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Neither thermosonication nor cold sonication is better than pasteurization for milk shelf life

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Abstract

High-power, low-frequency ultrasound has been suggested as a novel processing technique with the potential to extend milk shelf life via inactivation of bacteria and spores that survive standard pasteurization. The primary objective of this research was to determine whether short-duration (≤60 s) sonication treatment, in conjunction with pasteurization, can increase shelf life while producing no adverse aroma effect. Skim milk was inoculated with Paenibacillus amylolyticus, a spore-forming, thermotolerant and psychrophilic milk contamination bacterium. Milk was sonicated under 6 selected amplitude and time conditions, except for control. Both cold sonicated (C-S) and thermosonicated (T-S) milk and milk treatments were pasteurized; however, T-S milk was sonicated after pasteurization (72.5 ± 0.3°C; mean ± SD), whereas C-S milk was sonicated at 12.5 ± 5°C (mean ± SD) before pasteurization. Milk was refrigerated up to 50 d and total aerobic counts were enumerated on pasteurized control, C-S, and T-S milk weekly. Neither C-S nor T-S treatments reduced total aerobic counts to an equivalent level as pasteurization alone. Counts in pasteurized controls and C-S milk did not exceed 3.00 log cfu/mL for up to 50 d; counts in T-S milk exceeded 5.00 cfu/mL by d 36. Aroma qualities (cooked, lacks freshness, and rubbery) of 2 T-S treatment intensities [170 μm peak-to-peak (p-p) for 60s and 200 μmp-p for 10 s] and pasteurized controls were evaluated by a trained descriptive sensory panel. No significant differences were observed in cooked or lacks freshness aromas among samples. Only the milk treated with 170 μmp-p for 60 s had significantly higher rubbery aroma on d 1 compared with milk treated with 200 μmp-p for 10 s. Although the sensory effects of T-S on milk may not limit the commercial feasibility of cold sonication or thermosonication, conditions that differ from those used in the present study should be considered in the future. Neither C-S nor T-S were appropriate techniques for reducing bacterial count in fluid milk beyond standard pasteurization and, in fact, increased counts of spore-forming spoilage bacteria.

Keywords

quality, sensory, spores, ultrasound

Disciplines

Food Chemistry | Food Processing | Food Science | Human and Clinical Nutrition | Molecular, Genetic, and Biochemical Nutrition | Nutritional Epidemiology

Comments

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Neither Thermosonication nor Cold Sonication is better than Pasteurization for Milk Shelf Life

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INDUSTRIAL RELEVANCE TEXT

The objective of this research was to evaluate the potential for short-duration (≤60 s) sonication treatment, in conjunction with pasteurization, to increase milk shelf life while producing no adverse aroma effect. Whether sonicated at 12.5°C ± 5°C (cold sonication) before pasteurization, or immediately after pasteurization (72.5°C ± 0.3°C; thermosonication), total aerobic counts were not improved by the technology. Counts of spore-forming thermotolerant psychrophilic bacteria were higher than those enumerated after standard pasteurization.

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 pasteurization. The primary objective of this research was to determine whether short-duration (≤60 s) sonication treatment, in conjunction with pasteurization, can increase shelf life while producing no adverse aroma effect. Skim milk was inoculated with *Paenibacillus amylolyticus,* a

spore-forming, thermotolerant and psychrophilic milk contamination bacterium. Milk was sonicated under six selected amplitude and time conditions. Both cold sonicated (CS) and thermosonicated (TS) milk and milk treatments were pasteurized; however, TS milk was sonicated after pasteurization (72.5°C ± 0.3°C) while CS milk was sonicated at 12.5°C ± 5°C before pasteurization. Milk was refrigerated up to 50 d and total aerobic counts (TAC) were enumerated on pasteurized control, CS, and TS milk weekly. Neither CS nor TS treatments reduced TAC to an equivalent level as pasteurization alone. Counts in pasteurized controls and CS milk did not exceed 3.00 log CFU/mL for up to 50 days; counts in TS milk exceeded 5.00 CFU/mL by day 36. Aroma qualities (cooked, lacks freshness, and rubbery) of two TS treatment intensities (170$\mu m_{peak\text{-}to\text{-}peak(p\text{-}p)}$/60s and 200$\mu m_{p\text{-}p}$/10s) and pasteurized controls were evaluated by a trained descriptive sensory panel. No significant differences were observed in cooked or lacks freshness aromas among samples. Only the milk treated with 170$\mu m_{p\text{-}p}$/60s had significantly higher rubbery aroma on day 1 compared to milk treated with 200$\mu m_{p\text{-}p}$/10 s. Although the sensory effects of TS on milk may not limit the commercial feasibility of cold sonication or thermosonication, conditions that differ from those used in the present study should be considered in the future. Neither CS nor TS were appropriate techniques for reducing bacterial count in fluid milk beyond standard pasteurization, and in fact, increased counts of spore-forming spoilage bacteria.

Key words: quality, sensory, spores, ultrasound

INTRODUCTION

Fluid milk competes with beverages that have a long shelf life (Fromm et al., 2004). One way to increase fluid milk's marketability is to improve the quality and extend shelf life (Boor, 2001; Fromm et al., 2004). Shelf life can be extended through ultra-pasteurization or ultrahigh-temperature (UHT) processing and aseptic packaging, however quality is changed at the higher temperatures, because of Maillard or caramelization reactions (Clare et al., 2005) and an increase in sulfur compounds (Zabbia et al., 2012). These processes can result in off-flavors that are unacceptable to some consumers (Christensen and Reineccius, 1992). Standard pasteurization does not compromise the sensory quality and nutritional properties of milk (Gandy et al., 2008), but alternative processing techniques are sought to improve some aspects of milk flavor, shelf life, or other functionality. Some non-thermal technologies have emerged as alternative processes to minimize changes in sensory properties induced by extreme heating. Emerging technologies such as high pressure processing (Bilbao-Sainz et al., 2009; Borda et al., 2004; García-Risco et al., 2003), pulsed electric field (Bendicho et al., 2005), and ultrasound (Vercet et al., 2002) have been explored to investigate their potential to inactivate shelf life-limiting enzymes in milk but maintain milk quality.

Ultrasound is acoustic vibration by cyclic sound pressure waves of frequencies beyond the human hearing range, from 18 to 20 kHz (Mason, 1999; Patist and Bates, 2008). Higher-power ultrasound is typically defined as 16-100 kHz frequency and 10-1000 W/cm^2 power

density (Soria and Villamiel, 2010). Sonication has a complex mechanism, and therefore its wide-ranging effects on the treatment medium must be examined carefully. The power of an ultrasonic device is characterized by the amount of energy (Joules) passed through to the medium per second. Some researchers report ultrasonic treatments in terms of intensity, or Watts per area, however, Zisu et al. (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 is subject to change depending on the selected amplitude and duration of treatment. The technology relies on the application of pressure waves to a liquid food material, and alternating regions of high and low pressures, which induce cavitation and form gas/vapor bubbles (Nguyen and Anema 2010; Pingret et al., 2012). Some authors also use the term cavitation to describe the bubble growth and subsequent collapse, with considerable energy release, which induces localized extreme conditions and leads to bacterial cell death (Ashokkumar, 2010; Gogate, 2011; Wu et al., 2013; Juliano et al., 2014; Khanal et al., 2014a).

One reason behind the limited shelf life of milk is the presence of bacterial spores in milk that are unaffected by pasteurization, even at the temperatures of ultrapasteurization (Hantsis-Zacharov and Halpern 2007). Psychrotrophic strains such as spore-forming *Bacillus* and *Paenibacillus* are predominate the raw milk supply (Martin et al. 2011). They are common contaminants in the farm environment, often associated with soil, feed or manure, and the thermoduric psychrotrophs common to milk are also spore-forming bacteria (Meer et al., 1991). Spores, the dormant forms of bacteria, are resistant to extreme temperatures, acid, alkalinity, and

oxidizing agents (Khanal et al., 2014a). The main drawback of psychrotrophic strains in milk is their ability to produce extracellular enzymes, mainly proteases and lipases, which are responsible for spoiling milk and also finished or processed dairy products, as the extracellular enzymes can resist pasteurization and even UHT processing (Hantsis-Zacharov and Halpern, 2007). Furthermore, the pasteurization process may promote "activation" and result in more rapid outgrowth of some spore-forming bacteria (Huck et al., 2007). 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 destroy the thermo-tolerant spores of microorganisms such as *Bacillus* and *Paenibacillus*, researchers have turned to other technologies as a means of killing bacteria in dairy products.

The inactivation of bacteria using ultrasound was first initiated in the 1920s (Harvey and Loomis, 1929). Some researchers have shown the ability of high-power ultrasound to kill bacteria, inactivate enzymes, and improve the cheese- or yogurt-making process (Martini and Walsh, 2012; Reiner et al., 2009b; Shanmugam et al., 2012; Villamiel and de Jong, 2000). High-power ultrasound has proven to be useful in inactivating microorganisms (Wrigley and Lorca, 1992; Villamiel and de Jong, 2000; Cameron et al., 2008, 2009), suggesting potentials to extend shelf life of fluid milk. However, the effect of ultrasound alone has been considered ineffective for the inactivation of bacterial spores (Butz and Tauscher, 2002).

Some researchers observed higher inactivation of microorganisms and enzymes when ultrasound was combined with factors such as heat, or with heat and pressure (Lopez et al., 1994; Vercet et al., 2002; Manas et al., 2006; Czank et al., 2010). Villamiel and de Jong (2000) were among the first to promote the use of thermosonication (simultaneous ultrasound and thermal processing), reporting that a synergistic effect of heat and ultrasound was much higher for inactivating enzymes and reducing microbial load compared to ultrasound or heating alone. However, thermosonication has been associated with off-odor and off-flavor formation in milk, a phenomenon that has been studied but not entirely explained. The sensory quality of milk is of the utmost importance to consumers (Bus and Worsley, 2003), so the detrimental sensory effects of ultrasound must be overcome if ultrasound is to be taken seriously as an alternative processing method. Ultrasound energy can induce peroxide formation from water hydrolysis, which can lead to radical oxidation of milk lipids and off-flavor compounds (Chouliara et al., 2010; Reiner et al., 2009b; Marchesini et al., 2015). Some studies have suggested that even short periods of ultrasound treatment result in undesirable sensory attributes (Chouliara et al., 2010; Marchesini et al., 2015). Aroma compounds were studied from a sensory perspective by Chouliara and colleagues (2010), who found that panelists' acceptance of samples was lower for thermosonicated (TS at 200W, approximately 240μm_{p-p}, for 2 min) samples as compared to untreated milk. Both Reiner et al. (2009a) and Chouliara et al. (2010) cited a "foreign", "rubbery" or "burnt" chemical taste in TS samples, which panelists found objectionable. In

126 contrast, the use of thermosonication (152μm_{p-p}) for 1 to 3 min decreased 94% of plasmin
127 activity in raw skim milk and cream, and increased the microbial shelf life of skim milk without
128 sacrificing sensory quality (Vijayakumar et al., 2015).

129 Ultrasound treatments have been reported to damage cell membranes, causing them to
130 buckle inward to varying degrees, as well as causing spores to wrinkle and shrink (Cameron et
131 al., 2008). Static trials using batch ultrasonication were found effective in reducing *Bacillus*
132 spores in non-fat milk (Khanal et al., 2014a). Yet in some applications, ultrasound has been used
133 to stimulate bacterial growth. Khanal et al. (2014b) reported that increased ultrasonication
134 amplitude might induce sporulation rather increase endospore inactivation level. Low, sub-lethal
135 doses of ultrasound can bust up clumps of cells, increasing total counts or colony forming units
136 (Marchesini et al., 2015). Gao et al. (2014) found that the sensitivity to ultrasound does not
137 depend on the size, the Gram-status or the hydrophobicity of bacteria, but rather on the thickness
138 of the polysaccharide as well as the "softness" of the protein capsule, which is a highly-hydrated
139 layer external to the plasma membrane that is composed of homogeneous polysaccharides and
140 proteins that contribute to maintaining cellular integrity (Marchesini et al., 2015)

141 Research involving the effects of sonication or thermosonication on milk quality typically
142 employ treatment times exceeding one minute at various power or amplitude levels, which is not
143 practical in fast-paced commercial HTST operations. More research needs to be done to
144 determine the minimal amount of ultrasound treatment needed to induce desired microbial and
145 enzymatic changes to milk without damaging sensory quality. The purpose of this study was to
146 determine whether thermosonication (ultrasound treatment after pasteurization) or cold
147 sonication (ultrasound treatment in an ice bath followed by pasteurization), is more or less

effective at reducing aerobic bacteria compared to pasteurization alone, while not damaging milk sensory quality.

MATERIALS AND METHODS

Milk preparation and controls

Raw whole milk was obtained from the Iowa State University Dairy (Ames, IA) bulk tank at or below 4°C. Milk was immediately transported (drive time less than 10 minutes; canned milk temperature did not exceed 7°C) to the Iowa State University Center for Crop Utilization Research pilot plant, where it was separated into cream and skim fractions 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 6.07±0.31 log CFU/mL *Paenibacillus amylolyticus* (H7-0689; Cornell Milk Quality Institute, Ithaca, NY). Milk was refrigerated at 4°C for up to 1 h before processing (heat treatment and/or sonication). For each lot of milk, inoculated "raw spiked control" milk was stored in 10 mL sterile plastic snap-top tubes to determine the initial TAC in the unprocessed milk for enumeration during up to eight days of storage. After only eight days, spoilage was evident in raw spiked control milk, in the form of flocculation and confirmed by TAC exceeding 6 log CFU/mL.

For each lot of milk, raw spiked control skim milk (100 mL) was heated in a sanitized stainless steel bowl, covered with aluminum foil, over a hot plate set to 148.9°C. Milk was stirred with a sanitized rod approximately every 30 s, heated until 72.5°C ± 0.3°C, and held for 15 s. 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 treatments was replicated 3 times over the course of 12 weeks.

Sonicated Samples

For each lot of milk, 100 mL of raw spiked control milk was first pasteurized in the same manner as for the pasteurized control (Figure 1). However, the milk was transferred to a 300 mL capacity glass sonicating rosette cooling cell model 250 (All-Spec Industries Inc., Wilmington, NC) submerged in a 73°C water bath and temperature change (typically a drop of 1°C; Table 1) was recorded. The Branson 2000 (Branson Ultrasonics (Danbury, CT); 2200W max power, 20kHz 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 1. Sample temperatures were recorded at the start of heating, end of heating, start of sonication, and end of sonication. This milk is termed thermosonicated (TS).

For cold sonicated (CS) samples, raw spiked control milk (100 mL) was transferred to the sonication rosette set in an ice bath. Milk was subjected to the sonicated treatments listed in Table 1. After sonication, each milk sample was transferred to a sanitized stainless steel bowl and pasteurized as previously described. Sample temperatures were recorded at the start of sonication, end of sonication/start of heating, and end of heating. Cold (CS) and TS milk samples were divided into 10 mL aliquots and stored in sterile snap-top plastic tubes for weekly analysis for up to 50 days. Each of the sonicated treatments was replicated 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 (TAC). 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. 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. 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 on day one to confirm presence of live microorganisms and to ensure that the 5-log kill required for pasteurization was obtained (FDA, 2011). Petrifilm plates were aerobically 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, treatment effects are expressed as a difference between the log TAC of the treatment and the log TAC of the corresponding pasteurized control in Table 4.

Sensory Panel Training and Evaluation

The Institutional Review Board of Iowa State University (ISU) approved recruitment of human subjects for the trained panel. Because milk samples were not legally pasteurized, descriptive sensory analysis was based only on aroma. Nine panelists (eight females, one male), with prior descriptive analysis experience, were recruited from ISU. Group training sessions were held, at a round table in the Center for Crops Utilization sensory evaluation facility at ISU, 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 expected to be experienced during the study period. Approximately 15 mL of each sample was transferred into sanitized, opaque screw-top containers (ULINE, Pleasant Prairie, WI)—one for each panelist. Caps were labeled either with the product identity (early training days) or a 3-digit number (later training days). Panelists agreed that fresh pasteurized skim milk should be free of offensive off-notes such as sourness or oxidized, and the aroma should be clean, slightly sweet, and have a hint of characteristic milk fat richness. Panelists were acquainted with the aromas of treated milk. The first was raw skim milk heated to 72.5 ± 0.3°C for 15 s. The second was raw skim milk heated identically, then subjected to 200μm_{p-p} (approximately 165 W) ultrasonication for 60s 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 stored 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 debated which terms 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 for their lexicon and for more extensive training. Anchors, or references, were selected for each aroma and defined in relation to a 15-cm line scale (Table 2).

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.

To prepare for sensory evaluation, approximately 15 mL of each sample was transferred into sanitized, opaque screw-top containers (ULINE, Pleasant Prairie, WI)—one for each panelist and each day of evaluation (nine panelists; days 1, 3, 8, and 21 of storage). Each treatment was replicated three times. Panelists were given no more than six randomly presented samples (pasteurized controls or TS samples) at each evaluation session to minimize fatigue. No CS samples were evaluated because at the time of the sensory study, the CS study had not been conceived of yet, and by the time the CS study was conducted some panelists graduated. Similar to Vijayakumar et al. (2015), 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

Energy density differences and sensory data were analyzed using JMP (JMP Pro 11). A one-way 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 Tukey-Kramer adjustment for multiple comparisons and significance of $\alpha < 0.05$ using JMP (Version 11 Pro). Analysis of the mean log difference (MLD) between the TAC of thermosonicated or cold sonicated treatment and the TAC of the pasteurized control was performed by IBM SPSS Statistics (IBM Corp., V.24, New York, USA). Mean values of MLD in each treatment and storage day were compared by *t*-test and ANOVA with least significance

difference at $\alpha < 0.05$. Days 36 through 50 were not included in this model because of spoilage and/or data censoring from estimated counts.

RESULTS AND DISCUSSION

Sonicated Samples

The treatment conditions for CS and TS milk, along with mean initial and final temperatures, are summarized in Table 1. The values before and after sonication demonstrated that sonication generate little to no heat. Because sonication was performed in temperature-controlled situations (i.e., ice bath for CS; hot water bath for TS), the temperature changes of all milk samples before and after treatment were less than 6°C for CS and less than 4°C for TS. Thus, treatment differences in the present study were a result of the sound energy and subsequent cavitation and not of a bulk temperature increase.

Table 1 also summarizes the statistical analysis of mean energy density values for treatments. Energy density was calculated according to Zisu et al. (2013) by dividing the Joules of energy delivered to the sample by the sample volume. Energy density allows for a more direct comparison between treatments in terms of intensity, rather than simply expressing ultrasound treatments in terms of amplitude, wattage, or frequency. Despite initially selecting diverse ultrasound 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 1). This is in agreement with literature; prior research 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

CS and TS samples was not significant for 50µm$_{p-p}$/20s, 150µm$_{p-p}$/10s, and 200µm$_{p-p}$/10s treatments, but CS had a significantly higher energy density compared to TS for the 170µm$_{p-p}$/60s, 50µm$_{p-p}$/60s, and 100µm$_{p-p}$/30s treatments (Table 1). The CS 50µm$_{p-p}$/60s, 100µm$_{p-p}$/30s, and 200µm$_{p-p}$/10s, along with the TS 200µm$_{p-p}$/10s and 100µm$_{p-p}$/30s treatments, all delivered the same amount of energy, ranging from an average of 20.1 to 25.1 J/mL (Table 1). The CS 170µm$_{p-p}$/60 s was the most energy-dense, at 103.4 J/mL. The general energy density of TS treatments of the same time and amplitude were significantly lower than CS, averaging 79.6 J/mL. The lowest energy density was delivered by the 50µm$_{p-p}$/20s treatment, which did not differ significantly between CS and TS conditions (Table 1).

Since the majority of treatments were not significantly different from each other in terms of energy density, any differences in TAC between such energetically identical treatments may be attributed to temperature (CS vs. TS), 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., 2014a).

Total Aerobic Counts

The mean log TACs for all treatments, weekly through day 50 of storage, are included in Table 3. Raw spiked control milk (data not shown) was only plated to day 8, when spoilage became evident. A sample was judged spoiled when it reached 6 log CFU/mL or when protein coagulation (flocculation) was visible in the sample container (Fromm and Boor, 2004). Visibly

spoiled samples were not enumerated. On day 1, the pasteurized control had a mean TAC of 1.48±0.13 log CFU/mL, and all CS and TS treatments ranged between 1.39 to 1.79 log CFU/mL. These findings demonstrate how neither CS nor TS meaningfully modified initial counts compared to standard pasteurized control. A week later, mean counts for CS and TS milk ranged from 1.50 to 1.89 log CFU/mL, which were similar to pasteurized control (1.49±0.12 log CFU/mL). By day 15, TAC of CS and TS milk ranged from as low as 1.32 to 3.00 log CFU/mL, while pasteurized control remained similar to day one counts (1.51±0.18 log CFU/mL).

Day 22 is an important time point because it is the typical shelf life of pasteurized milk. By day 22, TAC for all CS and TS treatments, as well as the pasteurized control, were still less than 6 log CFU/mL, and none showed evidence of flocculation. However, several treatments showed mean log TACs approaching 4 to 5 log CFU/mL, which may have tasted spoiled to discerning consumers. The TS samples of 50μm_{p-p}/20s, 100μm/30s, and 170μm_{p-p}/60s had mean TACs of 4.65±0.12, 5.09±0.82, and 4.88±0.38 log CFU/mL, respectively. The CS treatments maintained lower TAC counts, ranging from a low of 1.34±0.13 log CFU/mL for 200μm_{p-p}/10s, to a high of 2.86±0.02 log CFU/mL for 170μm_{p-p}/60s. The lowest CS mean TAC was still higher than the pasteurized control, at 1.20±0.10 log CFU/mL.

By day 29, bacterial growth patterns became unpredictable, and mean TAC had to be estimated because bacterial growth either exceeded or was lower than the selected dilution level plated (Table 3). Many replicates experienced a large jump in TAC, greater than the week-to-week change seen earlier in shelf life, suggesting a bloom of psychrotrophic organisms in the final week of storage. This was particularly evident in TS samples, which all exceeded 4.30 log CFU/mL by day 36, and visibly spoiled by day 43. In contrast, all but one CS sample (150μm_{p-p}/10s) had counts lower than 3.00 log CFU/mL through day 50. The pasteurized controls

maintained counts below 2.00 log CFU/mL through day 50 (Table 3), demonstrating the effectiveness of our laboratory pasteurization conditions.

Mean log difference (MLD) for TAC

Milk naturally contains a variable amount of bacteria based on the cleanliness of the milking conditions, dairy workers, sanitation, and storage conditions (Huck et al., 2008; Ranieri and Boor, 2009). Therefore, a simple mean obtained from replications of treatments on milk with different initial bacterial counts does not accurately represent treatment effects. Because experiments were conducted over 12 weeks using a different batch of milk each week, and each batch of milk had its own set of controls, data were transformed in relation to the pasteurized control corresponding to the batch of milk from which that treatment originated. In Table 4, data from days 1 to 22 are presented as the mean log difference (MLD) between the treatment TAC and the TAC of the pasteurized control from the same milk batch. This procedure allows control for milk batch as a source of random variation. A negative MLD value indicates that the treatment had a lower TAC than the pasteurized control, meaning that the sonicated treatment was more effective than pasteurization alone; positive numbers mean the opposite.

In all cases through day 15 (except one), MLD were within 1 log of respective pasteurized controls. Additionally, only one TS treatment (100$\mu m_{p\text{-}p}$/30s; day 1) and five CS treatments, (50$\mu m_{p\text{-}p}$/60s, 100$\mu m_{p\text{-}p}$/30s, 150$\mu m_{p\text{-}p}$/10s, 170$\mu m_{p\text{-}p}$/60s, and 200$\mu m_{p\text{-}p}$/10s; day 15) had a negative MLD. Every other CS and TS treatment yielded milk with positive MLD-higher counts than their respective pasteurized controls. These results confirm that neither CS nor TS were more effective than pasteurization at reducing milk TAC or extending milk microbial shelf life (Table 4).

Statistical analysis revealed that on day 22, the MLD from control of all TS and one CS sample were significantly higher than the MLD from control of almost all other samples on previous days ($p < 0.05$). One reason for this phenomenon can be explained by the findings of Ranieri et al. (2009). Although their research did not focus on ultrasound technology, the authors found that higher pasteurization temperatures (85.2°C instead of 72.9°C) led to increased sporulation and eventual cell growth among contaminating Gram-positive bacteria during subsequent storage. Gram-negative microorganisms such as *E. coli* have a more flexible cell membrane compared to the more rigid wall of Gram-positive bacteria. It has been observed that ultrasound is more effective in destroying Gram-negative bacteria than Gram-positive bacteria (Gao et al., 2014). Our TS samples were not pasteurized at a higher temperature, but some samples experienced a small bulk temperature increase or, more importantly, localized extreme temperature resulting from cavitation. The localized stress potentially induced conditions for sporulation and later germination. Khanal et al. (2014b) applied this theory to ultrasonication and found similar results-the treatments can simply lead to sporulation rather than destroying cells.

Thermal treatments such as pasteurization and UHT are capable of killing most spoilage and pathogenic bacteria, but they show a limited effectiveness on thermoduric spore-formers and their spores (Lewis and Deeth, 2009). Sporulation is a mechanism of survival for bacterial cells in response to adverse conditions including stress and starvation. Spores form as an end product of the sporulation process, which results in mother cell lysis to release spores (Setlow and Johnson, 2012). Multiple layers are then formed around the spore, between their inner membrane and outer membrane, which include a cell wall, a thick peptidoglycan cortex with a complex protein coating (Setlow and Johnson, 2012). Beaman and Gerhardt (1986) evaluated the factors affecting spore heat resistance and found that thermal adaptation can impact spore resistance by

reducing the water content and increasing wet density, and by mineralization where calcium re-mineralized protoplasts were drier, and hence, were more heat resistant (Beaman et al., 1982; Beaman and Gerhardt, 1986). Spores can be converted under adverse and stressful conditions, then resist severe heat treatments, radiations, chemicals, and high pressure, which make them capable to survive under unfavorable conditions (Setlow, 2006; Henriques and Moran, 2007; Burgess et al., 2010). Once the conditions become favorable, spores convert themselves to vegetative cells by activating themselves first, then germinating, and multiplying (Setlow, 2003).

The present findings further substantiate the observation that a bloom of psychrotrophic bacteria occurred in TS samples between days 15 and 22. These results lead us to believe that while pasteurization killed some cells, it also injured some cells and induced spore formation of yet other cells. Subsequent ultrasonication, we hypothesize, caused germination of spores (some of those acquired from the environment and some of those added to the milk), which enabled earlier outgrowth of vegetative cells in the milk between days 15 and 22. Although none of the CS or TS treatments could be considered effective compared to the pasteurized controls on days 1 through 22, the significant differences between CS and TS treatments in their effect on TAC help explain the microbiology. Our initial research was designed to only look at TS. However, after seeing the TS results, we designed the CS experiments, hypothesizing that ultrasound could be used to germinate spores and/or damage vegetative cells enough to make them vulnerable to heat, and that subsequent pasteurization would kill them. Unfortunately, the results obtained in this study only partially support that hypothesis. If fully supported, CS would have had a greater effect on reducing TAC compared to pasteurization. CS was more effective than TS, but not better than pasteurization alone (Tables 3 and 4).

To isolate the impact of energy density in the present study, a regression of MLD on energy density (J/mL) was conducted for CS and TS (figures not included; energy density values are included in Table 4). Only 10% of the variability in TS MLD from pasteurized control could be explained by the treatment energy density. For CS MLD from pasteurized control, 49% of the variability could be explained by the energy density. For both CS and TS, the least energy-dense treatments (50μm_{p-p}/20s) were not the least effective treatments, but the most energy-dense treatments (170μm_{p-p}/60s) were among the least effective treatments (least effective of all CS and 2nd least effective for TS). Khanal et al. (2014a) reported that an increase of amplitude from 91.2 to 114μm_{p-p} did not result in a significant effect in spore inactivation. This, coupled with our findings, indicates that energy density alone is not directly related to the impact of ultrasonicaton on cells. It is likely that amplitude, time, and energy density are all important factors to consider when choosing ultrasonication settings.

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 CS and TS 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 spore-forming bacteria were responsible for the increase in TAC and observed milk spoilage. Gram-negative bacteria present in raw milk may have outcompeted *Paenibacillus* or *Bacillus* present because of their faster growth (Ranieri and Boor, 2009). In future studies, the identity of the microorganisms presented before and after

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

Sensory Evaluation

The trained panelists' mean scores for cooked, rubbery, and lacks freshness aromas are displayed for each treatment (Table 5). Mean ratings of cooked aroma were low (did not exceed 3.8 on a 15-cm line scale) throughout storage; and there were no significant differences between the panelists' ratings among treatments or across days ($p > 0.05$). Although the cavitation heat and pressure generated by ultrasound energy itself is capable of denaturing whey protein and producing sulfhydryl aromas (Juliano et al., 2014), the intensity of the cooked aroma was neither extreme (above 10.0 on 15-cm line scale) nor intensified by the TS treatments selected in the present work.

Similar to the cooked attribute, there were no significant differences among mean scores for the intensity of lacks freshness aroma among samples (Table 5). Additionally, the amount of lacks freshness aroma did not significantly increase during refrigerated storage (21 days) ($p > 0.05$). The low mean scores (below 2.0 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 low TACs up to 22 days support the absence of lacks freshness aromas from bacterial sources.

Unlike the other attributes in question, the rubbery aroma, did vary significantly among treatments (Table 5). One day post-treatment, the 170$\mu m_{p\text{-}p}$/60s milk yielded a mean score of 4.5 out of 15. This did not significantly differ from the score of the pasteurized control samples (2.1) but was significantly higher than the mean score for the 200$\mu m_{p\text{-}p}$/10s sample (1.3; $p < 0.05$). The rubbery aroma in all samples dissipated over time. For the pasteurized control, it took until

day 8; for the 170µm$_{p-p}$/60s, it took until day 21. 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. 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 the rubbery aroma than others. Some panelists identified strong cooked aromas as rubbery or vice versa. However, statistical analysis determined that no one panelist skewed data than any other, so no data were discarded.

Mean rubbery aroma scores were similar to but slightly lower than those observed by Vijayakumar et al. (2015), who used similar ultrasonic amplitude conditions but longer treatment times (up to 3 min). Additionally, the present work demonstrates that the rubbery aroma dissipated relatively rapidly during refrigerated storage. 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. 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), indicating that the rubbery aroma in the ultrasonicated samples may originate from heat-induced oxidation of lipids into volatile compounds instead of a radical mechanism.

While the sensory effects of TS on milk may not limit the commercial feasibility of cold sonication or thermosonication, conditions that differ from those used in the present study should be considered in future studies to ensure extended microbial shelf life. 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 ultrasound research of fluid milk.

Although the majority of ultrasound treatments are only proven in the laboratory, ultrasound has numerous applications in the dairy industry, ranging from microbial reduction to tailoring ingredient functionality (Zisu and Chandrapala, 2015). As ultrasonic processing is a relatively new field of endeavor in dairy research, the availability of industrial scale or even pilot-scale equipment is still quite limited (Ashokkumar et al., 2010). Nowadays, the best opportunities for adoption of this technology would seem to be as an adjunct process in an existing processing line of the dairy industry. Ultrasound has not currently been used to widespread acceptance in fluid milk for processing and/or preservation, in part because of the limited knowledge on the effects upon shelf-limiting enzymes, sensory and other quality parameters (Ashokkumar et al., 2010; Zisu and Chandrapala, 2015).

If ultrasound is to be applied to a dairy processing operation, it will be important to consider all of the 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 milk fat globule size, and changes to the casein micelle structure must all be considered. Some of these changes may be 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. Residual fat tends to be more susceptible to radical reactions because it may not be contained within intact milkfat globules (Frankel, 1980; Walstra et al., 1999). 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% amplitude (170μm_{p-p}/60s) 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 might perceive a rubbery-smelling product which could inhibit future purchasing.

CONCLUSION

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, but may induce undesirable flavors and aromas under certain treatment conditions. The mild treatments selected for the present study may be applicable to commercial applications from a sensory standpoint since a low-level objectionable rubbery aroma dissipated very quickly. However, bacteria counts

511 in milk treated with thermosonication and cold sonication were significantly higher than
512 pasteurized control milk spiked with *Paenibacillus amylolyticus* throughout all 22 days of
513 storage, particularly for TS samples. This research demonstrated that thermosonication induces
514 vegetative cells of anaerobic spore-forming bacteria to form heat-resistant spores, enabling
515 higher rates of subsequent spoilage than standard pasteurization. Integration of TS with HTST,
516 under the conditions of this study, is not a feasible means of extending milk shelf life. Cold
517 sonication may be an appropriate method, but more research is needed to optimize the conditions
518 and understand the effect of CS and subsequent heating, including the identity of surviving
519 microorganisms, to ensure effectiveness at eliminating bacteria and extending the shelf life of
520 fluid milk.
521
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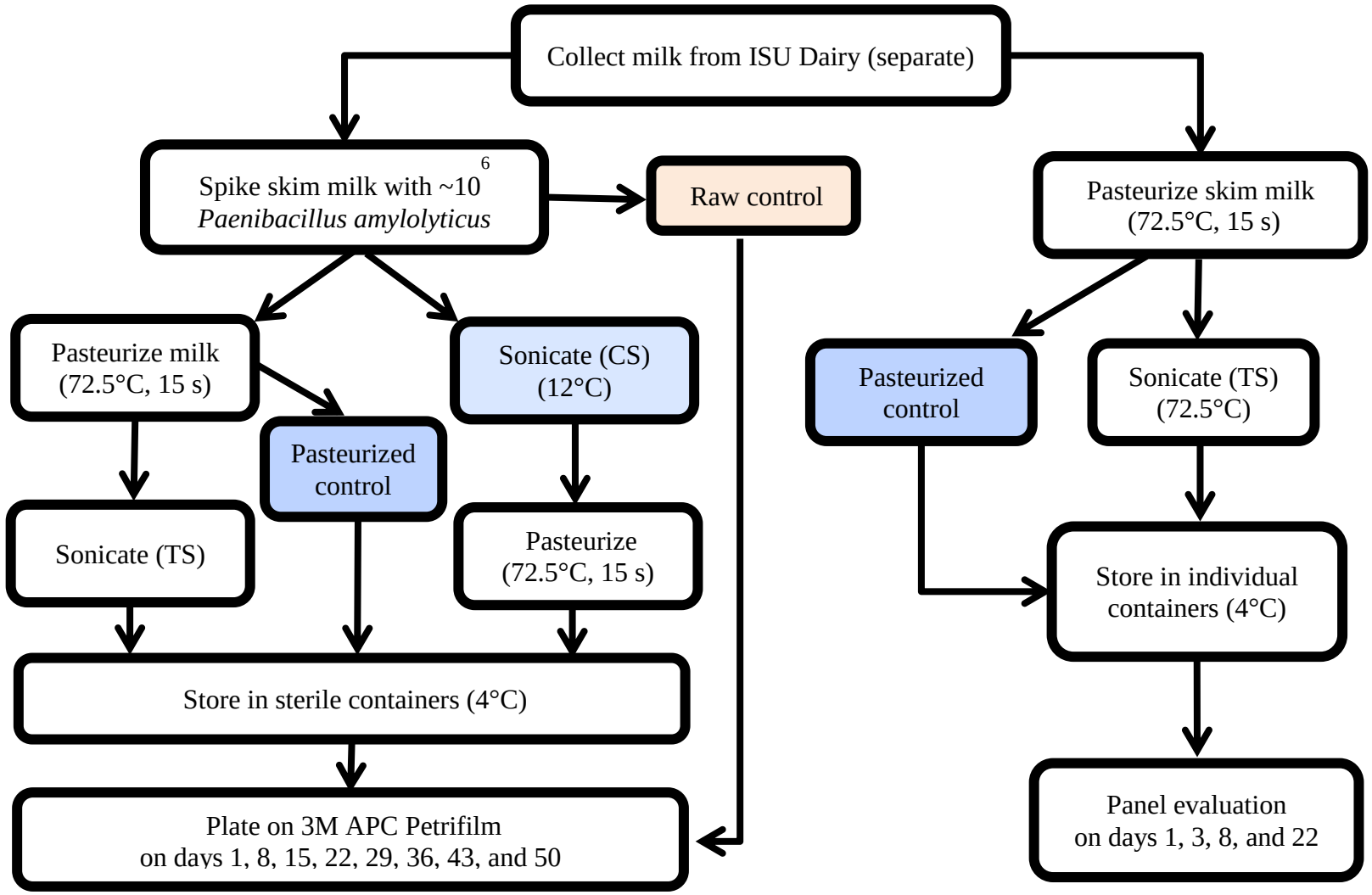

Figure 1. Process for fluid milk collection, sonication and pasteurization; conducted in triplicate.

Table 1. Treatment conditions for TS and CS skim milk, including statistical analysis of energy density and average temperature before and after treatment for sonication settings.

Amplitude (%)	Amplitude (μm_{p-p})	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)
TS treatments								
21	50	20	15.3	n/a	72.6	71.1	71.5	6.2^{G}
21	50	60	12.2	n/a	72.8	70.0	70.6	19.2^{DE}
42	100	30	10.9	n/a	72.2	69.6	71.0	20.2^{CDE}
63	150	10	14.6	n/a	72.8	71.8	71.5	11.2^{FG}
72	170	60	14.1	n/a	72.5	71.2	74.5	79.6^{B}
84	200	10	11.4	n/a	72.4	70.4	71.6	19.8^{CD}
CS treatments								
21	50	20	10.4	9.1	72.3	n/a	n/a	8.5^{G}
21	50	60	8.7	7.8	72.2	n/a	n/a	25.1^{C}
42	100	30	10.5	9.3	72.9	n/a	n/a	24.7^{CD}
63	150	10	15.1	11.4	72.9	n/a	n/a	15.0^{EF}
72	170	60	11.7	17.5	73.2	n/a	n/a	103.4^{A}
84	200	10	9.9	9.8	73.0	n/a	n/a	22.7^{CD}
	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

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

TS: Thermosonicated CS: Cold sonicated, n/a: Not applicable

$^{A-G}$: Energy density values with differing letters statistically differ ($p < 0.05$)

722 **Table 2. Sensory terms and anchors for 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 and conventional skim (50/50 mixture) = score of 5 Fairlife skim milk = score of 10
Rubbery	The rubber and chemical aroma of rubber bands.	Rubber bands in skim milk = score of 5 Rubber bands = score of 15
Lacks Freshness	Milk that is spoiling or has absorbed unpleasant off-aromas from the milking environment. Described with terms such as acid/sour, barny, stale, dirty, or unclean.	Raw milk stored 3 days = score of 5 Raw milk stored 8 days = score of 15

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736 **Table 3. Least squares mean log total aerobic bacteria count for pasteurized, TS, or CS milk stored up to 50 days (±standard**
737 **error), from linear mixed model. All values are average of 3 observations unless noted. First number (50, 100, 150, 170, 200)**
738 **indicates treatment amplitude (μm_{p-p}), second number (20, 60, 30, 10) indicates treatment time (s).**

Treatment	Day 1	Day 8	Day 15	Day 22	Day 29	Day 36	Day 43	Day 50
50/20 TS	1.69±0.21	1.89±0.003	2.61±0.76	4.65±0.12	6.37±0.16*	> 5.90 E	S	S
50/60 TS	1.44±0.20	1.60±0.13	2.11±0.56	2.76±1.08	> 2.00 E	5.43±0.58*	S	S
100/30 TS	1.39±0.23	1.52±0.16	3.00±0.58*	5.09±0.82*	5.89±0.01*	6.28±0.07*	S	S
150/10 TS	1.72±0.09	1.79±0.10	2.62±0.76	3.55±0.46	< 3.60 E	> 4.30 E	S	S
170/60 TS	1.64±0.13	1.76±0.10	2.73±0.66	4.88±0.38*	6.12±0.18*	> 5.30 E	S	S
200/10 TS	1.48±0.16	1.66±0.11	2.29±0.91	2.76±0.89	> 2.00 E	4.79±0.66*	S	S
50/20 CS	1.77±0.11	1.84±0.04	2.35±0.69	2.40±0.48*	> 3.00 E	< 2.00 E	2.59±0.91	2.85±1.13
50/60 CS	1.56±0.15	1.59±0.17	1.39±0.19	1.92±0.36	1.32±0.11	2.22±0.39*	1.40±0.18*	1.24±0.09
100/30 CS	1.50±0.18	1.61±0.15	1.37±0.29	1.45±0.11	2.12±0.64	2.92±1.02*	1.58±0.03*	1.09±0.28
150/10 CS	1.79±0.20	1.84±0.12	1.71±0.11	1.88±0.01*	3.76±1.56*	< 3.70 E	< 3.00 E	< 3.00 E
170/60 CS	1.71±0.17	1.75±0.13	2.30±0.46	2.86±0.02	< 2.90 E	1.69±0.07*	> 3.3 E	1.64±0.01*
200/10 CS	1.47±0.23	1.50±0.18	1.32±0.13	1.34±0.13	1.59±0.32	1.30±0.24*	0.83±0.12	1.80±0.99
Pasteurized control	1.48±0.13**	1.49±0.12**	1.51±0.18**	1.20±0.10**	1.18±0.28**	1.39±0.14**	1.30±0.09**	1.13±0.16**

739 TS: Thermosonicated, CS: Cold sonicated

740 *2 observations, **4 observations

741 E: Estimated value, S: Spoiled sample

Table 4. One-way ANOVA analysis of mean log difference (MLD) from pasteurized control for TAC of milk treated with ultrasound before (cold sonication, CS) or after (thermosonciation, TS) pasteurization (± standard error), for milk stored 1, 8, 15 and 22 days. First number (50, 100, 150, 170, 200) indicates treatment amplitude (μm_{p-p}), 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^{g}	0.098 ± 0.064^{FG}	0.197 ± 0.004^{FG}	0.283 ± 0.072^{EFG}	$3.238\pm0.202^{*A}$
50/60 TS	19.2^{de}	0.042 ± 0.069^{FG}	0.179 ± 0.042^{FG}	0.648 ± 0.311^{CDEF}	1.630 ± 1.020^{BC}
100/30 TS	20.1^{cde}	-0.004 ± 0.104^{FG}	0.096 ± 0.022^{FG}	1.310 ± 0.503^{BCDE}	$4.022\pm0.863^{*A}$
150/10 TS	11.2^{fg}	0.133 ± 0.071^{FG}	0.104 ± 0.105^{FG}	0.300 ± 0.088^{EFG}	$2.135\pm0.794^{*B}$
170/60 TS	79.6^{b}	0.056 ± 0.035^{FG}	0.073 ± 0.100^{FG}	0.407 ± 0.448^{DEFG}	$3.455\pm0.466^{*A}$
200/10 TS	19.7^{cd}	0.088 ± 0.051^{FG}	0.237 ± 0.045^{EFG}	0.820 ± 0.661^{CDEF}	1.626 ± 0.802^{BC}
50/20 CS	8.5^{g}	0.184 ± 0.070^{FG}	0.149 ± 0.043^{FG}	0.027 ± 0.051^{FG}	$0.980\pm0.834^{*CDEF}$
50/60 CS	25.1^{c}	0.165 ± 0.038^{FG}	0.168 ± 0.075^{FG}	-0.077 ± 0.240^{FG}	0.791 ± 0.282^{CDEF}
100/30 CS	24.7^{cd}	0.101 ± 0.082^{FG}	0.185 ± 0.038^{FG}	-0.099 ± 0.267^{FG}	0.323 ± 0.039^{EFG}
150/10 CS	15.0^{ef}	0.204 ± 0.049^{FG}	0.144 ± 0.122^{FG}	-0.610 ± 0.801^{G}	$0.452\pm0.015^{*DEFG}$
170/60 CS	103.4^{a}	0.126 ± 0.007^{FG}	0.066 ± 0.134^{FG}	-0.023 ± 0.244^{FG}	1.435 ± 0.026^{BCD}
200/10 CS	22.7^{cd}	0.075 ± 0.134^{FG}	0.071 ± 0.055^{FG}	-0.145 ± 0.248^{FG}	0.207 ± 0.060^{FG}

TS: Thermosonicated, CS: Cold sonicated

$^{a-g}$Energy density values with differing letters statistically differ ($p < 0.05$)

$^{A-G}$ Mean log differences with differing letters statistically differ ($p < 0.05$)

*2 observations

Table 5. Mean trained panelist ratings (n = 9; 15-cm line scale ± standard deviation) of cooked, lacks freshness and rubbery aroma attributes of skim milk subjected to thermosonication or pasteurization. First number (170 or 200) indicates treatment amplitude (μm_{p-p}), second number (60 or 10) indicates treatment time (s).

Thermosonicated milk treatments	Day 1	Day 3	Day 8	Day 21
		Cooked		
170/60	3.8 ± 3.8^{A}	3.4 ± 2.6^{A}	2.6 ± 2.3^{A}	3.3 ± 2.7^{A}
200/10	2.7 ± 2.4^{A}	3.1 ± 2.7^{A}	3.2 ± 2.6^{A}	2.7 ± 3.1^{A}
Pasteurized control	2.4 ± 2.6^{A}	3.3 ± 2.6^{A}	2.3 ± 2.3^{A}	2.4 ± 2.4^{A}
		Lacks Freshness		
170/60	0.8 ± 1.6^{A}	0.4 ± 0.7^{A}	1.5 ± 2.0^{A}	1.3 ± 2.4^{A}
200/10	1.1 ± 2.1^{A}	0.9 ± 1.7^{A}	1.5 ± 2.4^{A}	1.9 ± 2.2^{A}
Pasteurized control	1.8 ± 2.6^{A}	1.1 ± 1.3^{A}	1.5 ± 2.0^{A}	1.6 ± 2.1^{A}
		Rubbery		
170/60	4.5 ± 4.9^{A}	3.2 ± 4.1^{AB}	2.0 ± 3.0^{AB}	0.8 ± 1.8^{B}
200/10	1.3 ± 2.0^{B}	1.2 ± 2.3^{ABC}	0.9 ± 1.8^{B}	1.0 ± 1.9^{B}
Pasteurized control	2.1 ± 3.1^{AB}	2.0 ± 3.1^{AB}	1.9 ± 2.9^{B}	1.6 ± 2.6^{B}

$^{A, B}$ Values with differing letters within the same aroma category statistically differ ($p < 0.05$)