenge of the Spores**

Although UHT may kill all vegetative cells in the milk, including spoilage microorganisms that might survive HTST pasteurization, recent research has shown that spores can survive UHT as well (Khanal et al., 2014; Scheldeman et al., 2006). A spore is a dormant form of bacterial life that has ceased metabolic processes and retreated within a protective capsule called the spore coat. Spores are able to survive harsh environmental conditions including extreme pH, high salinity, oxidizing agents, and very hot or cold temperatures (Khanal et al., 2014). They are produced by certain Gram positive rod-shaped bacteria including those from the genera *Bacillus*, *Clostridium*, and related organisms. Spores pose a threat because they can germinate, or begin growing into vegetative cells, while food is stored. A product that was initially found to have a low bacterial count could suddenly be home to millions of bacteria in a matter of days.

*Bacillus cereus* has traditionally been the sporeforming bacteria of concern in the food industry, especially since it is capable of causing foodborne illness. *Bacillus cereus* has been implicated in illnesses related to contaminated rice, milk, meat products, and many prepared foods (Evelyn et al., 2015). However, other *Bacillus* species are causing more problems for the dairy industry. *Bacillus licheniformis* may not sicken anyone directly, but it is capable of producing proteolytic enzymes and bitter taste that might make a consumer *want* to be sick. *B. licheniformis* is one of the most common contaminating organisms found in pasteurized milk that has not been re-contaminated by Gram negatives such as *Pseudomonas* (Fromm and Boor, 2004;

Ranieri and Boor, 2009). There are 71 isolates of *B. licheniformis* known to cause spoilage in dairy products (Lucking et al., 2013). *Pseudomonas* grow much faster than *Bacillus*, so in cases where post-pasteurization contamination (PPC) has occurred, *B. licheniformis* and other Gram positive spore-formers will be only a minor, overshadowed presence (Fromm and Boor, 2004).

If pasteurization goes to plan, that is time for *Bacillus* and relatives to shine. The spores that have gone through pasteurization unscathed now face no competition to their continued growth as vegetative cells. At this point, a race for proliferation begins with *Bacillus* and their close relatives, *Paenibacillus*. Although only six *Paenibacillus* isolates have been associated with milk spoilage to date, particularly *Paenibacillus amylolyticus*, they have strong growth characteristics at refrigerator temperatures (4°C) while *Bacillus* usually do not (Ivy et al., 2012; Lucking et al., 2013). Another important differentiation between these two groups is ability to metabolize lactose; *Paenibacillus* isolates generally have  $\beta$ -galactosidase activity, while most *Bacillus* do not (Ivy et al., 2012). Thus, *Paenibacillus* is logically more likely to thrive in the lactose-rich environment of milk.

Because *Bacillus* of many kinds are more abundant in the milking environment than *Paenibacillus*, more of their spores and vegetative cells contaminate the milk on the farm (Huck et al., 2008). A microbial succession takes place during transportation in the refrigerated tanker truck to the dairy processing plant. *Paenibacillus*' ability to grow at these low temperatures allows it to overtake *Bacillus*. However, *Paenibacillus* is not as heat resistant as *Bacillus*, and fewer spores survive the pasteurization process (Lucking et al., 2013). *Bacillus* has the edge at the beginning of pasteurized milk shelf life. After packaging and cooling, the succession must begin again. By day 14 of refrigeration, *Paenibacillus* spp. are likely the most abundant

microorganisms in pasteurized milk (Huck et al., 2008; Martin et al., 2011; Ranieri et al, 2009; Ranieri and Boor, 2009).

### **Using Ultrasound Energy to Control Bacterial Growth**

Since heat is not able to destroy the thermo-tolerant spores of microorganisms such as *Bacillus licheniformis* and *Paenibacillus amylolyticus*, scientists have turned to alternative sources of energy besides thermal. Sound waves have been successfully used for imaging and diagnostic purposes for many years, but a different, higher-power form of US can be used to induce physical and chemical changes within a fluid. This type of sound energy is typically defined as 16-100 kHz frequency and 10-1000 W/cm<sup>2</sup> power density (Soria and Villamiel, 2010). High-power US has been shown to be an effective homogenization technique, can alter protein structure and functionality, and there is evidence that it can also kill bacteria and inactivate enzymes (Bermudez-Aguirre et al., 2008; Cameron et al., 2008; Herceg et al., 2012; Villamiel and deJong, 2000). Sonication has a complex mechanism, and therefore its wide-ranging effects on the treatment medium must be examined carefully.

Terminology is an important part of ultrasonics research, so it is useful to define several terms before discussing their technicalities. Sound waves are described in terms of their frequency (cycles per s, or Hertz [Hz]) and amplitude (height of wave, measured in microns [ $\mu\text{m}$ ]). The power (Wattage) of an ultrasonic device is characterized by the amount of energy (Joules) passed to the medium per s. Some researchers report their ultrasonic treatments in terms of intensity, or Watts per area. However, Zisu (2013) chose to use energy density, or Joules per volume liquid. The energy density is a result of treatment at a set frequency and power defined by the ultrasonic device, and subject to change depending on the selected amplitude and duration of treatment. With that foundation laid, the mechanics of the process can be explained.

When a sound wave travels through a liquid, its energy moves as a series of compressions and rarefactions (Ashokkumar, 2010). Depending on the temperature of the liquid, its density or solids content, the vapor pressure, and the shape of the vessel the liquid is in, a number of bubbles are produced. In a fluid that is under pressure, at an elevated temperature, or has a low dissolved gas content, fewer bubbles are formed (Ashokkumar, 2010; Juliano et al., 2014; Khanal et al., 2014).

The bubbles in the fluid undergo a rapid growing and shrinking process as the sound energy passes through. Eventually, these bubbles can either explode or collapse violently, causing pockets of high temperature and pressure in the fluid. This process is called cavitation, and it creates a small-scale but intense fluid movement. This essentially acts as a churn for the liquid. Shear force capable of disrupting membranes and cleaving water into peroxide radicals is generated (Juliano et al., 2014; Marchesini et al., 2012). Although extreme heat results at the site of cavitation, the overall temperature of the fluid does not increase as much as in these localized spots. This allows for a “spot treatment” effect, where the temperature is over 5000 K in the gas of the bubble but less than 2000 K in the liquid phase immediately surrounding it (Ashokkumar, 2010).

Sonication can generate peroxide radicals from water molecules under certain processing conditions (Ashokkumar 2010; Feril and Kondo, 2005). Radical formation has been associated with the number of cavitation bubbles in a fluid and their size (Juliano et al., 2014). Most ultrasonic food processing is done between 20-24 kHz frequency. Above this frequency range, smaller cavitation bubbles are generated, but in larger numbers. They collapse faster and less violently than the large bubbles of 20 kHz sonication (Juliano et al., 2014; Wu et al., 2012). In fact, these small bubbles generated by high frequency sound waves are also called “standing”



bubbles because they grow and implode so quickly and regularly that they appear not to move at all, in contrast to the erratic life cycle of transient bubbles caused by lower frequencies (Ashokkumar, 2010). Interestingly, the creation of radicals seems to be tied more closely with the number of cavitation bubbles in the fluid, not to the intensity of their collapse. Therefore, higher frequency US results in more free radicals (Ashokkumar, 2010; Juliano et al., 2014). Reactive oxygen species formed by this process can be extremely detrimental to the sensory quality of milk, and their effects are discussed further in the following section.

### **Ultrasound's Effects on Milk**

Ultrasound has been used to successfully alter protein structure and functionality to aid in processing of several dairy foods (Arzeni et al., 2012). The quality of both cheese and yogurt depend on the ability of milk proteins to form a network. Yogurt processing involves a preheating step, which removes some water and denatures whey proteins ( $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin). The unfolded proteins have exposed reaction sites and side chains and are then more easily formed into a gel. US is also capable of denaturing whey, but without the increase in hydrophobic and disulfide interactions that are caused by heating and result in undesirable or premature clumping (Reiner et al, 2009; Zisu et al., 2010). Reiner and colleagues (2009) also found that US whey denaturation for yogurt production was a more energy-efficient process than heating alone, using 66% of the energy required for the conventional method.

Whey protein is popularly used in nutritional supplements and fitness beverages, and US has applications in those processes as well. Martini and fellow researchers (2012) used a 15W, 15 minute US treatment to improve the solubility and clarity of whey, while simultaneously eliminating cardboard and malty odors from the solution. Other researchers had similar success

using US to treat liquid whey before drying to create more stable whey protein or milk protein powders (Chandrapala et al., 2012; O'Sullivan et al., 2014; Zisu et al., 2010).

Ultrasound's effect on casein is less dramatic. Casein naturally exists in milk as micelles, or spherical arrangements of  $\alpha$ - and  $\beta$ -caseins with  $\kappa$ -casein and colloidal calcium phosphate (CCP) on the hydrophilic exterior of the micelle. According to Shanmugam and colleagues (2012),  $\beta$ -casein is most affected by 15 min of US at 90W and 180W, while  $\alpha$ - and  $\kappa$ -casein required further treatment in order to denature. Although  $\kappa$ -casein at the micelle surface was eventually disturbed by the US treatment, the micelle remained intact because of the CCP's stable interactions (Shanmugam et al., 2012).

Besides the main nutritional proteins casein and whey, milk is also host to native enzymes that can be affected by sonication as well. Some of these enzymes can have injurious effects on milk quality. Plasmin, or milk protease, is one such enzyme. Plasmin refers to the active component of the zymogen plasminogen. Plasminogen is cleaved into action by a system of activators, which in turn are regulated by a system of activator inhibitors. Plasminogen is found on the casein micelle, while the inhibitors are in the whey. During pasteurization, the plasmin inhibitors are destroyed, and the plasmin is then free to actively degrade casein throughout storage, resulting in age gelation defects (Politis et al., 1993). Ultrasound has the potential to remedy this problem by destroying plasmin activity. Villamiel and deJong (2000) demonstrated the use of TS for inactivation of alkaline phosphatase,  $\gamma$ -glutamyltranspeptidase, and lactoperoxidase. Previous work by a colleague at Iowa State showed promising results for the reduction of plasmin activity via TS as well, but the processing times in that study ranged from 1 to 3 min, which would not be feasible for integration with HTST processing in a dairy plant (Vijayakumar et al., 2015).

One of the most useful applications of US for protein alteration lies in its ability to reduce particle sizes. This is especially relevant for another milk component as well: fat.

Homogenization is a well-studied application for US, as it is an efficient method of breaking down the milk fat globule membrane (MFGM) and evenly distributing smaller milk fat globules throughout the serum (Villamiel and DeJong, 2000). However, US may reduce fat particle size a bit too much. Some studies show a reduction in pH after US, which may be tied to the release of free fatty acids (FFA) (Bermudez-Aguirre et al., 2008). Native milk lipase or bacterial lipases can easily work upon these FFA to produce short-chain FA associated with rancid aroma and flavor. FFA are also more susceptible to oxidation, especially by free radicals (Juliano et al., 2014).

During the fracture of milk fat globules, the MFGM becomes pitted, which creates increased surface area (Marchesini et al., 2012). Casein micelles can bind the membrane, resulting in clusters of protein and fat. This is a desirable event if milk is being used for cheesemaking, but not so great for fluid milk production. Bacteria may also latch onto the damaged MFGM, making them more difficult to kill by heat or sonication itself (Bermudez-Aguirre et al., 2008). The protective effect of fat will be discussed more in reference to US's action against bacteria.

The final macronutrient component of milk is lactose. Ultrasound's interaction with lactose has not been well-studied. Some research shows that US is able to release  $\beta$ -galactosidase from cells that can hydrolyze lactose into its monosaccharide components: glucose and galactose (Kardos and Luche, 2001). The authors also theorize that the ultrasonic energy itself can cleave the glycosidic bond of the disaccharide. However, these experiments were performed at the high

frequency of 200 kHz, which is generally out of range for most food processing applications (Kardos and Luche, 2001).

### **Microbial destruction via ultrasound**

Ultrasound has three basic mechanisms for killing bacteria: disrupting or thinning cell membranes, extreme (localized) heat, and free radical damage (Herceg et al., 2012). The antimicrobial effects of US, especially in conjunction with heat, have been demonstrated by several researchers over the past 15 years (Cameron et al., 2008; Christen et al., 2012; Czank et al., 2010; Chouliara et al., 2010; Evelyn et al., 2015; Herceg et al., 2012; Villamiel and DeJong, 2000). Villamiel and DeJong (2000) were among the first to promote the use of TS, reporting that the combination of heat and US was much more effective for inactivating enzymes and reducing microbial load compared to US or heating alone. Since then, scientists have been trying to determine whether US is a viable alternative or adjunct to traditional thermal processing such as HTST.

There is no question that bacteria are affected by US; however, the nuances of that effect are complex and must be examined. Cameron and colleagues (2008) used a transmission electron microscope to observe *Escherichia coli*, *Lactobacillus acidophilus*, and *Saccharomyces cerevisiae* that had been treated for 2 and 5 min with US at 25°C. They found that *E. coli*, the Gram negative organism, was the most damaged. It appeared that the outer cell membrane had been sucked into the cell and formed tiny sacs, as if around cavitation bubbles. The yeast, the only eukaryote in the study, ended the treatment with few organelles left intact. The Gram positive *L. acidophilus* appeared protected by its peptidoglycan cell wall and was able to resist serious damage for up to 5 min of treatment, as opposed to 2 min for the other organisms (Cameron et al., 2008). This study supports findings by Herceg et al. (2012) and Czank et al.

(2010), which concluded that Gram negative organisms are more susceptible to US than Gram positive.

The complete story may be more complicated. As previously mentioned, the amplitude, power, and duration of US can have a profound effect on the outcome. Since so few researchers are using the same treatment conditions, and many do not report the energy delivered to the sample, it is impossible to compare results accurately. It has also been suggested that it is not Gram status, but rather the shape of the cell that is important. Or, it could be the thickness of the polysaccharide and protein capsule outside of some cell membranes that has a protective effect (Marchesini et al., 2015).

To further complicate matters, US can also be used to stimulate bacterial growth. Low, sub-lethal doses of US can bust up clumps of cells, increasing total counts (Marchesini et al., 2015). US has potential to stimulate fermentation starter cultures and speed their action (Barukcic et al., 2014). Most unsettling, US can induce spore germination, leading to increased vegetative cell counts (Khanal et al., 2014).

Mild heat has been shown to increase the resistance of spores; in colloquial terms, what doesn't kill you makes you stronger (Scheldeman et al., 2006). Fortunately, the opposite may be true for US. Khanal and colleagues (2014) found that their US treatment of 1 to 10 min at 91 and 114  $\mu\text{m}$  amplitude in an ice bath was not able to destroy spores, but it was able to damage them enough to make them susceptible to thermal destruction. This is a promising finding, and one that supports the hypothesis behind the second part of the microbiology study in this project.

### **Ultrasound's sensory and milk quality effects**

Dairy fat is an important component of milk flavor and consumer acceptance (Bus et al., 2002). Therefore, any alteration to the integrity of the dairy fat can greatly change the

acceptability of the product. As previously mentioned, US can generate free radicals, and lipids are susceptible to radical oxidation by these compounds. In addition, the extreme heat and pressure created by cavitation can induce redox reactions independent of radical mechanisms (Juliano et al., 2014; Reiner et al., 2009). This second scenario may be more likely due to the large activation energy of radical lipid oxidation and the need for a high US frequency in order to form appreciable numbers of free radicals. More research is needed to discern which mechanism is more common under the US conditions used in food processing.

Either way, many reactions occur during US treatment that may result in sensory changes to the milk. Several researchers have reported a rubbery, plastic, or burnt aroma associated with US (Chouliara et al., 2010; Juliano et al., 2014; Reiner et al., 2009; Vijayakumar et al., 2015). This aroma was attributed to volatile lipid oxidation products, but only recently were the identity of some of these smelly compounds discovered (Marchesini et al., 2015).

Preliminary work by Reiner and colleagues (2009) used gas chromatography-mass spectroscopy (GC-MS) to examine pasteurized and homogenized 1.5% fat milk that had been treated with 2.5 to 20 min of 24 kHz US at 45°C. The researchers found that sonicated samples contained 1-hexene, 1-octene, 1-nonene, benzene, toluene, *n*-hexanal, and *n*-heptanal, among other volatiles. However, they were not able to identify one specific compound responsible for the rubbery aroma. Chouliara and others attempted to correlate GC results with a sensory panel. Panelists noted burnt, foreign, and chemical aromas in the milk which rendered them unacceptable to consumers. The US treatment was done at a shorter duration, lower temperature, and lower power compared to Reiner's study, but off aromas were still evident. However, they were also not able to pinpoint which volatile was to blame for the odor (2010).

Most recently, Marchesini and colleagues (2015) were able to find a statistically significant correlation between certain volatile compounds and specific aromas identified by a trained sensory panel. GC-MS analysis showed the rubbery smell was associated primarily with  $\delta$ -dodecalactone; metallic flavor was due to dodecanoic acid; burnt flavor resulted from octanoic acid; and what the panelists called “sharp” was from decanoic acid, methyl ester. All of the flavors and aromas originated from a combination of these compounds, but the statistical analysis showed strong correlations between certain compounds and sensory attributes (Marchesini et al., 2014). The authors of this study concluded that ultrasonication’s damage to sensory quality outweighed its benefits as an antimicrobial.

If these aforementioned rubbery-smelling volatiles are the result of lipid oxidation, then it follows that skim milk (containing less than 0.5% milkfat) would be less susceptible to this kind of adverse effect. That might not be the case. The trace amounts of fat in skim milk are usually in a less intact form than the whole globules seen in whole milk, and therefore can be more easily oxidized (Juliano et al., 2014). In that case, it may be more important to control the heat of sonication. As previously mentioned, the volatiles may not be the result of radical oxidation but rather the intensity of the heat and pressure of cavitation.

It is evident that the detrimental sensory effects of US pose a major concern and must be overcome if US is to be taken seriously as an alternative processing method. More research is needed to narrow down the sonication parameters that result in the least damage to sensory quality while still achieving the desired treatment effect.

**REFERENCES**

- Arzeni C, Martinez K, Zema P, Arias A, Perez OE, Pilosof AMR. 2012. Comparative study of high intensity ultrasound effects on food proteins functionality. *J. Food Eng.* 108: 463-472.
- Ashokkumar, M. 2010. The characterization of acoustic cavitation bubbles—An overview. *Ultrasonics Sonochem.* 18: 864-872.
- Barukcic I, Jakopovic KL, Herceg Z, Karlovic S, Bozanic R. 2015. Influence of high intensity ultrasound on microbial reduction, physic-chemical characteristics and fermentation of sweet whey. *Innov Food Sci Emerging Tech.* 27: 94-101.
- Bermudez-Aguirre D, Mawson R, Barbosa-Canovas GV. 2008. Microstructure of fat globules in whole milk after thermosonication treatment. *J. Food Sci.* 73:E325-332.
- Braem G, De Vliegher S, Verbist B, Heyndrickx M, Leroy F, and De Vuyst L. 2012. Culture-independent exploration of the teat apex microbiota of dairy cows reveals a wide bacterial species diversity. *Vet. Microbiol.* 157: 383-390.
- Bus A, Worsley A. 2003. Consumers' sensory and nutritional perceptions of three types of milk. *Pub. Health Nutr.* 6(2): 201-208.
- Cameron M, McMaster LD, Britz TJ. 2008. Electron microscopic analysis of dairy microbes inactivated by ultrasound. *Ultrasonics Sonochem.* 15: 960-964.
- Chandrapala J, Zisu B, Kentish S, Ashokkumar M. 2012. The effects of high-intensity ultrasound on the structural and functional properties of a-lactalbumin, b-lactoglobulin and their mixtures. *Food Research Int.* 48: 940-943.
- Chouliara E, Georgogianni KG, Kanellopoulou N, Kontominas MG. 2010. Effect of ultrasonication on microbiological, chemical, and sensory properties of raw, thermized and pasteurized milk. *Int. Dairy J.* 20: 307-313.
- Christen L, Tat Lai C, Hartmann PE. 2012. Ultrasonication and the quality of human milk: variation of power and time of exposure. *J. Dairy Res.* 79: 361-366.
- Czank C, Simmer K, Hartmann PE. 2010. Simultaneous pasteurization and homogenization of human milk by combining heat and ultrasound: effect on milk quality. *J. Dairy Res.* 77: 183-189.



- Evelyn E, Silva FVM. 2015. Thermosonication versus thermal processing of skim milk and beef slurry: Modeling the inactivation kinetics of psychrotrophic *Bacillus cereus* spores. *Food Res. Int.* 67: 67-74.
- Fairlife. 2015. Our Process. [www.fairlife.com/our-process](http://www.fairlife.com/our-process). Accessed June 2015.
- Farm-to-Consumer Legal Defense Fund (FTCLDF). 2013. State-by-state review of raw milk laws. [www.farmtoconsumer.org](http://www.farmtoconsumer.org). Accessed June 2015.
- Feril LB, Kondo T. 2005. Major factors involved in the inhibition of ultrasound-induced free radical production and cell killing by pre-sonication incubation or high cell density. *Ultrasonics Sonochem.* 12(5): 353-357.
- Food and Drug Administration. 2011. Pasteurized Milk Ordinance. Department of Health and Human Services.
- Fromm HI and Boor KJ. 2004. Characterization of pasteurized fluid milk shelf-life attributes. *J. Food Sci.* 69(8): M207-M214.
- Hayes W, White CH, Drake MA. 2002. Sensory aroma characteristics of milk spoilage by *Pseudomonas species*. *J Food Sci.* 67: 448-454.
- Herceg Z, Jambrak AR, Lelas V, Thagard SM. 2012. The effect of high intensity ultrasound treatment on the amount of *Staphylococcus aureus* and *Echerichia coli* in milk. *Food Tech Biotech.* 50(1): 46-52.
- Huck JR, Sonnen M, Boor KJ. 2008. Tracking heat-resistant, cold-thriving fluid milk spoilage bacteria from farm to packaged product. *J.Dairy Sci.* 91(3): 1218-1228.
- Ivy RA, Ranieri ML, Martin NH, den Bakker HC, Xavier BM, Wiedmann M, Boor KJ. 2012. Identification and characterization of psychrotolerant sporeformers associated with fluid milk production and processing. *Appl. Env. Microbiol.* 78(6):1853-1864.
- Jenness, R. 1988. Milk Composition. The Fundamentals of Dairy Chemistry. 3<sup>rd</sup> ed. Aspen Publishers, Gaithersburg, MD.
- Juliano P, Torkamani AE, Leong T, Kolb V, Watkins P, Ajlouni S, Singh TK. 2014. Lipid oxidation volatiles absent in milk after selected ultrasound processing. *Ultrasonics Sonochem.* 21: 2165-2175.

- Kardos N, Luche J-L. 2001. Sonochemistry of carbohydrate compounds. *Carbohydrate Res.* 332: 115-131.
- Khanal SN, Anand S, Muthukumarappan K. 2014. Evaluation of high-intensity ultrasonication for the inactivation of endospores of 3 bacillus species in nonfat milk. *J Dairy Sci.* 97(10): 5952-5963.
- Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Buchele G, Weber J, Sozanska B, Danielewicz H, Horack E, van Neerven RJJ, Heederik D, Lorenzen PC, vonMutius E, Braun-Fahrlander C. 2011. The protective effect of farm milk consumption on childhood asthma and atopy: The GABRIELA study. *J Allergy Clin Immunol.* 128(4): 766-773.
- Lucking G, Stoeckel M, Atamer Z, Hinrichs J, Ehling-Schulz M. 2013. Characterization of aerobic spore-forming bacteria associated with industrial dairy processing environments and product spoilage. *Int. J. Food Microbiol.* 166: 270-279.
- MacDonald LE, Brett J, Kelton D, Majowicz SE, Snedeker K, Sargeant JM. 2011. A systematic review and meta-analysis of the effects of pasteurization on milk vitamins, and evidence for consumption and other health-related outcomes. *J. Food Protect.* 74(11): 1814-1832.
- Marchesini F, Balzan S, Montemurro F, Fasolato L, Andrighetto I, Segato S, Novelli E. 2012. Effect of ultrasound alone or ultrasound coupled with CO<sub>2</sub> on the chemical composition, cheese-making properties and sensory traits of raw milk. *Innov. Food Sci. Emerging Tech.* 16: 391-397.
- Marchesini G, Fasolato L, Novelli E, Balzan S, Contiero B, Montemurro F, Andrighetto I, Segato S. 2015. Ultrasonic inactivation of microorganisms: A compromise between lethal capacity and sensory quality of milk. *Innov. Food Sci. Emerging Tech.* 29: 215-221.
- Martin NH, Ranieri ML, Murphy SC, Ralyea RD, Wiedmann M, and Boor KJ. 2011. Results from raw milk microbiological tests do not predict the shelf-life performance of commercially pasteurized fluid milk. *J. Dairy Sci.* 94(3): 1211-1222.
- Martini S, Walsh MK. 2012. Sensory characteristics and functionality of sonicated whey. *Food Res. Int.* 49: 694-701.
- O'Sullivan J, Arellano M, Pichot R, Norton I. 2014. The effect of ultrasound treatment on the structural, physical, and emulsifying properties of dairy proteins. *Food Hydrocolloids.* 42:386-396.

- Pediatrics. 2014. Consumption of raw or unpasteurized milk and milk products by pregnant women and children. *Pediatrics*. 133: 175-179.
- Politis I, Zavisian B, Barbano DM, Gorewit RC. 1993. Enzymatic assay for the combined determination of plasmin plus plasminogen in milk: Revisited. *J. Dairy Sci.* 76: 1260-1267.
- Quigley L, O'Sullivan O, Stanton C, Beresford TP, Ross RP, Fitzgerald GF, Cotter PD. 2013. The complex microbiota of raw milk. *FEMS Microbiol. Rev.* 37: 664-698.
- Ranieri ML and Boor KJ. 2009. Short communication: Bacterial ecology of high-temperature, short-time pasteurized milk processed in the United States. *J. Dairy Sci.* 92(10): 4833-4840.
- Ranieri ML, Huck JR, Sonnen M, Barbano DM, Boor KJ. 2009. High temperature, short time pasteurization temperatures inversely affect bacterial numbers during refrigerated storage of pasteurized fluid milk. *J. Dairy Sci.* 92(10): 4823-4832.
- Reiner J, Noci F, Cronin DA, Morgan DJ, Lyng JG. 2009a. Characterisation of volatile compounds generated in milk by high intensity ultrasound. *Int. Dairy J.* 19: 269-272.
- Reiner J, Noci F, Cronin DA, Morgan DJ, Lyng JG. 2009b. The effect of thermosonication of milk on selected physiochemical and microstructural properties of yoghurt gels during fermentation. *Food Chem.* 114: 905-911.
- Scheldeman P, Herman L, Foster S, Heyndrickx M. 2006. *Bacillus sporothermodurans* and other highly heat-resistant spore formers in milk. *J. Appl. Microbiol.* 101: 542-555.
- Shanmugam A, Chandrapala J, Ashokkumar M. 2012. The effect of ultrasound on the physical and functional properties of skim milk. *Innov. Food Sci. Emerging Technol.* 16: 251-258.
- Soria A C, Villamiel M. 2010. Effect of ultrasound on the technological properties and bioactivity of food: a review. *Trends Food Sci. Technol.* 21: 323-331.
- Stewart H, Dong D, Carlson A. 2013. Why are Americans consuming less fluid milk? A look at generational differences in intake frequency. *USDA ERS Report* 149.
- Ternstrom A, Lindberg AM, Molin G. 1993. Classification of the spoilage flora of raw and pasteurized bovine milk, with special reference to *Pseudomonas* and *Bacillus*. *J. Appl. Bacteriol.* 75: 25-34.

- Vijayakumar S, Grewell D, Annandarajah C, Benner L, Clark S. In Press. Quality characteristics and plasmin activity of thermosonicated skim milk and cream. *J Dairy Sci.*
- Villamiel M and de Jong P. 2000. Influence of high-intensity ultrasound and heat treatment in continuous flow on fat, proteins, and native enzymes of milk. *J. Agri. Food Chem.* 48: 472-478.
- Weisbecker, A. 2007. A legal history of raw milk in the United States. *J. Environ. Health.* 69: 62-64.
- Wu X, Joyce EM, Mason TJ. 2012. Evaluation of the mechanisms of the effect of ultrasound on *Microcystis aeruginosa* at different ultrasonic frequencies. *Water Res.* 46: 2851-2858
- Zisu B, Bhaskaracharya R, Kentish S, Ashokkumar M. 2010. Ultrasonic processing of dairy systems in large scale reactors. *Ultrasonics Sonochem.* 17: 1075-1081.
- Zisu B, Schleyer M, Chandrapala J. 2013. Application of ultrasound to reduce viscosity and control the rate of age thickening of concentrated skim milk. *Int. Dairy J.* 31: 41-43.

**CHAPTER 3: SHORT-DURATION COLD SONICATION AND  
THERMOSONICATION AFFECT THE TOTAL AEROBIC COUNT OF MILK  
SUPPLEMENTED WITH *PAENIBACILLUS AMYLOLYTICUS***

**Benner, L<sup>1</sup>, Clark, S.<sup>2\*</sup>**

<sup>1</sup>Dr. Pepper Snapple Group. Plano, TX 75024

<sup>2</sup>Department of Food Science and Human Nutrition, Iowa State University, IA 50010

\*Corresponding author, milkmade@iastate.edu

**ABSTRACT**

High-power, low-frequency ultrasound has been suggested as a novel processing technique with potential to extend milk shelf life via inactivation of bacteria and spores that survive standard high temperature short time pasteurization. Long-duration sonication yields off-aroma formation and undesired physical and chemical effects. The objective of this study was to examine the effect of short-duration ( $\leq 60$  s) sonication treatment on bacterial counts. Skim milk was inoculated with spore-forming psychrotolerant spoilage bacteria *Paenibacillus amylolyticus*. Milk was sonicated under six selected amplitude and time condition, both before (cold sonication, CS) and after (thermosonication, TS) pasteurization (72°C for 15 s). Milk was stored up to 50 d and total aerobic count (TAC) of bacteria was determined weekly. Neither TS nor CS treatments successfully reduced TAC to an equivalent level as pasteurization alone. Evidence from this and other recent research suggests short-duration ultrasound under the conditions studied to date may not be an appropriate technique for reducing bacterial count in fluid milk beyond that of pasteurization.

**Key words:** spores, dairy, ultrasound

## INTRODUCTION

Heat is the conventional method for milk preservation. The industry standard, high temperature short time pasteurization (72°C for  $\geq 15$  s), kills pathogenic and spoilage bacteria, resulting in a shelf life of 21-28 days (FDA, 2011). Further heat treatment ( $\geq 135^\circ\text{C}$ ) and aseptic packaging can extend shelf life past 6 months without refrigeration, but once the milk is opened, its shelf life is no more than that of HTST (FDA, 2011). One reason behind the short shelf life of milk is the presence of bacterial spores in the milk that are unaffected by pasteurization, even at high temperatures. A spore is a dormant form of bacteria that is resistant to extreme temperatures, acid, alkalinity, and oxidizing agents (Khanal et al., 2014). *Bacillus* and *Paenibacillus* are the most abundant sporeformers that contaminate milk (Martin et al., 2011). These closely-related, Gram positive, psychrotrophic rods are differentiated by *Paenibacillus*' ability to metabolize lactose and grow at refrigerator (4°C) temperatures. Consequently, if *Paenibacillus* spores are present, they can germinate and proliferate during refrigerated storage, leading to spoiled, bitter-tasting milk (Fromm and Boor, 2004; Rainieri and Boor, 2009).

Since heat alone is not able to kill these spores, researchers are turning to high-power ultrasound as a means of killing bacteria in dairy products while decreasing the need for intense heat treatments that may harm milk quality from a sensory, functional, and nutritional standpoint (Bermudez-Aguirre et al., 2008; Chouliara et al., 2010; Czank et al., 2010; Evelyn et al., 2015; Herceg et al., 2012; Vijayakumar et al., 2015; Villamiel and DeJong, 2000). Ultrasound utilizes acoustic energy to create cavitation, or tiny bubbles that expand and collapse, resulting in localized high temperature and pressure that is capable of thinning cell membranes and producing free radicals (Ashokkumar, 2010; Herceg et al., 2012). Electron microscope examination of *Bacillus coagulans* and *B. licheniformis* spores exposed to ultrasound (1 to 10

min at 92 and 114  $\mu\text{m}$  amplitude) revealed that the spore coat was damaged and made more susceptible to thermal damage (Khanal et al., 2014). Some researchers have found that ultrasound is only an effective method for bacterial inactivation when performed in conjunction with heat (thermosonication [TS]) (Czank et al., 2010, Christen et al., 2012, Herceg et al., 2012, Villamiel and DeJong, 2000). However, heat treatment may only strengthen spores (Scheldeman et al., 2006). If so, TS may make the spore contamination problem worse by simply germinating spores instead of killing them (Khanal et al., 2014).

Most previous research with ultrasound has been on the order of 30 seconds to several minutes (Cameron et al., 2008; Christen et al., 2012; Czank et al., 2010; Chouliara et al., 2010; Evelyn et al., 2015; Herceg et al., 2012; Villamiel and DeJong, 2000). However, long duration may not be feasible in the dairy industry, since high temperature short time (HTST) treatments are typically 15 to 25 seconds.

The purpose of the present study was to examine the effect of two different methods of short-duration ultrasound treatment on skim milk samples that were inoculated with *Paenibacillus amylolyticus*, one spore-forming, thermophilic and psychrophilic bacteria of concern (Huck et al., 2008). The objective was to determine whether ultrasound treatment after pasteurization (TS) was more or less effective at reducing the total aerobic bacteria count compared to pasteurization alone, or ultrasound treatment in an ice bath followed by pasteurization (cold sonication [CS]).

## MATERIALS AND METHODS

### Milk preparation

Raw whole milk was obtained from the Iowa State University Dairy (Ames, IA) bulk tank. Milk was immediately transported to the Iowa State University Center for Crop Utilization Research pilot plant, where it was separated into cream and skim fractions (Figure 3.1) using a centrifugal cream separator (Varidrive Motor, US Electrical Motors, Inc., Milford, CT; 1750 rpm). Skim milk was collected in sterile containers. Approximately 1600 mL raw skim milk was inoculated with up to 6 log CFU/mL *Paenibacillus amylolyticus* (H7-0689; Cornell Milk Quality Institute, Ithaca, NY). Milk was refrigerated at 4°C for up to 1 hour before processing.

### Controls

For each lot of milk, inoculated “raw control” milk was stored in 10 mL sterile plastic snap-top tubes for enumeration during up to eight days of storage, in order to determine the initial concentration of total aerobic bacteria present in the unprocessed milk. After eight days, spoilage was evident in raw control milk, in the form of flocculation and confirmed by bacterial counts exceeding 6 log CFU/mL.

For each lot of milk, raw skim milk (100 mL) spiked with *P. amylolyticus* was heated in a sanitized stainless steel bowl over a hot plate set to 300°C. Milk was stirred with a sanitized rod approximately every 30 s, heated until reaching 72° C ± 1°C, and held for 15 s. The milk was transferred to a sonicating rosette submerged in a 73°C water bath and temperature change was recorded. For the “pasteurized controls”, the milk was immediately divided into 10 mL sterile plastic snap-top tubes. One tube was allocated to be opened for analysis weekly, during storage for up to 50 days. Each of the replications was conducted 3 times over the course of 12 weeks.



### **Thermosonicated Samples**

For each lot of milk, 100 mL raw skim milk spiked with *P. amylolyticus* was pasteurized in the same manner as for the pasteurized control. However, after the milk was transferred to a 300 mL capacity sonicating rosette submerged in a 73°C water bath and temperature change was recorded, the Branson 2000 (2200 W max power, 20 kHz frequency) 1:8 titanium sonicating horn with 1.1.5 booster was lowered 2 to 3 cm into the milk for sonication under the conditions listed in Table 3.1. Sample temperatures were recorded at start of heating, end of heating, start of sonication, and end of sonication (Table 3.1). Thermosonicated milk was divided into 10 mL aliquots and stored in sterile snap-top plastic tubes for weekly analysis for up to 50 days. Each of these TS treatments was repeated 3 times over the course of 12 weeks.

### **Cold Sonicated Samples**

Raw skim milk (100 mL) spiked with *P. amylolyticus* was transferred to a sonication rosette set in an ice bath. Milk was subjected to the ultrasound treatments listed in Table 3.2. After sonication, each milk sample was transferred to a sanitized stainless steel bowl and pasteurized as previously described. Sample temperatures were recorded at start of sonication, end of sonication/start of heating, and end of heating (Table 3.2). Cold-sonicated milk was divided into 10 mL aliquots and stored in sterile snap-top plastic tubes for weekly analysis for up to 50 days. Each of these ultrasound treatments was repeated 3 times over the course of 12 weeks.

### **Total Aerobic Counts**

The concentration of viable aerobic bacteria in each milk sample was determined by performing total aerobic counts. Preliminary work for this project, as well as published research (Blackburn et al., 1995; Casillas-Buenrostro et al., 2012), confirmed that aerobic plate count

Petrifilm (3M, Minneapolis, MN) delivers accurate and reproducible results comparable to brain-heart infusion (BHI) agar pour plates recommended by Martin (personal correspondence, 2015). Colonies on Petrifilm plates were also easier to enumerate due to their bright red appearance. In contrast, pour plates inoculated with undiluted milk samples were difficult to count accurately as a result of the milk's opaque and hazy appearance. In addition, Petrifilm was chosen because of its use in the dairy industry as a rapid and space-efficient quality control technique. Therefore, all TAC was performed on 3M® Petrifilm Aerobic Plate Count plates (Fisher Scientific, Pittsburgh, PA) for this study.

Pasteurized control and all sonicated milk samples were plated undiluted on day one of storage. Raw control dilutions of  $10^{-4}$  to  $10^{-6}$  were plated to confirm presence of live microorganisms and to ensure that the 5-log kill required for pasteurization was obtained (FDA, 2011). Plates were incubated at 32°C for enumeration after 96 h. Total aerobic count was expressed in terms of colony forming units per milliliter and log-transformed (log CFU/mL) for readability. Because each set of samples had a different initial bacterial count, expressing results as average log counts does not necessarily represent the effect of the treatment. Therefore, all treatment effects are expressed as a difference between the log TAC of the treatment and the log TAC of the corresponding pasteurized control. A positive value indicates a higher average TAC compared to pasteurization, while a negative change means that the treatment was more effective than pasteurization alone.

### **Statistical Analysis**

Energy density differences were analyzed using JMP (JMP Pro 11). A one-way ANOVA was performed with Tukey-Kramer adjustment for multiple comparisons and significance of  $\alpha \leq 0.05$ . Analysis of microbial counts was done using SAS (version 9.4). A linear mixed model

(Glimmix) was used for days 1 through 22 of storage, with treatment and day as fixed effects and set and set-treatment interaction were random effects. Days 36 through 50 were not included in this model due to data censoring from estimated counts.

## RESULTS AND DISCUSSION

Table 3.1 summarizes the treatment conditions for TS and CS. Mean initial and final temperatures were recorded to monitor the amount of heat generated by US. Because sonication was performed in temperature-controlled situations (i.e. ice bath and hot water bath), the temperature change of all milk samples before and after treatment was less than 4°C for TS and less than 6°C for CS. This is a very minimal increase, implying that any treatment differences were a result of the sound energy and subsequent cavitation instead of a bulk temperature increase. Table 3.1 gives a statistical analysis and summary of these values. Energy density was calculated according to Zisu et al. (2013) by dividing the Joules of energy delivered to the sample by the sample volume. This allows a more direct comparison between treatments in terms of their intensity, rather than simply expressing US treatments in terms of their amplitude, wattage, or frequency.

Despite initially selecting diverse US treatments based on amplitude and duration, energetically, many treatments were very similar. Statistical analysis of the energy density for all treatments reveals that in general, more energy was transferred to the CS samples than the TS samples for a given treatment (Table 3.1). This is in agreement with literature; prior research has reported that as the temperature of a fluid increases, so does its vapor pressure, leading to less violent cavitation and therefore less energy transfer (Herceg et al., 2012; Juliano et al., 2014). The difference in energy density between the TS and CS samples was not significant for 50  $\mu\text{m}/20$  s, 150  $\mu\text{m}/10$  s, and 200  $\mu\text{m}/10$  s treatments, but CS had a significantly higher energy

density compared to TS for the 170  $\mu\text{m}$  /60 s, 50  $\mu\text{m}$  /60 s, and 100  $\mu\text{m}$  /30 s treatments (Table 3.1). As mentioned, a number of the treatments' energy densities were statistically identical to each other. The CS 50  $\mu\text{m}$  /60 s, 100  $\mu\text{m}$  /30 s, and 200  $\mu\text{m}$  /10 s, along with the TS 200  $\mu\text{m}$  /10 s and 100  $\mu\text{m}$  /30 s treatments, all delivered the same amount of energy, ranging from an average of 20.1 to 25.1 J/mL (Table 3.1). The CS 170  $\mu\text{m}$  /60 s was the most energy-dense, at 103.4 J/mL. The TS treatments of the same time and amplitude were significantly lower than CS, averaging 79.6 J/mL. The lowest energy was delivered by the 50  $\mu\text{m}$ /20 s treatment, which did not differ significantly between CS and TS conditions. (Table 3.1)

Since the majority of treatments were not significantly different from each other in terms of energy density, any differences in total aerobic count between such energetically identical treatments may be attributed to temperature (TS vs. CS), amplitude or treatment time, rather than the amount of energy delivered. Christen et al. (2012) theorized that exposure time—not amount of ultrasonic power—was the most important factor for inactivation of *Escherichia coli*. Marchesini et al. (2015) also found that ultrasound duration was significant in relation to *E. coli*, *Pseudomonas fluorescens*, and *Staphylococcus aureus* kill. Other researchers have found that amplitude is important because of an increase in area being affected by sonic energy as amplitude increases (Khanal et al., 2014).

The mean log total aerobic bacteria counts (TAC) for all treatments through day 22 of storage are included in Table 3.2, along with the standard error. Raw milk (data not shown) was only plated to day 8, when spoilage became evident. A sample was judged spoiled when it reached 6 logs TAC/mL or when protein coagulation (flocculation) was visible in the sample container (Fromm and Boor, 2004). Spoiled samples were not enumerated.

On day 1, the pasteurized control had a mean TAC of 1.49 log CFU/mL, while all TS and CS treatments ranged between 1.47 to 1.71 log CFU/mL. No appreciable differences were seen between treatments or TS and CS conditions. A similar pattern continued up to day 15; the TAC of treatment means were slightly higher those that of the pasteurized controls (1.42 log CFU/mL to 4.69 log CFU/mL versus 1.23 log CFU/mL for pasteurized control).

Day 22 is an important time point because it is a typical pasteurized milk shelf life. By day 22, all TS and CS treatments, as well as the pasteurized control, were still less than 6 log CFU/mL, and none showed evidence of flocculation. However, several treatments had a higher mean log TAC approaching 4 to 5 log CFU/mL, which may have tasted spoiled to discerning consumers. 50  $\mu$ m/20 s TS had a TAC of 4.69 log CFU/mL, and the 170  $\mu$ m/60 s TS sample averaged 4.17 log CFU/mL. The CS treatments maintained lower counts ranging from a low of 1.42 log CFU/mL for 200  $\mu$ m/10 s to a high of 3.45 log CFU/mL for 150  $\mu$ m/10 s. The lowest CS TAC is still higher than the pasteurized control at 1.23 log CFU/mL.

By day 29, bacterial growth patterns became unpredictable, and mean TAC had to be estimated due to bacterial growth exceeding that of the predicted dilution level, or due to very little growth occurring (Table 3.3). Many replicates experienced large jump in TAC greater than the week-to-week change seen earlier in shelf life. The TS treatments were particularly prone to this. On day 29, the 50  $\mu$ m /20 s TS TAC was estimated at greater than 4.7 log CFU/mL, while the 170  $\mu$ m/60 s averaged less than 5.7 log CFU/mL. The lowest TS TAC was for 50  $\mu$ m/60 s and 200  $\mu$ m/10 s, both estimated at greater than 2 log CFU/mL. In contrast, the CS treatments' TAC ranged from 1.32 log CFU/mL to greater than 3.5 log CFU/mL. Statistical analysis was not performed for any of the mean log TAC values in Tables 3.2 and 3.3.

Because experiments were conducted over 12 weeks using a different batch of milk each week, each batch of milk had its own set of controls. Milk naturally contains a variable amount of bacteria based on the cleanliness of the milking conditions and dairy workers (Huck et al., 2008; Ranieri and Boor, 2009). Therefore, a simple mean created from treatments with different initial bacterial counts are not an accurate representation of the treatment effect. Data were transformed in terms of the pasteurized control corresponding to the batch of milk from which that treatment originated. Data from days 1 to 22 are presented as the log difference between the treatment TAC and the TAC of the pasteurized control from the same milk batch (Table 3.5). This procedure allows control for milk batch as a source of random variation. A negative log difference value indicates that the treatment had a lower TAC than the control, meaning that the US treatment was more effective than pasteurization alone. Only the 100  $\mu\text{m}/30$  s TS treatment on day 1, 50  $\mu\text{m}/60$  s CS on day 15, 100  $\mu\text{m}/30$  s CS on day 15, 170  $\mu\text{m}/60$  s CS on day 15, and 200  $\mu\text{m}/10$  s CS on day 15 had negative mean log differences. This means that the majority of both TS and CS treatments increased the TAC compared to the pasteurized control by anywhere from less than 0.1 log to as much as 3 to 4 logs, in the case of day 22 170  $\mu\text{m}/60$  TS and 100  $\mu\text{m}/30$  TS, respectively.

Statistical analysis using a mixed linear model (Glimmix) determined that the only significant differences between the pasteurized control and US treatments were on day 22 for all TS treatments as well as one CS treatment: 170  $\mu\text{m}/60$  s (Table 3.4). But again, these differences were all *increases* in TAC compared to pasteurized control instead of decreases. Obviously, this was not the intended treatment effect. The findings lead us to believe that US, particularly TS, stimulated spore germination rather than killing bacteria. One reason for this phenomenon was explained by Ranieri et al. (2009). Although their research did not focus on US technology, the

authors found that higher pasteurization temperatures (85.2°C instead of 72.9°C) led to increased spore formation and eventual cell growth among Gram positive contamination bacteria during subsequent storage. Our TS samples were not pasteurized at a higher temperature, but some experienced a small bulk temperature increase due to sonication (Table 3.1), and the localized extreme temperature resulting from cavitation. Khanal et al. (2014) applied this theory to US and found similar results: US treatments can simply lead spores to germinate faster rather than destroying them.

Although none of the TS or US treatments could be considered effective compared to the pasteurized controls on days 1 through 22, there were some significant differences between treatments in their effect on TAC. ANOVA was used to analyze the mean log differences from control between treatments on days 1 through 22 (Table 3.7) along with the mean energy density for each treatment for easy reference. The least energy-dense treatments (50  $\mu\text{m}/20$  s, both TS and CS) were surprisingly not the least effective treatments. However, 50  $\mu\text{m}/20$  s was the second-least effective, having the second-highest positive log difference from control. A mean difference from control could not be calculated for 50  $\mu\text{m}/20$  s CS on day 22 due to a lack of complete data on that day. The 100  $\mu\text{m}/30$  s TS treatment was the numerically highest mean difference from control on day 22, but was statistically similar to 50  $\mu\text{m}/20$  s TS, 150  $\mu\text{m}/10$  s TS, 170  $\mu\text{m}/60$  s TS, and 170  $\mu\text{m}/60$  s CS. Interestingly, these treatments all had statistically identical mean log difference values, but their energy density values are not identical. This may indicate that energy density alone is not directly related to the impact of the US treatment. It is possible that amplitude, time, and energy density are all important factors to consider when choosing US treatment settings.

Ultrasound treatments have been reported to damage cell membranes, causing them to buckle inward in varying degrees, as well as causing spores to wrinkle and shrink (Cameron et al., 2008; Khanal et al., 2014). Gram-negative microorganisms such as *E. coli* have a more flexible cell membrane compared to the more rigid wall of a Gram-positive cell. Using an electron microscope, Cameron et al. (2008) found that ultrasound was more harmful to Gram-negative cells for this reason. Despite seeing the damage inflicted by ultrasound, it is difficult to say whether it was the amount of energy, the treatment duration, or the US amplitude that had the most effect on the extent of the damage. Also, since the bacteria present in milk in the present study were not identified, it is impossible to say whether the majority of the population consisted of primarily of the supplemented *Paenibacillus* or other bacteria .

Numerically, it could be stated that 100  $\mu\text{m}/30$  s TS was the least effective treatment, while 200  $\mu\text{m} /10$  s CS, 100  $\mu\text{m}/30$  s CS, and 50  $\mu\text{m} /60$  CS were the most effective, having a mean log difference from pasteurized control of less than 1. In this case, all three of these treatments were energetically similar. However, the statistical analysis demonstrates that the variability among treatment groups was high, leading to few statistical differences between treatments overall.

One impediment to statistical analysis in this experiment was the lack of concrete data available past day 22 of storage for all samples. Bacterial growth was unpredictable on days 29, 36, 43, and 50. This may be because the TAC increased beyond the capacity of the milk's nutrients to support further growth, which caused a sudden surge then an unpredictable drop in TAC after day 29 in most samples.

In summary, although TS is touted by many researchers (Czank et al., 2010; Herceg et al., 2012; Vijayakumar et al., 2015; Villamiel and DeJong, 2000, etc.), the effect may be



different for sporeforming bacteria than for other microorganisms. We hypothesize, similar to Khanal et al. (2014), that during TS treatment's pasteurization step, vegetative bacteria are killed, but the thermophilic organisms, possibly including *P. amylolyticus* and other bacteria, are not killed. We hypothesize that spore-forming microorganisms form spores during the pasteurization, then during the subsequent non-lethal US dose, the spores germinate. With no further kill step, the vegetative cells are free to grow during refrigerated storage.

We reasoned that CS would have a greater effect on reducing TAC compared to TS. We predicted that ultrasound could be used to germinate spores or damage bacteria enough to make them vulnerable to heat (Cameron et al., 2008), and that subsequently pasteurization would kill them. Unfortunately, the results obtained in this study do not support that hypothesis. Five of the six CS treatments were statistically identical to the control on day 22, and the sixth treatment had a significantly higher TAC. It is possible that more time is needed between the sonication and heating steps, or that more severe sonication or pasteurization conditions are needed.

Under both TS and CS treatments conducted in the present study, it is also possible that thermophilic microorganisms such as *Bacillus sporothermodurans* or *Geobacillus stearothermophilus* were present, stimulated by ultrasound, and survived pasteurization (Casillas-Buenrostro et al., 2012). Because no isolation of microorganisms or biochemical tests were done in this study, it is impossible to conclude whether Gram-positive sporeformers were responsible for the observed milk spoilage. Although carefully avoided, Gram-negative contamination could have occurred at some point, in which case they may have outcompeted any *Paenibacillus* or *Bacillus* present because of their faster growth (Ranieri and Boor, 2009). In future studies, the identity of the microorganisms present before and after ultrasonication should

be determined in order to illuminate the best method for treating the specific type of cell or spore.

## CONCLUSION

Short-duration sonication ( $\leq 60$ s), under the conditions of this study (up to 200  $\mu\text{m}$  and 103.4 J/mL), are not an effective means of reducing total aerobic count (TAC) of milk supplemented with *Paenibacillus amylolyticus* compared to pasteurization. Cold sonication (CS) delivered more acoustic energy to a sample than thermosonication (TS) under the same amplitude and time conditions. However, no significant reductions in TAC resulted from any CS or TS treatment. Despite its energy density being significantly higher than the other treatments, the CS 170 $\mu\text{m}$ /60 s treatment resulted in significantly higher TAC compared to pasteurized control on day 22. US treatments may activate the heat-resistant spores of psychrotrophic microorganisms, which survive pasteurization, resulting in a higher TAC in treatments compared to pasteurized controls. Integration of TS with HTST, under the conditions of this study, is not a feasible means of extending milk shelf life. Cold sonication may be an appropriate method, but more research is needed on the effect of CS and subsequent heating, including the identity of surviving microorganisms to ensure effectiveness at eliminating bacteria from milk and extending milk shelf life beyond that of HTST pasteurization.

## REFERENCES

- Ashokkumar, M. 2010. The characterization of acoustic cavitation bubbles—An overview. *Ultrasonics Sonochem.* 18: 864-872.
- Bermudez-Aguirre D, Mawson R, Barbosa-Canovas GV. 2008. Microstructure of fat globules in whole milk after thermosonication treatment. *J. Food Sci.* 73:E325-332.

- Blackburn C de W, Baylis CL, Petitt SB. 1995. Evaluation of Petrifilm methods for enumeration of aerobic flora and coliforms in a wide range of foods. *Letters in Applied Microbiology*, 22, 137-140.
- Casillas-Buenrostro RM, Heredia NL, Benesh DL, Garcia S. 2012. Efficacy of 3M Petrifilm aerobic count plates for enumerating *Bacillus sporothermodurans* and *Geobacillus stearothermophilus* in UHT milk. *Int. Dairy J.*, 25, 147-149.
- Chouliara E, Georgogianni KG, Kanellopoulou N, Kontominas MG. 2010. Effect of ultrasonication on microbiological, chemical, and sensory properties of raw, thermized and pasteurized milk. *International Dairy Journal*, 20, 307-313.
- Christen L, Tat Lai C, Hartmann PE. 2012. Ultrasonication and the quality of human milk: variation of power and time of exposure. *Journal of Dairy Research*, 79, 361-366.
- Czank C, Simmer K, Hartmann PE. 2010. Simultaneous pasteurization and homogenization of human milk by combining heat and ultrasound: effect on milk quality. *Journal of Dairy Research*, 77, 183-189.
- Evelyn E, Silva FVM. 2015. Thermosonication versus thermal processing of skim milk and beef slurry: Modeling the inactivation kinetics of psychrotrophic *Bacillus cereus* spores. *Food Research International*, 67, 67-74.
- Food and Drug Administration. 2011. Pasteurized Milk Ordinance. Department of Health and Human Services.
- Fromm HI and Boor KJ. 2004. Characterization of pasteurized fluid milk shelf-life attributes. *Journal of Food Science*, 69(8), M207-M214.
- Herceg Z, Jambrak AR, Lelas V, Thagard SM. 2012. The effect of high intensity ultrasound treatment on the amount of *Staphylococcus aureus* and *Echerichia coli* in milk. *Food Technology and Biotechnology*, 50 (1), 46-52.
- Huck JR, Sonnen M, Boor KJ. 2008. Tracking heat-resistant, cold-thriving fluid milk spoilage bacteria from farm to packaged product. *Journal of Dairy Science*, 91(3), 1218-1228.
- Juliano P, Torkamani AE, Leong T, Kolb V, Watkins P, Ajlouni S, Singh TK. 2014. Lipid oxidation volatiles absent in milk after selected ultrasound processing. *Ultrasonics Sonochemistry*, 21, 2165-2175.

- Khanal SN, Anand S, Muthukumarappan K. 2014. Evaluation of high-intensity ultrasonication for the inactivation of endospores of 3 bacillus species in nonfat milk. *J Dairy Sci.* 97(10): 5952-5963.
- Marchesini G, Fasolato L, Novelli E, Balzan S, Contiero B, Montemurro F, Andrighetto I, Segato S. 2015. Ultrasonic inactivation of microorganisms: A compromise between lethal capacity and sensory quality of milk. *Innovative Food Science and Emerging Technologies*, 29, 215-221.
- Martin NH. 2015. Personal correspondence. Cornell University, Ithaca, NY.
- Martin NH, Ranieri ML, Murphy SC, Ralyea RD, Wiedmann M, and Boor KJ. 2011. Results from raw milk microbiological tests do not predict the shelf-life performance of commercially pasteurized fluid milk. *Journal of Dairy Science*, 94(3), 1211-1222
- Ranieri ML and Boor KJ. 2009. Short communication: Bacterial ecology of high-temperature, short-time pasteurized milk processed in the United States. *Journal of Dairy Science*, 92 (10), 4833-4840.
- Scheldeman P, Herman L, Foster S, Heyndrickx M. 2006. *Bacillus sporothermodurans* and other highly heat-resistant spore formers in milk. *Journal of Applied Microbiology*, 101, 542-555.
- Vijayakumar S, Grewell D, Annandarajah C, Benner L, Clark S. 2015. Quality characteristics and plasmin activity of thermosonicated skim milk and cream. *J Dairy Sci.* In press.
- Villamiel M and de Jong P. 2000. Influence of high-intensity ultrasound and heat treatment in continuous flow on fat, proteins, and native enzymes of milk. *Journal of Agricultural Food Chemistry*, 48, 472-478.

**Table 3.1. Sonication settings, average temperature before and after treatment, and average energy density for thermosonicated and cold sonicated skim milk, including statistical analysis of energy density.**

Amplitude (%)	Amplitude (μm)	Treatment Time (s)	Initial T (°C)	T after CS (°C)	T after HTST (°C)	T before TS (°C)	T after TS (°C)	Mean Energy density (J/mL)
21%	50	20	15.3	n/a	72.6	71.1	71.5	6.2 <sup>G</sup>
21%	50	60	12.2	n/a	72.8	70.0	70.6	19.2 <sup>DE</sup>
42%	100	30	10.9	n/a	72.2	69.6	71.0	20.2 <sup>CDE</sup>
63%	150	10	14.6	n/a	72.8	71.8	71.5	11.2 <sup>FG</sup>
72%	170	60	14.1	n/a	72.5	71.2	74.5	79.6 <sup>B</sup>
84%	200	10	11.4	n/a	72.4	70.4	71.6	19.8 <sup>CD</sup>
21%	50	20	10.4	9.1	72.3	n/a	n/a	8.5 <sup>G</sup>
21%	50	60	8.7	7.8	72.2	n/a	n/a	25.1 <sup>C</sup>
42%	100	30	10.5	9.3	72.9	n/a	n/a	24.7 <sup>CD</sup>
63%	150	10	15.1	11.4	72.9	n/a	n/a	15.0 <sup>EF</sup>
72%	170	60	11.7	17.5	73.2	n/a	n/a	103.4 <sup>A</sup>
84%	200	10	9.9	9.8	73.0	n/a	n/a	22.7 <sup>CD</sup>
	Raw	Control	11.6	n/a	n/a	n/a	n/a	n/a
	Pasteurized	Control	10.9	n/a	73.3	n/a	n/a	n/a

3 HTST: High temperature short time pasteurization conditions (72°C, 15 sec)

4 TS: Thermosonication

5 CS: Cold sonication treatment conducted while sonication vessel was submerged in ice bath

6 n/a: Not applicable

7 <sup>A-G</sup>: Energy density values with the same letters are statistically identical

**Table 3.2. Least squares mean log total aerobic bacteria count for milk stored 1, 8, 15 and 22 days  $\pm$  standard error, from linear mixed model. All values are average of 3 observations unless noted. First number (50, 100, 150, 170, 200) indicates treatment amplitude, second number (20, 60, 30, 10) indicates treatment time (s).**

<b>Treatment</b>	<b>Day 1</b>	<b>Day 8</b>	<b>Day 15</b>	<b>Day 22</b>
50/20 TS	1.59 $\pm$ 0.438	1.80 $\pm$ 0.438	2.53 $\pm$ 0.438	4.69 $\pm$ 0.438
50/20 CS	1.69 $\pm$ 0.438	1.75 $\pm$ 0.438	2.27 $\pm$ 0.438	1.92 $\pm$ 0.528*
50/60 TS	1.54 $\pm$ 0.438	1.68 $\pm$ 0.438	2.19 $\pm$ 0.438	2.84 $\pm$ 0.438
50/60 CS	1.63 $\pm$ 0.438	1.67 $\pm$ 0.438	1.47 $\pm$ 0.438	2.00 $\pm$ 0.438
100/30 TS	1.47 $\pm$ 0.438	1.60 $\pm$ 0.438	2.50 $\pm$ 0.438	3.81 $\pm$ 0.438
100/30 CS	1.58 $\pm$ 0.438	1.68 $\pm$ 0.438	1.52 $\pm$ 0.438	1.53 $\pm$ 0.438
150/10 TS	1.71 $\pm$ 0.438	1.71 $\pm$ 0.438	2.54 $\pm$ 0.438	3.46 $\pm$ 0.438
150/10 CS	1.71 $\pm$ 0.438	1.76 $\pm$ 0.438	2.54 $\pm$ 0.438	3.45 $\pm$ 0.438
170/60 TS	1.61 $\pm$ 0.438	1.68 $\pm$ 0.438	2.65 $\pm$ 0.438	4.17 $\pm$ 0.438
170/60 CS	1.63 $\pm$ 0.438	1.67 $\pm$ 0.438	2.22 $\pm$ 0.438	2.49 $\pm$ 0.438
200/10 TS	1.56 $\pm$ 0.438	1.74 $\pm$ 0.438	2.37 $\pm$ 0.438	2.82 $\pm$ 0.438
200/10 CS	1.55 $\pm$ 0.438	1.57 $\pm$ 0.438	1.40 $\pm$ 0.438	1.42 $\pm$ 0.438
Pasteurized control	1.49 $\pm$ 0.313***	1.56 $\pm$ 0.313***	1.89 $\pm$ 0.313***	1.23 $\pm$ 0.341**

\* 2 observations

\*\* 5 observations

\*\*\* 6 observations

**Table 3.3. Mean and estimated mean log total aerobic bacteria count for milk stored 29, 36, 43, and 50 days. First number (50, 100, 150, 170, 200) indicates treatment amplitude, second number (20, 60, 30, 10) indicates treatment time (s).**

<b>Treatment</b>	<b>Day 29</b>	<b>Day 36</b>	<b>Day 43</b>	<b>Day 50</b>
50/20 TS	> 4.7 E	> 5.9 E	S	S
50/20 CS	> 3 E	< 2 E	2.59	2.85
50/60 TS	> 2 E	> 5.6 E	S	S
50/60 CS	1.32	< 1.72 E	< 1.13 E	1.24
100/30 TS	> 3.6 E	> 6.2 E	S	S
100/30 CS	2.12	< 2.91 E	< 3 E	< 2.5 E
150/10 TS	< 3.6 E	> 4.4 E	S	S
150/10 CS	> 3.5 E	< 3.7 E	< 3 E	< 3 E
170/60 TS	< 5.7 E	> 5.3 E	S	S
170/60 CS	< 2.9 E	> 2.5 E	> 3.3 E	> 3.1 E
200/10 TS	> 2 E	> 4 E	S	S
200/10 CS	1.5	< 1.2 E	< 0.81 E	1.79
Pasteurized control	> 1.56 E	< 2.01 E	2.44	1.86

**E: Estimated value**

**Table 3.4. Treatments that differ significantly from pasteurized control (PC) in mixed linear model (Day 22)**

Treatment	Log Difference from PC	SE	p value
50/20 TS	3.42	0.533	<0.0001
50/60 TS	1.57	0.5267	0.0038
100/30 TS	2.54	0.527	<0.0001
150/10 TS	2.19	0.533	<0.0001
170/60 TS	1.22	0.533	<0.0001
170/60 CS	2.90	0.533	0.0247
200/10 TS	1.55	0.527	0.0043

TS: Thermosonicated treatment

CS: Cold sonicated treatment

**Table 3.5. One-way ANOVA analysis of mean log difference from control for TAC of milk treated with ultrasound before (CS) or after (TS) pasteurization  $\pm$  standard error, for milk stored 1, 8, 15 and 22 days. First number (50, 100, 150, 170, 200) indicates treatment amplitude, second number (20, 60, 30, 10) indicates treatment time (s).**

Treatment	Energy Density (J/mL)	Day 1	Day 8	Day 15	Day 22
50/20 TS	6.2 <sup>G</sup>	0.087 $\pm$ 0.362 <sup>CDE</sup>	0.197 $\pm$ 0.362 <sup>CDE</sup>	0.283 $\pm$ 0.362 <sup>CDE</sup>	3.24 $\pm$ 0.443 <sup>AB</sup>
50/20 CS	8.5 <sup>G</sup>	0.187 $\pm$ 0.362 <sup>CDE</sup>	0.147 $\pm$ 0.362 <sup>CDE</sup>	0.0233 $\pm$ 0.362 <sup>CDE</sup>	ND
50/60 TS	19.2 <sup>DE</sup>	0.067 $\pm$ 0.362 <sup>CDE</sup>	0.180 $\pm$ 0.362 <sup>CDE</sup>	0.657 $\pm$ 0.362 <sup>CDE</sup>	1.66 $\pm$ 0.362 <sup>BCD</sup>
50/60 CS	25.1 <sup>C</sup>	0.160 $\pm$ 0.362 <sup>CDE</sup>	0.170 $\pm$ 0.362 <sup>CDE</sup>	-0.063 $\pm$ 0.362 <sup>DE</sup>	0.82 $\pm$ 0.362 <sup>CDE</sup>
100/30 TS	20.1 <sup>CDE</sup>	-0.010 $\pm$ 0.362 <sup>CDE</sup>	0.0967 $\pm$ 0.362 <sup>CDE</sup>	1.44 $\pm$ 0.443 <sup>BCDE</sup>	4.06 $\pm$ 0.443 <sup>A</sup>
100/30 CS	24.7 <sup>CD</sup>	0.103 $\pm$ 0.362 <sup>CDE</sup>	0.177 $\pm$ 0.362 <sup>CDE</sup>	-0.0133 $\pm$ 0.362 <sup>DE</sup>	0.35 $\pm$ 0.362 <sup>CDE</sup>
150/10 TS	11.2 <sup>FG</sup>	0.210 $\pm$ 0.362 <sup>CDE</sup>	0.103 $\pm$ 0.362 <sup>CDE</sup>	0.303 $\pm$ 0.362 <sup>CDE</sup>	2.14 $\pm$ 0.443 <sup>ABCD</sup>
150/10 CS	15.0 <sup>EF</sup>	0.210 $\pm$ 0.362 <sup>CDE</sup>	0.143 $\pm$ 0.362 <sup>CDE</sup>	0.210 $\pm$ 0.362 <sup>E</sup>	0.455 $\pm$ 0.443 <sup>CDE</sup>
170/60 TS	79.6 <sup>B</sup>	0.110 $\pm$ 0.362 <sup>CDE</sup>	0.0733 $\pm$ 0.362 <sup>CDE</sup>	0.407 $\pm$ 0.362 <sup>CDE</sup>	3.00 $\pm$ 0.627 <sup>ABC</sup>
170/60 CS	103.4 <sup>A</sup>	0.123 $\pm$ 0.362 <sup>CDE</sup>	0.0667 $\pm$ 0.362 <sup>CDE</sup>	-0.020 $\pm$ 0.362 <sup>DE</sup>	1.47 $\pm$ 0.627 <sup>ABCDE</sup>
200/10 TS	19.7 <sup>CD</sup>	0.087 $\pm$ 0.362 <sup>CDE</sup>	0.237 $\pm$ 0.362 <sup>CDE</sup>	0.833 $\pm$ 0.362 <sup>CDE</sup>	1.63 $\pm$ 0.362 <sup>BCD</sup>
200/10 CS	22.7 <sup>CD</sup>	0.073 $\pm$ 0.362 <sup>CDE</sup>	0.0700 $\pm$ 0.362 <sup>CDE</sup>	-0.130 $\pm$ 0.362 <sup>DE</sup>	0.23 $\pm$ 0.362 <sup>CDE</sup>

<sup>A-G</sup>Energy density values with the same letters are statistically identical

<sup>A-E</sup>Mean log differences with the same letters are statistically identical

ND: not determined



## CHAPTER 4: DESCRIPTIVE SENSORY EVALUATION OF SHORT-DURATION THERMOSONICATED MILK

Benner, L<sup>1</sup>, Clark, S.<sup>2\*</sup>

<sup>1</sup>Dr. Pepper Snapple Group, Plano, TX 75024

<sup>2</sup>Department of Food Science and Human Nutrition, Iowa State University, IA 50010

\*Corresponding author, milkmade@iastate.edu

### ABSTRACT

Raw skim milk, maintained at 72°C, was treated with 20 J/mL and 80 J/mL ultrasound (thermosonicated) and stored for up to 21 days at 4°C. A descriptive sensory panel (9 panelists) identified cooked, lacks freshness, and rubbery as aromas of importance in pasteurized and thermosonicated milk, and were trained to identify intensity. The aromas of thermosonicated and pasteurized samples were evaluated on days 1, 3, 8, and 21 of storage. No significant differences were seen in cooked or lacks freshness aromas among samples or over time. The milk treated at 80 J/mL had a significantly higher offensive rubbery aroma on day 1 compared to milk treated at 20 J/mL. The sensory effects of thermosonation on milk may not limit the commercial feasibility of the technology, but must seriously be considered.

**Key words: ultrasound, aroma, quality**

## INTRODUCTION

The sensory quality of milk is of the utmost importance to consumers (Bus and Worsley, 2002). Alternative processing techniques have been explored for their potential to increase milk shelf life, but their effect on sensory attributes has only recently been studied (Chouliara et al., 2010; Marchesini et al., 2015; Martini et al., 2010; Reiner et al., 2009).

Ultrasound is an alternative processing technique that has been suggested as a way to decrease microbial load and inactivate milk enzymes such as plasmin, which can degrade casein and contribute to the undesirable consequence known as age gelation, or increased viscosity during refrigerated storage. Thermosonication (TS) is the combined treatment of heat and high-power ultrasound (16-100 kHz frequency and 10-1000 W/cm<sup>2</sup> power density (Soria and Villamiel, 2010)), and various studies have shown that heat and ultrasound have a beneficial synergistic effect (Czank et al., 2010; Villamiel and DeJong, 2000).

However, TS has been associated with off-odor and off-flavor formation in milk, a phenomenon that has been studied but not entirely explained. Ultrasound energy can induce peroxide formation from water hydrolysis, which can lead to radical oxidation of lipids present in the milk. Reiner and colleagues (2009) used gas chromatography/mass spectrometry (GC/MS) analysis to detect volatile hydrocarbons such as benzene, toluene, and various dienes and alkenes resulting from treatment times up to 20 min with 400W sonication in previously pasteurized milk. Aroma compounds were studied from a sensory perspective by Chouliara and colleagues (2010), who found that panelists' acceptance of samples was lower for thermosonicated samples as compared to untreated milk. Both Reiner et al. (2009) and Chouliara et al. (2010) cited a "rubbery" or "burnt" chemical taste in thermosonicated samples, which panelists found

objectionable. Chouliara and colleagues (2010) also evaluated milk sample aromas and found that taste was a more sensitive method for detecting undesirable volatiles than smell.

More recent work has sought to explain the origins of these undesirable taste and aroma compounds. GC/MS analysis, in combination with a trained sensory panel, was used to show a significant association between certain compounds and flavors in milk sonicated in an ice bath (24 kHz, 400 W power, treated at 70% and 100% for 50-300s). Specifically,  $\delta$ -dodecalactone was correlated with rubbery aroma and taste, while burnt was associated with octanoic acid, and metallic with dodecanoic acid. Other compounds such as decanoic acid methyl ester, hexanoic acid, and  $\delta$ -decalactone were also present and contributed to the treated milk's flavor (Marchesini et al., 2015).

These studies, as well as most other research involving the effects of sonication or TS on milk quality, employ treatment times exceeding one minute at various power or amplitude levels. Although favorable microbiological results were obtained at these long durations, ultrasound treatment on a large scale would not be feasible if several minutes were required to treat the product. Current HTST pasteurization in dairy plants involves heating milk to above 72°C for at least 15 s. In order to be a commercially feasible processing technique in a plant environment, ultrasound treatments would likewise need to be a matter of seconds, not minutes.

The objective of the present study was to determine whether TS treatments of 10 and 60 s would affect the aroma quality of skim milk. Because TS is not an approved pasteurization process, human subjects were trained to evaluate only the odor of thermosonicated skim milk samples. The trained descriptive analysis panel sniffed pasteurized and sonicated milk samples for up to 21 days of refrigerated storage to determine whether the rubbery aroma was detectable, and if so, whether it persisted throughout normal HTST pasteurized milk shelf life.

## MATERIALS AND METHODS

### Panel Recruitment and Training

Nine panelists (eight female, one male) were recruited from Iowa State University. All had prior descriptive analysis experience. Group training sessions were held for one hour per week for five weeks, with two additional individual practice sessions held at the panelists' convenience.

The first training session focused on identifying the typical milk aroma profile. Panelists agreed that fresh pasteurized skim milk should be free of offensive off-notes such as sourness, and the aroma should be clean, slightly sweet, and have a hint of characteristic dairy fat richness. Then, panelists smelled three sample treatments. The first was raw skim milk heated to  $72 \pm 1^\circ\text{C}$  for 15 s. The second was raw skim milk heated identically, then subjected to 200  $\mu\text{m}$  ultrasonication for 60 s (100 J/mL) to provide an extreme sonication example. The final sample was raw skim milk that had been collected from the dairy farm bulk tank three days prior. Panelists were guided through generating terms to describe the aromas they detected in these three samples. Attributes such as sour, acid, barny, goaty, earthy, dirty, and lacks freshness were attributed to the raw milk. The pasteurized sample was deemed cooked, nutty, toasted, sweet aromatic, caramel, eggy, and custardy. The thermosonicated sample shared many of the same descriptors as the pasteurized milk, but it was additionally noted to be burnt, plastic, rubbery, and chemical.

During the second session, all of the terms generated at the first training were compiled and examined. Similar or redundant terms were eliminated, and panelists selected terms that were most appropriate and easily understood. Duplicate milk samples to the ones smelled at the first training session were evaluated, and panelists reassessed the validity of the terms in

question. Ultimately, the terms cooked, rubbery, and lacks freshness were chosen, and anchors were selected for each aroma and defined in relation to a 15-cm line scale (Table 4.1). Anchors are a reference by which to judge the intensity of the corresponding aroma manifested in a sample.

The third through fifth training sessions were opportunities for panelists to practice sample evaluation in a group setting. Panelists sniffed samples and discussed their observations until consensus was reached. There were two additional 30-min individual sniffing sessions held to test within- and between-panelists consistency, without discussion.

### **Sample Preparation**

Raw milk was purchased from the Iowa State University Dairy Farm (Ames, IA), separated with a centrifugal cream separator (Varidrive Motor, US Electrical Motors, Inc., Milford, CT; 1750 rpm) to remove cream, and the skim milk was stored at 4°C in opaque, sanitized bottles for up to three days before processing.

Two TS treatments and a pasteurized control were prepared. The pasteurized control was prepared by heating raw skim milk in a glass beaker set on a hot plate with magnetic stir bar to 72 ±1°C for 15 s. Thermosonicated samples were pasteurized as described, transferred to sonication rosettes submerged in a 73°C water bath, and subjected to 170 µm ultrasound for 60 s (72% power, 80 J/mL) or 200 µm for 10 s (84% power, 20 J/mL). A Branson 2000 sonication unit providing 2200W max power and 20 kHz frequency was used with a 1:1.5 booster and 1:8 titanium horn. Sample temperature did not exceed 73°C during processing. After treatment, approximately 15 mL of each was transferred into sanitized, opaque screw-top containers (ULINE, Pleasant Prairie, WI)—one for each panelist and day of evaluation. Each treatment was replicated three times.

## Sample Evaluation

Each treatment was evaluated by the nine panelists on day 1, 3, 8, and 21 of storage. Panelists were given no more than six randomly presented samples at each evaluation session. Panelists were asked to place a vertical mark on a 15-cm line indicating the intensity of the cooked, rubbery, and lacks freshness attributes they detected in the sample. The distance from zero to the marked segment was measured in cm.

## Statistical Analysis

ANOVA was performed to analyze both the differences in mean sensory scores for each aroma between treatments on each day, and for the difference between days for each treatment using JMP (Version 11 Pro). Tukey-Kramer adjustment for multiple comparisons was utilized and significance was set at  $\alpha=0.05$ .

## RESULTS AND DISCUSSION

The panelists' mean scores for cooked, rubbery, and lacks freshness aromas are displayed for each treatment (Table 4.2). At  $\alpha=0.05$ , there were no significant differences found between the panelists' ratings of the cooked attribute for the 80 J/mL, 20 J/mL and pasteurized control samples. Intensity of the cooked attribute also did not vary by day of storage; there were no significant differences in cooked aroma between day 1 and day 21 for any of the treatments. Although the results are not statistically significant, the pasteurized control was rated less cooked than the two thermosonicated samples for all evaluation days except Day 3, when it averaged slightly more than the 20 J/mL sample. Part of the higher-intensity cooked aroma in the thermosonicated samples can be explained by longer exposure to 72°C heat in the water bath during sonication. In addition, the cavitation heat and pressure generated by ultrasound energy

itself is capable of denaturing whey protein and producing sulfhydryl aromas (Juliano et al., 2014). Cooked was the predominant aroma identified by sensory panelists in this study, but the intensity of the cooked aroma was neither extreme (mean scores below 7.0 on 15-cm line scale) nor intensified by the TS treatments selected in the present work. Cooked flavor in milk is due to the denaturation of whey proteins, specifically  $\beta$ -lactoglobulin, and consequent formation of sulfhydryl groups, dimethyl sulfide, or methanethiol (Christensen and Reineccius, 1992; Hutton and Patton, 1952). These give the milk an eggy or custard smell that can range from sweet to acrid. However, these aromas are not considered offensive at a low level, and Maillard browning caramelized smells often appear in cooked samples as well (Christensen and Reineccius, 1992).

Similar to the cooked attribute, there were no significant differences between the intensity of lacks freshness aroma in the two thermosonicated samples and pasteurized control (Table 4.2). Additionally, the amount of lacks freshness aroma did not significantly increase as length of refrigerated storage (21 days) increased ( $\alpha=0.05$ ). The low mean scores (below 3.0 on 15-cm line scale) demonstrate that the pasteurization process and the TS treatments selected in the present study enabled milk to smell fresh for up to 21 days, which is the typical shelf life of pasteurized milk.

The rubbery aroma, unlike the other attributes in question, did vary significantly among treatments (Table 4.2). The 80 J/mL milk yielded a mean score of 4.5 out of 15 on the first day of storage. This was significantly higher than the mean score on day 1 for the 20 J/mL sample (1.3), but was statistically identical to the score of the pasteurized control samples (2.1) because of high standard deviations. The 80 J/mL milk also had higher rubbery aroma on day 1, than the same freshly-opened product on day 21, and both the pasteurized control and 20 J/mL treatments freshly-evaluated on days 8 and 21. No other significant differences were noted between

treatments, on the same days of evaluation, beyond day 1, indicating that the rubbery attribute faded rapidly. Additionally, it should be noted that the rubbery aroma never exceeded 5 on a 15-cm line scale, suggesting that the mild treatments selected for the present study may be applicable to commercial applications from a sensory standpoint. Similar to other investigations, TS induced a rubbery off-aroma in milk. Mean rubbery aroma scores are similar to but slightly lower than those observed by Vijayakumar et al. (2015) using similar ultrasonic amplitude conditions but longer treatment times (up to 3 min). Additionally, the present work demonstrates that with short-duration, the rubbery aroma dissipated relatively rapidly. Since short-duration TS milk may not be distinguishable from pasteurized milk by the time that consumers receive the milk (generally within 3 days of processing), short-duration TS may be appropriate for industry applications from a sensory standpoint.

The aromas produced by TS are distinct in origin from traditional cooked aromas, but as the results of the present study demonstrate, they are not easily distinguished by a trained sensory panel. Standard deviations for rubbery were greater than the average rubbery rating for all samples over all evaluation days. Despite training, some panelists were more sensitive to rubbery than others. Some panelists identified strong cooked aromas as rubbery or vice versa. However, statistical analysis determined that no one panelist skewed data more than any other, so no data were discarded. In contrast to pasteurization, ultrasound energy produces off-flavors resulting from radical or cavitation-induced-heat damage to milk components, specifically fat (Juliano et al., 2014). This experiment used high-power, low-frequency sonication (20 kHz). At this frequency, the size of cavitation bubbles formed in a fluid such as milk are larger and less numerous than what would be present at a higher frequency. The number of free radicals generated is correlated with both the number of bubbles and the violence of their collapse. Large bubbles collapse more



violently than small bubbles, but the end result is fewer free radicals (Marchesini et al., 2012; Juliano et al., 2014). This indicates that the rubbery aroma in the ultrasonicated samples may originate from heat-induced oxidation of lipids into volatile compounds instead of a radical mechanism. If ultrasound is to be applied to a dairy processing operation, it will be important to consider the sensory effects of the treatment. Milk is a complex fluid and its components are subject to damage from acoustic cavitation. The possibilities of lipid oxidation, whey denaturation, reduction of milkfat globule size, and changes to the casein micelle structure should all be considered. Some of these changes are beneficial or desired. However, except for homogenization effects, physical changes may not be desirable in fluid milk intended for direct consumption, where consumers crave a clean-tasting, refreshing beverage with characteristic fresh dairy flavor. Further research on ultrasound treatment of fluid dairy milk is needed to illuminate the line between improved functionality or stability and sensory quality.

Although this study evaluated skim milk, skim milk is not entirely fat-free. This residual fat tends to be more susceptible to radical reactions because it may not be contained within intact milkfat globules. Additionally, indigenous milk lipases or those produced by contaminating psychrotolerant bacteria can contribute to volatile formation during refrigerated shelf life, exacerbating the off-flavor problem (Juliano et al., 2014). For the most sensitive of consumers, the results of this study demonstrate that even a mild TS treatment of 72% power for 60 s (80 J/mL) can cause a rubbery aroma, which might be objectionable during early shelf life. Although the rubbery odor faded significantly within 21 days, the most sensitive consumer could perceive a rubbery-smelling product.

## CONCLUSION

Trained panelists were able to detect a significantly higher rubbery aroma in skim milk that was thermosonicated for 60 s at 170 $\mu$ m (80 J/mL) compared to milk treated at 200 $\mu$ m for 10 s (20 J/mL) within a day of treatment. The rubbery aroma never exceeded 5 on a 15-cm line scale, suggesting that the mild treatments selected for the present study may be applicable to commercial applications, from a sensory standpoint. However, the research demonstrates that even mild thermosonication treatments of one minute or less can have a noticeable detrimental effect on sensory quality of milk that may offend the most sensitive of consumers.

## REFERENCES

- Bus A, Worsley A. 2003. Consumers' sensory and nutritional perceptions of three types of milk. *Pub. Health Nutr.* 6(2): 201-208.
- Chouliara E, Georgogianni KG, Kanellopoulou N, Kontominas MG. 2010. Effect of ultrasonication on microbiological, chemical, and sensory properties of raw, thermized and pasteurized milk. *Int. Dairy J.* 20: 307-313.
- Czank C, Simmer K, Hartmann PE. 2010. Simultaneous pasteurization and homogenization of human milk by combining heat and ultrasound: effect on milk quality. *Journal of Dairy Research*, 77, 183-189.
- Juliano P, Torkamani AE, Leong T, Kolb V, Watkins P, Ajlouni S, Singh TK. 2014. Lipid oxidation volatiles absent in milk after selected ultrasound processing. *Ultrasonics Sonochem.* 21: 2165-2175.
- Marchesini F, Balzan S, Montemurro F, Fasolato L, Andrighetto I, Segato S, Novelli E. 2012. Effect of ultrasound alone or ultrasound coupled with CO<sub>2</sub> on the chemical composition, cheese-making properties and sensory traits of raw milk. *Innov. Food Sci. Emerging Tech.* 16: 391-397.
- Marchesini G, Fasolato L, Novelli E, Balzan S, Contiero B, Montemurro F, Andrighetto I, Segato S. 2015. Ultrasonic inactivation of microorganisms: A compromise between lethal capacity and sensory quality of milk. *Innov. Food Sci. Emerging Tech.* 29: 215-221.

- Martini S, Walsh MK. 2012. Sensory characteristics and functionality of sonicated whey. *Food Res. Int.* 49: 694-701.
- Reiner J, Noci F, Cronin DA, Morgan DJ, Lyng JG. 2009a. Characterisation of volatile compounds generated in milk by high intensity ultrasound. *Int. Dairy J.*19: 269-272.
- Soria A C, Villamiel M. 2010. Effect of ultrasound on the technological properties and bioactivity of food: a review. *Trends Food Sci. Technol.* 21: 323-331.
- Vijayakumar S, Grewell D, Annandarajah C, Benner L, Clark S. In Press. Quality characteristics and plasmin activity of thermosonicated skim milk and cream. *J Dairy Sci.*
- Villamiel M and de Jong P. 2000. Influence of high-intensity ultrasound and heat treatment in continuous flow on fat, proteins, and native enzymes of milk. *Journal of Agricultural Food Chemistry*, 48, 472-478.

**Table 4.1. Terms and anchors for the aroma attributes of thermosonicated skim milk.**

Term	Description	Anchors
Cooked	Characteristic of heated milk, encompassing a range of aromas from slight sweet/caramel to toasted nuts to custard/egg.	Fairlife skim milk = score of 10 50/50 Fairlife skim and conventional skim= score of 5
Rubbery	The rubber and chemical aroma of rubber bands	Rubber bands = score of 15 Rubber bands in skim milk= score of 5
Lacks Freshness	Milk that is spoiling or has absorbed unpleasant off-aromas from the milking environment. Described with terms such as acidic, barny, stale, dirty, or unclean	Raw milk stored 3 days=score of 5 Raw milk stored 8 days=score of 15

**Table 4.2. Average panelist rating (on 15-cm line scale) of cooked, lacks freshness and rubbery aroma attributes of skim milk subjected to thermosonication or pasteurization.**

Treatments	Day 1	Day 3	Day 8	Day 21
<b>Cooked</b>				
170µm/60s (80 J/mL)	3.8±3.8 <sup>A</sup>	3.4±2.6 <sup>A</sup>	2.6±2.3 <sup>A</sup>	3.3±2.7 <sup>A</sup>
200 µm /10s (20 J/mL)	2.7±2.4 <sup>A</sup>	3.1±2.7 <sup>A</sup>	3.2±2.6 <sup>A</sup>	2.7±3.1 <sup>A</sup>
Pasteurized control	2.4±2.6 <sup>A</sup>	3.3±2.6 <sup>A</sup>	2.3±2.3 <sup>A</sup>	2.4±2.4 <sup>A</sup>
<b>Lacks Freshness</b>				
170µm/60s (80 J/mL)	0.8±1.6 <sup>A</sup>	0.4±0.7 <sup>A</sup>	1.5±2.0 <sup>A</sup>	1.3±2.4 <sup>A</sup>
200µm/10s (20 J/mL)	1.1±2.1 <sup>A</sup>	0.9±1.7 <sup>A</sup>	1.5±2.4 <sup>A</sup>	1.9±2.2 <sup>A</sup>
Pasteurized control	1.8±2.6 <sup>A</sup>	1.1±1.3 <sup>A</sup>	1.5±2.0 <sup>A</sup>	1.6±2.1 <sup>A</sup>
<b>Rubbery</b>				
170µm/60s (80 J/mL)	4.5±4.9 <sup>A</sup>	3.2±4.1 <sup>AB</sup>	2.0±3.0 <sup>AB</sup>	0.8±1.8 <sup>B</sup>
200 µm /10s (20 J/mL)	1.3±2.0 <sup>B</sup>	1.2±2.3 <sup>ABC</sup>	0.9±1.8 <sup>B</sup>	1.0±1.9 <sup>B</sup>
Pasteurized control	2.1±3.1 <sup>AB</sup>	2.0±3.1 <sup>AB</sup>	1.9±2.9 <sup>B</sup>	1.6±2.6 <sup>B</sup>

<sup>A, B</sup> Values with the same letter, within the same aroma category, are statistically identical

## CHAPTER 5: GENERAL CONCLUSIONS

Dairy processing with high-power, low-frequency ultrasound is an emerging field of research, and many complexities have yet to be teased out. Some studies have shown that ultrasound is capable of increased bacterial kill compared to pasteurization alone. It has applications in increasing protein functionality and performance of cheese and whey. However, ultrasound also affects the sensory quality of milk and produces undesirable flavors and aromas under certain treatment conditions.

The results of this research demonstrate the difficulty of performing consistent ultrasound treatments. Under the same frequency, amplitude, and time duration, all selected cold sonication (CS) treatments delivered numerically or significantly more energy to a sample compared to thermosonication (TS). Mean TAC was significantly higher than pasteurized control for all TS treatments (and one CS treatment) on day 22, which indicates that ultrasound likely stimulated the proliferation of aerobic spoilage bacteria, rather than killing them. This is especially of concern if ultrasound induced germination of the spores of thermophilic bacteria, which would not be killed by subsequent pasteurization. Although this study did not identify specifically which bacteria were present in milk before and after US treatments, none of the treatments were an effective means of decreasing total aerobic count (TAC) beyond that of high temperature short time pasteurization $\geq$

The trained panel aroma study uncovered no discernable differences in cooked or lacks freshness attributes between pasteurized control and TS treatments over 21 days of storage. The 80 J/mL TS treatment imparted a significantly more rubbery aroma to skim milk on day 1, which dissipated over time, but could offend the most sensitive of milk consumers.

Although it seems that ultrasound may be a “bust” as a way to extend fluid milk shelf life and improve quality, it is still a field full of unknowns that merit study. Future research should focus on standardizing the way ultrasound treatment conditions are reported, as well as examining the effect of temperature and amplitude on bacterial counts and sensory quality. Heat and ultrasound have been shown to have a complicated synergistic or antagonistic relationship depending on the study conditions, and more work should be done to ameliorate the consistency issues in ultrasonics research and evaluating the feasibility of the technology for dairy foods applications.