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Effect of thermosonication in a laboratory-scale continuous system on the survival of thermophilic bacteria and indigenous microbiota in milk

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Abstract

Fluid foods are typically heat-treated to eliminate pathogens and reduce microbial counts. Coupling thermosonication with heat may reduce the microbial load in fluid foods and enhance the product quality during storage. This study evaluated the effect of thermosonication along with heat in a laboratory-scale continuous system, on the survival of *Geobacillus stearothermophilus* in milk, at two different settings (setting 1: 27.7 s total heating time with or without 11.9 s of sonication; setting 2: 20.3 s total heating time with or without 7.1 s of sonication). This study also investigated the effect of thermosonication along with heat on indigenous microbiota in raw milk; and milk quality was assessed by pH, free fatty acid (FFA) content, and casein/total protein (CN/TP) content during storage at the two different settings. Overall, thermosonication with heat resulted in higher log reductions for *G. stearothermophilus*; but, the reduction was not significant overall. Thermosonication with heat significantly decreased the indigenous microbiota in milk as compared to heat alone at both settings. Longer residence times (setting 1) had significantly higher log reductions at week 0, and treatment samples had significantly higher reductions than control during storage time at both the settings. Treatment samples at setting 1 had significantly higher pH and CN/TP, and lower FFA content at week 4, as compared to the control. Thermosonication using practical residence times along with heat may reduce milk microbiota. Results from this study need to be verified in a scale-up study employing pasteurization conditions.

Introduction

High temperature short time (HTST; at 72°C for 15 s) pasteurized milk has a shelf life of up to three weeks (Gandy *et al.,* 2008; FDA, 2017). Pathogens are destroyed, and vegetative bacteria are reduced as a result of pasteurization, thus extending raw milk shelf life. About 19% of dairy products are lost at retail and consumer level due to their tendency to spoil (Gunders, 2012). Pasteurized milk quality and shelf life is limited mostly due to thermophilic organisms which may grow at both room and refrigeration temperatures, and reduce the overall dairy product quality (Sørhaug and Stepaniak, 1997; Fromm and Boor, 2004; Ledenbach and Marshall, 2009; Ivy *et al.,* 2012).

Thermophilic bacteria have been associated with spoilage of raw, HTST pasteurized, and ultra-high temperature (UHT) pasteurized milk along with canned milk products (Kalogridou-Vassiliadou, 1992; Burgess *et al.,* 2010; Lücking *et al.,* 2013). Elimination or reduction of thermally resistant organisms that are responsible for milk product spoilage cannot be achieved by increasing pasteurization temperatures up to 85°C (Sørhaug and Stepaniak, 1997; Gandy *et al.,* 2008; Ivy *et al.,* 2012). Gandy *et al.* (2008) showed that increasing pasteurization temperatures up to 85°C did not lead to an increase in milk shelf life. Use of alternative technologies such as sonication may help achieve a higher quality product by reducing the microbiota in pasteurized milk.

Sonication is the application of sound waves at frequencies ≥ 20 kHz in a fluid medium. When sonication is applied with heat, it is referred to as thermosonication. Sonication can result in cavitation. Cavitation and bubble collapse can generate very high temperatures (≈4726°C) and pressures (≈2,000 atm) locally in the fluid medium (Suslick, 1998). Applications of sonication are being explored in different areas in food processing such as emulsification, filtration, viscosity modification, improvement of whey protein heat stability, improvement of meat tenderness, and inactivation of spoilage microorganisms (Knorr *et al.,* 2004; Chemat *et al.,* 2011; Chandrapala *et al.*, 2012; Ganesan *et al.,* 2015; Beatty and Walsh, 2016; Sutariya *et al.,* 2018; Deshpande and Walsh, 2018). Sonication has been researched to

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improve milk quality and shelf life by destruction of indigenous microbiota or added pathogens, with the majority of studies being in a batch system (Bermúdez-Aguirre *et al.,* 2009a; 2009b; Villamiel *et al.,* 2009).

Many studies showing reduction in thermophilic bacteria using batch sonication have been reported. *Bacillus subtilis* cells were inactivated after up to 15 min of sonication (Joyce *et al.,* 2003). Milk inoculated with *Anoxybacillus flavithermus* (5 - 6 log CFU/mL) showed a 1.1-log reduction after 1 min of cold batch sonication, and after 10 min, a 4-log reduction was seen (Khanal *et al.,* 2014). Comparatively, Khanal *et al.* (2014), reported a log reduction of 1.27 of *A. flavithermus* after pasteurizing at 63°C for 30 min. Lim *et al.* (2019) showed that thermosonication and cold sonication along with pasteurization in batch sonication system (for 10 - 60 s) did not significantly reduce the milk microbial numbers when inoculated with *Paenibacillus amolyticus*. Thermosonication has shown success in reducing microbiota in fluid systems other than milk. Batch thermosonication (40 - 60% amplitude) for 2 - 10 min at 30 - 50°C significantly reduced the total microbial population in quince juice (Yıkmış *et al.,* 2019).

A 2.9-log reduction in overall milk microbiota was seen after 1.7 min of sonication in a continuous system; however, the effect of thermosonication was not evaluated (Villamiel and De Jong, 2000). Khanal *et al.* (2014) showed that when cold batch sonication for 1 min was used with batch pasteurization, the log reductions in *A. flavithermus* cells were doubled. Most of the previously reported studies did not use sonication at practical residence times along with use of additional heat which could yield comparable or higher reductions as compared to sonication alone. A majority of the studies have been in a batch system as well. Thus, the effect of shorter and practical residence times (≤ 1 min) for thermosonication in a continuous system combined with heat on milk microbiota should be investigated.

Based on previous reports, this study aimed at evaluating the effect of thermosonication with heat treatment using a plate heat exchanger (PHE) in a laboratory-scale continuous system on *Geobacillus stearothermophilus* (thermophilic bacterium) and indigenous microbiota reductions. The first objective of this study was to evaluate the effect of thermosonication with heat on the survival of *G. stearothermophilus* at two different residence times. In this part of the study, the effect of location of the sonicator (before or after the PHE) was also evaluated. The second objective of this study was to evaluate the effect of thermosonication with heat on the survival of indigenous microbiota in raw milk during storage. This experiment was designed to be a preliminary study which can be used to design future scale up studies. Free fatty acid (FFA) content, casein as a percentage of total protein (CN/TP) content, and pH were evaluated during storage to see if they can be used as indicators of milk quality in future studies.

Materials and methods

Experimental design

This study was designed with two components, first was to evaluate the effect of thermosonication with heat as compared to heat alone in a laboratory-scale continuous system on *G. stearothermophilus* reductions; second, to evaluate the effect of thermosonication with heat as compared to heat alone in a laboratory-scale continuous flow system on indigenous microbiota in raw milk and milk quality during storage. Samples that received only heat are termed as control, and samples that received thermosonication with heat are termed as treatment.

To determine the effect of thermosonication (PHE set at 72°C, sonicator set at 72°C at 90% amplitude) on reductions of *G. stearothermophilus* cells in milk in a laboratory-scale continuous flow system, 2% fat sterile milk (Gossner Foods, Logan, UT, USA) was processed under control or treatment conditions for different residence times by adjusting the flow rate to give two different settings (Table 1).

Table 1. Mean $\log_{10}$ reduction of *G. stearothermophilus* cells after control or treatment at each setting.

Setting	Residence time in PHE[1] (s)	Residence time in sonicator[1] (s)	Total residence time[1] (s)	Exit temperature (°C)	Control reductions[2]	Treatment reductions[3]
Plate heat exchanger-sonicator						
1	15.8	11.9	27.7	71	0.37 ± 0.05^{abc}	0.45 ± 0.06^{ad}
2	13.2	7.1	20.3	67	0.05 ± 0.01^{c}	0.26 ± 0.04^{bc}
Sonicator-plate heat exchanger						
1	15.8	11.9	27.7	71	0.25 ± 0.02^{bc}	0.54 ± 0.04^{d}
2	13.2	7.1	20.3	67	0.13 ± 0.01^{bc}	0.35 ± 0.02^{abd}

[1]Equipment at 72°C, [2]Control: treated with heat alone with sample flowing through the PHE and sonicator with sonicator off, and [3]Treatment: with sample flowing through the PHE and sonicator with sonicator on. Values with different letters are significantly different. Values are mean ± S.D.

The effect of the sonicator's position on microbial reductions was evaluated as well, by placing the sonicator before or after the PHE for the *G. stearothermophilus* experiments. All experiments at each residence time were performed in triplicate. To evaluate the effect of thermosonication (PHE set at 72°C, sonicator set at 72°C at 90% amplitude) on reductions of indigenous microbiota in a laboratory-scale continuous system, raw whole milk (obtained from Aggie Creamery, Utah State University, Logan, UT, USA) was processed under control or treatment conditions for different residence times as described previously to achieve two settings. A photographic and schematic representation of the experimental setup is shown in Figure 1.

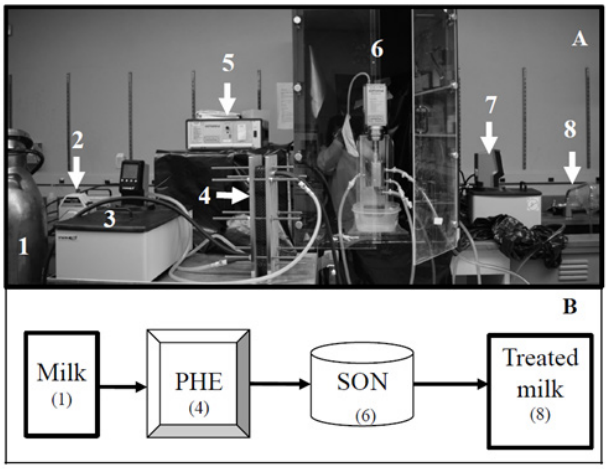

Figure 1. Assembly used for milk experiments shown in a photographic (A) and schematic format (B). PHE: plate heat exchanger; SON: sonicator vessel; 1: milk at 20 - 25°C; 2: pump; 3: circulating water bath for PHE; 4: PHE set at 72°C; 5: transducer; 6: sonication vessel set at 72°C; 7: circulating water bath for sonicator; and 8: treated milk collection.

At setting 1, samples received 27.7 s total heating time with or without 11.9 s of sonication, with all equipment set at 72°C. At setting 2, samples received 20.3 s total heating time with or without 7.1 s of sonication, with all equipment set at 72°C. All experiments at each residence time were performed in at least triplicates. The inlet and outlet temperatures of milk were monitored. For G. stearothermophilus experiments, the inlet temperature of the milk was 60°C, while outlet temperature measured was 71 and 67°C, at setting 1 and 2, respectively. Whereas, the inlet temperature for raw milk was between 20 - 25°C, and the outlet temperature for indigenous microbiota experiments were 63 and 57°C, at setting 1 and 2, respectively.

Growth of G. stearothermophilus cells

G. stearothermophilus spores were germinated using 0.1 mL of stock solution (NAMSA *G. stearothermophilus*, 2.4×10^6 in 0.1 mL; VWR, Atlanta GA, USA) inoculated into 10 mL of sterile water. The diluted stock was incubated for 10 min in an 80°C water bath to germinate the spores. For growing vegetative cells, 25 mL of TSB was inoculated with 1 mL of germinated bacteria in a sterile 250 mL Erlenmeyer flask covered with sterile foil, and incubated at 55°C aerobically for 24 h in a shaker at 100 rpm. The optical density (OD) was measured at 600 nm using a spectrophotometer (Bio Spec-1601, Shimadzu, USA), and was found to be approximately 0.57 after 24 h, which corresponded to 7 log CFU/mL as determined by plating on tryptic soy agar (TSA: VWR; Atlanta GA, USA) (Beatty and Walsh, 2016). A subculture was grown by inoculating 25 mL of TSB with 0.1 mL of culture grown from germinated cells in a sterile 250 mL Erlenmeyer flask covered with sterile foil. Cells were grown aerobically at 55°C in a shaker at 100 rpm for 15 h. Freezer stocks were made by inoculating 20 mL of TSB containing 30% (w/v) glycerol with 2 mL of subculture and aliquoted and stored in 2 mL cryo-vials at -6°C (Beatty and Walsh, 2016).

Cultures for experiments were grown by inoculating 600 mL TSB with 2.4 mL of freezer stock in 1 L Erlenmeyer flasks, and incubating at 55°C in a shaker at 100 rpm for 15 h. OD at 600 nm was checked after every overnight growth, and the culture was used when the OD was greater than 0.70. Overnight growth from the flasks was transferred to sterile 500 mL centrifuge bottles, which were then centrifuged at 500 rpm for 20 min at 10°C. Pellets containing cells were obtained, and the filtrate was discarded. Pellets were resuspended in 400 mL of sterile 2% fat milk at room temperature. This milk was then transferred to the pot with 3,600 mL of pre-heated 2% fat milk (at 60°C), and an initial concentration of 7.37 ± 3.89 log CFU/mL was obtained.

Heating and thermosonication conditions for G. stearothermophilus experiments

Milk was prepared by heating 3,600 mL of 2% fat sterile milk to 60°C in a pot (cleaned and wiped with ethanol before use), and inoculated with *G. stearothermophilus* before being treated either with control or treatment conditions. To simulate continuous operation at a laboratory scale, milk was pumped using a Masterflex 7529 pump (Cole-Palmer, Vernon Hills, IL, USA) at different flow rates described previously. A PHE was used for heating milk, and the temperature of the PHE was maintained using a circulating water bath. PHE was placed either before or after the sonicator to evaluate the sonicator's

location on *G. stearothermophilus* reductions. The sonication flow cell had a 160 mL volume resulting in different residence times (time sample was exposed to sonication or heating) at different flow rates. The flow rate also affected the heating residence time through the plate heat exchanger. The stainless-steel flow cell of the sonicator has a water jacket and was connected to a water bath to maintain the temperature of the sample during thermosonication.

Samples were thermosonicated (Heischler UIP500hd, Ringwood, NJ, USA) at 90% amplitude, and temperature of the flow cell was maintained at 72°C using a circulating water bath. All equipment was rinsed with detergent (Conquest, EcoLab, Saint Paul, MN, USA) followed by washing with hot water and sanitizer solution (Exelerate CIP Solution, EcoLab, Saint Paul, MN, USA) after each experiment, and with hot water between different treatments to avoid cross-contamination. Pre-treatment samples were placed on ice until ready to plate for time zero. The remaining sample was then subjected to control or treatment conditions. After being processed with control or treatment conditions, samples were collected in sterile 50 mL tubes, and placed on ice before microbial plating. This entire procedure was performed for each experiment and its replicates.

Heating and thermosonication conditions for indigenous microbiota experiments

Raw milk (received at 4°C) was heated to 20 - 25°C by placing sealed hot water bottles (between 85 - 90°C) in the milk can and occasional stirring over 1 - 2 h. The raw milk received had microbial counts of 2.03 ± 1.04 log CFU/mL, and the warming step was performed to increase the initial microbial counts to 4.41 ± 2.36 log CFU/mL, so that the effects of heat and thermosonication could be evaluated.

Experiments were performed using the same assembly and procedures used for *G. stearothermophilus* experiments, unless stated otherwise. The PHE was placed before the sonicator for indigenous microbiota experiments. Pre-treatment samples were placed on ice until ready to plate for time zero and the remaining sample was then subjected to control or treatment conditions. For each replicate, eight samples of 200 mL of milk were collected post processing in sterile containers and transferred to a cold room at 4°C for various analyses over time.

The energy density (J/mL) for the samples sonicated in the laboratory-scale continuous flow system was calculated (Chandrapala *et al.,* 2014). The power readings ranged from 168 to 180 W with an average of 174. 7 W. The energy density calculated at setting 1 (11.9 s residence time) was 12.99 J/mL, and setting 2 (7.1 s residence time) was 7.75 J/mL.

Microbial and pH evaluations

For *G. stearothermophilus* experiments, dilutions of samples were made in sterile phosphate buffered saline (PBS) and plated on TSA and incubated for 24 - 48 h in a humidified incubator at 55°C to determine microbial reductions. Duplicate measurements were done for each sample.

For indigenous microbiota experiments, dilutions of samples were made in sterile PBS, and plated on standard plate count agar (SPC; for total aerobic bacteria) and TSA (for indigenous thermophilic bacteria). Plates were incubated for 24 - 48 h in a humidified incubator at 32°C (for SPC) and 55°C (for TSA). Plating was done each week, followed by pH measurements and the milk sample containers were transferred to the freezer at -30°C for remaining evaluations. The pH of samples was measured at 4°C after standardization of the pH meter (Orion 3-star pH meter, Thermo Fisher Scientific, Waltham, MA, USA) with buffers of pH 4 and 7. Duplicate measurements were done for each sample.

The storage containers were then transferred to freezer at -30°C and stored for quality parameters testing as described below for FFA and CN/TP. Analyses during storage were stopped when sample appeared to be spoiled with CFU/mL > 6 log, or when visible curdling of milk was observed (Fromm and Boor, 2004; Lim *et al.,* 2019).

Free fatty acid (FFA) content

Lipolysis in milk samples was evaluated by measuring the increase in free fatty acid content from week 0 through week 4. The FFA content was measured using the copper soap method described in Shipe *et al.* (1980), as modified by Ma *et al.* (2003). Milk was thawed using a combination of a water bath at 20°C and microwave while keeping the temperature below 10°C. Reagents were prepared as described by Shipe *et al.* (1980). Color was measured in cuvettes after mixing at 440 nm within 1 h using a spectrophotometer (BioSpec-1601, Shimadzu, Kyoto, Japan). Blanks were prepared by using deionized (DI) water instead of milk.

A standard curve was obtained by preparing six known concentrations of palmitic acid (0, 50, 100, 150, and 200 µg/mL) and mixed with 0.1 mL of 0.7 N HCl and 1 mL of DI water, and a standard curve was plotted with absorbance measured at 440 nm. FFA content in milk was calculated in µg/mL from the standard curve. The final value is reported in meq FFA/kg of milk abbreviated as FFA/kg, and was calculated as:

$$\text{meq } FFA = \frac{\frac{\mu g \text{ of FFA} \times 0.001 \text{ mg}/\mu g}{256.43 \text{ mg/meq}}}{\text{g of milk} \times 0.001 \text{ kg/g}}$$

Duplicate reactions were conducted for each sample.

Casein/total protein (CN/ TP) content

Casein (CN) as a percentage of total protein (TP) was measured at weeks 0, 2, and 4 to evaluate the proteolysis activity during storage of milk (Ma *et al.,* 2003). Milk was thawed using a combination of a water bath at 20°C and a microwave as described above. Whole milk samples were used for total protein (TP) measurements. Non-casein nitrogen (NCN) content was analyzed using whey portions from the whole milk.

Whey portions of milk were obtained by mixing 20 mL milk sample with 20 mL DI water in a 50 mL test tube, which was then kept at 37°C for 30 min in a water bath. Two mL of acetic acid solution (10% v/v) was added, and the mixture was vortexed for 30 s, followed by incubation for 10 min. Two mL of 1 M sodium acetate was then added to the test tube, and the mixture was cooled to 20°C in an ice bath. DI water was added to the 50 mL calibration mark, and the test tubes were centrifuged at 3,500 rpm for 15 min. The filtrate (whey) was collected for further analyses, and casein was obtained as a pellet. Whole milk and whey samples were then refrigerated and sent to Utah State University Analytical Laboratories (USUAL; Logan, UT, USA) for nitrogen content analyses performed using combustion. TP was calculate using nitrogen content from whole milk, and CN was calculated from TP-NCN. CN/TP is reported by taking a mean and standard deviation of three replicates.

Statistical analysis

Three replicates were used for *G. stearothermophilus* experiments. A total of eight replicates were used for indigenous microbiota experiments, and outliers were identified using semi-studentized residual plot of microbial evaluation. Two replicates were identified as outliers and eliminated from all analyses performed. Mixed model ANOVA was used to test for statistical significance ($\alpha = 0.05$) for a repeated measures design in SAS 9.4. Ryan, Einot, Gabriel, Welsh Studentized Range Q (REGWQ) test, and Tukey HSD for *post-hoc* analysis in SAS 9.4 were performed within each setting.

ANOVA followed by Tukey HSD was used to evaluate the effect of thermosonication and sonicator position on reduction of *G. stearothermophilus* cells. Also, the effect of thermosonication, treatment time, and storage duration on microbial quality of milk were evaluated using ANOVA, followed by Tukey HSD. One-way ANOVA followed by Tukey HSD were used to analyze the log reductions at week 0. ANOVA followed by REGWQ test was used to determine if thermosonication and storage time had a significant effect on the pH, FFA, and CN/TP content of the milk during storage at each setting.

Results and discussion

Effect of thermosonication in a laboratory-scale continuous system on thermophilic bacteria

The log reductions in *G. stearothermophilus* cells (initial counts of 7.37 ± 3.89 log CFU/mL) for control and treatment conditions are shown in Table 1, when the sonicator was placed before and after the PHE. Setting 1 having longer residence times had the highest log reductions at both sonicator positions. The highest log reduction seen was 0.54 for treatment samples at setting 1. Thermosonication increased the log reductions of *G. stearothermophilus* cells at both settings as compared to the control conditions. For example, at PHE- sonicator position, control had log reductions of 0.37 ± 0.05 (setting 1) and 0.05 ± 0.01 (setting 2), whereas treatment had higher log reductions of 0.45 ± 0.05 (setting 1) and 0.26 ± 0.04 (setting 2) as compared to control at each setting. The only significant increase in log reductions due to thermosonication was seen for sonicator-PHE samples at setting 1. Overall, the sonicator's location did not have a significant effect on control and treatment log reductions of *G. stearothermophilus* cells (p-value = 0.18). The outlet temperature was not affected by the sonicator's location.

The findings for *G. stearothermophilus* reductions are comparable to the ones reported in previous studies for thermophilic or facultative thermophilic bacteria in a continuous system. Villamiel and De Jong (2000) studied the effect of thermosonication in a continuous system on *Streptococcus stearothermophilus* inoculated in growth media. They reported, that after 22.5 and 56.3 s of thermosonication, log reductions of 0.1 and 0.2 were observed for *S. stearothermophilus*. The results in this study are similar to those by Villamiel and De Jong (2000) where higher log reductions were observed at longer residence times as compared to shorter residence times. When comparing to a batch sonication study of *A. flavithermus* in milk (Khanal *et al.,* 2014), lower log reductions for *G. stearothermophilus* were seen in this continuous

system study. Khanal *et al.* (2014) reported a 1.27 log reduction in *A. flavithermus* cells when heat alone was applied for 30 min at 63°C. With cold sonication alone, a 1.1-log reduction was seen after 1 min of treatment time, and after application of sonication for 10 min, a 4-log reduction in *A. flavithermus* cells was observed (Khanal *et al.,* 2014).

This study showed that applying 11.9 s of thermosonication (in addition to 15.9 s of heating) did not significantly reduce *G. stearothermophilus* cells as compared to heat alone. The highest log reduction was 0.54 for treatment and 0.37 for control samples. *G. stearothermophilus* was used as a representative microorganism for thermophilic bacteria, and thermosonication may not decrease thermophilic bacterial cells associated with dairy foods under the conditions used in this study.

Effect of thermosonication in a laboratory-scale continuous system on indigenous milk microbiota during storage

Log reductions for week 0 after raw whole milk (initial microbial count of 4.41 ± 2.36 log CFU/mL) were processed either under control or treatment conditions at setting 1 and 2 are depicted in Table 2. Setting 1 had the highest log reductions in both control and treatment conditions. At setting 1, the log reductions were 1.38 and 1.91 for control and treatment, respectively. Whereas, for setting 2, the log reductions were 0.74 and 1.21 for control and treatment, respectively.

Table 2. Mean $\log_{10}$ reductions of indigenous microflora at week 0, after processing, at two different settings as described in Table 1.

Setting	Exit temperature (°C)	Control reductions[1]	Treatment reductions[2]
1	63	1.38 ± 0.04[a]	1.91 ± 0.06[b]
2	57	0.74 ± 0.13[c]	1.21 ± 0.07[d]

[1]Control: treated with heat alone with sample flowing through the PHE and sonicator with sonicator off, and [2]Treatment: with sample flowing through the PHE and sonicator with sonicator on. Values with different letters are significantly different. Values are mean ± S.E.

The log reductions for both control and treatment conditions during milk storage at settings 1 and 2 are shown in Figure 2. The log reductions decreased during storage as the microbial count increased. End of analyses during storage was determined when samples appeared to be spoiled with visible milk curdling (Fromm and Boor, 2004; Lim *et al.,* 2019). During storage, setting 1 could be analyzed for four weeks as compared to three weeks for setting 2. Significantly higher log reductions were seen for treatment samples at week 1 (1.29 logs), 2 (0.85 logs), and 3 (0.37 logs) at setting 1, and at setting 2 significant reductions were observed until week 1 (0.64 logs). Setting 1 control and treatment samples had an average microbial count of 4 log CFU/mL after four weeks, whereas for setting 2 it was 4 log CFU/mL after three weeks. Flow rate, thermosonication, and storage period had a significant three-way interactive effect on log reductions (p-value < 0.0001).

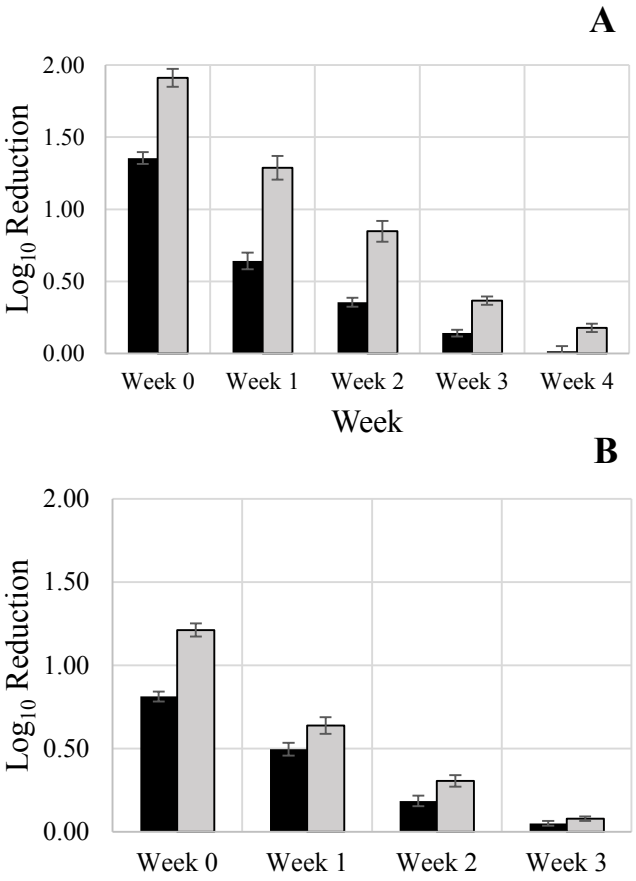

Figure 2. Log_{10} reductions (mean ± S.E) of indigenous microorganisms in whole milk over storage after being processed with control (solid black bar) or treatment (solid grey bar), at setting 1 (A) and setting 2 (B). *Signifies significant difference as compared to control.

The raw milk had an initial thermophilic bacterial count of 2.03 ± 1.04 log CFU/mL prior to any treatment. No thermophilic bacteria were observed at setting 1 after control or treatment processes from week 0 to 4. At setting 2, 1.21 ± 0.82 log CFU/mL of thermophilic bacteria was recorded at week 0 for control samples and 1.06 ± 0.56 CFU/mL for treatment samples. No growth of thermophilic bacteria was observed after week 1. Due to such lower microbial numbers for

thermophilic bacteria in milk, any interpretation of thermosonication effect on indigenous thermophilic bacteria in milk would be speculative.

Villamiel and De Jong (2000) applied thermosonication to raw milk at 76°C for 102.3 s in a continuous system, and reported a 3.1 log reduction in overall microbial count. Cameron *et al.* (2008) observed a 2-log reduction for *Listeria monocytogenes* after applying sonication for 10 min in pasteurized milk. Gera and Doores (2011) showed that sonication effectively inactivated the non-thermophilic bacteria *Escherichia coli* and *Listeria* at 30 - 35°C, after more than 2.5 and 7 - 8 min of treatment time, respectively. Similarly, in the current continuous system study, thermosonication along with heat significantly reduced the total microbial count as compared to control alone. The log reductions observed immediately after treatment are lower than reported values, which could be due to shorter residence times or application of low heat conditions in this study.

Thus, thermosonication of 11.9 s (after 15.8 s of heating) along with heat employed in the current study did improve the microbial quality of milk by significantly reducing the microbial numbers after treatment as compared to control. Although treatment samples maintained the lower microbial count during storage, no significant difference was observed at week 4 (setting 1) and week 3 (setting 2) between control and treatment samples. Thus, future studies should consider applying thermosonication with pasteurization for enhanced microbial reduction.

Effect of thermosonication with heat in a laboratory-scale continuous system on overall milk quality during storage

The pH of whole milk for both control and treatment at settings 1 and 2 are given in Tables 3 and 4, respectively. A decrease in pH could be a result of acids produced by microorganisms or due to lipolysis (Bermúdez-Aguirre *et al.,* 2009b). The pH of milk decreased during storage for both control and treatment at both the settings. At setting 1, pH of both control and treatment were not significantly different at week 0, with pH of 6.82 ± 0.02 for control, and 6.85 ± 0.02 for treatment. After four weeks, setting 1 control samples (6.29 ± 0.03) had significantly lower pH than treatment samples (6.43 ± 0.04). Meanwhile, at setting 2, pH of both control and treatment samples were not significantly different, with week 0 pH values (6.73 ± 0.01 for control, and 6.80 ± 0.03 for treatment) decreasing gradually to week 3 (6.35 ± 0.02 for control, and 6.39 ± 0.03 for treatment).

Similar to this study, Bermúdez-Aguirre *et al.* (2009b) observed no significant differences in pH between batch pasteurized and thermosonicated (for 30 min) in milk during 2-week storage, and a decrease in pH was observed for both the samples during storage. The pH value of setting 1 treatment samples after four weeks (6.4) was comparable to the value of 6.4 reported by Bermúdez-Aguirre *et al.* (2009b) after two weeks. Therefore, thermosonication with heat resulted in improving the pH of milk during storage as compared to heat alone at setting 1.

The extent of lipolysis was evaluated using the FFA content of milk for both control and treatment samples. FFA content over time is shown in Table 3 for setting 1 and Table 4 for setting 2. It is known that microorganisms in milk release lipases; therefore, the higher FFA content may be due to microbial lipases. The FFA content of treatment were lower at setting 1 as compared to setting 2 from week 1 to 3. There was no significant difference between the FFA content of both control and treatment samples at week 0 at each setting. Also, the FFA content significantly increased for both the control and treatment as the weeks progressed at each setting. At setting 1, after four weeks, thermosonication samples had significantly lower FFA content of 0.24 ± 0.02 meq FFA/kg as compared to 0.34 ± 0.01 for control. At setting 2, after three weeks, treatment samples had an FFA content of 0.24 meq FFA/kg similar to 0.26 for control.

To compare if milk used in this study was still acceptable based on the FFA content, values from this study were compared to previously published data. In previous research, the FFA content of commercially pasteurized (HTST) milk after 2.5-week storage was 0.25 meq FFA/kg (Fromm and Boor, 2004). In this study, treatment samples had an FFA content of 0.24 meq FFA/kg after four weeks at setting 1, and three weeks at setting 2, which is comparable to the value reported by Fromm and Boor (2004) after 2.5 weeks. Thus, thermosonication along with heat did significantly improve the FFA content of milk samples as compared to heat alone for setting 1.

During milk storage, the extent of proteolysis occurring in milk samples was tested using the CN/TP content at weeks 0, 2, and 4 and is shown in Table 3 for setting 1 and Table 4 for setting 2. A decrease in the CN/TP represents an increase in the proteolysis of milk proteins as a result of proteases released from microorganisms. Overall, the CN/TP ratio decreased for both control and thermosonication at each setting. At each setting, treatment samples had a higher CN/TP ratio as

Table 3. pH, free fatty acid (FFA), and casein/total protein (CN/TP) content of whole milk during storage after control or treatment at setting.

Week	pH		FFA (FFA/kg)		CN/TP	
	Control[1]	Treatment[2]	Control[1]	Treatment[2]	Control[1]	Treatment[2]
0	6.82 ± 0.02^{a}	6.85 ± 0.02^{a}	0.09 ± 0.01^{a}	0.09 ± 0.01^{a}	0.89 ± 0.04^{ab}	0.92 ± 0.01^{a}
1	6.67 ± 0.03^{b}	6.81 ± 0.02^{a}	0.11 ± 0.01^{ab}	0.12 ± 0.01^{ab}	-	-
2	6.52 ± 0.03^{cd}	6.67 ± 0.04^{cb}	0.18 ± 0.01^{cd}	0.17 ± 0.01^{bc}	0.85 ± 0.04^{b}	0.89 ± 0.02^{ab}
3	6.41 ± 0.03^{de}	6.55 ± 0.04^{cbd}	0.23 ± 0.01^{def}	0.20 ± 0.01^{cde}	-	-
4	6.29 ± 0.03^{e}	6.43 ± 0.04^{d}	0.34 ± 0.02^{f}	0.24 ± 0.01^{e}	0.74 ± 0.06^{c}	0.85 ± 0.03^{b}

[1]Control: treated with heat alone with sample flowing through the PHE and sonicator with sonicator off, and [2]Treatment: with sample flowing through the PHE and sonicator with sonicator on. (-): Measurements not taken. Values with different letters are significantly different within each parameter tested. Values are mean ± S.D.

Table 4. pH, free fatty acid (FFA), and casein/total protein (CN/TP) content of whole milk during storage after control or treatment at setting 2.

Week	pH		FFA (FFA/kg)		CN/TP	
	Control[1]	Treatment[2]	Control[1]	Treatment[2]	Control[1]	Treatment[2]
0	6.73 ± 0.01^{ab}	6.80 ± 0.03^{a}	0.11 ± 0.01^{a}	0.10 ± 0.01^{a}	0.87 ± 0.03^{a}	0.89 ± 0.04^{a}
1	6.62 ± 0.03^{bc}	6.72 ± 0.03^{ab}	0.13 ± 0.01^{a}	0.12 ± 0.01^{a}	-	-
2	6.49 ± 0.04^{cd}	6.55 ± 0.05^{cd}	0.19 ± 0.01^{ab}	0.18 ± 0.01^{ab}	0.83 ± 0.02^{a}	0.85 ± 0.03^{a}
3	6.35 ± 0.02^{e}	6.39 ± 0.03^{de}	0.26 ± 0.01^{b}	0.24 ± 0.01^{b}	-	-

[1]Control: treated with heat alone with sample flowing through the PHE and sonicator with sonicator off, and [2]Treatment: with sample flowing through the PHE and sonicator with sonicator on. (-): Measurements not taken. Values with different letters are significantly different within each parameter tested. Values are mean ± S.D.

compared to control for both the settings. Only at setting 1 did treatment samples have a significantly higher CN/TP content of 0.85 ± 0.03 as compared to 0.74 ± 0.07 of control after four weeks. Fromm and Boor (2004) when analyzing HTST pasteurized milk during storage for proteolysis, reported that the CN/TP content decreased from 0.87 to 0.85 in 2.5 weeks. After four weeks in this study, at setting 1, samples treated with thermosonication had CN/TP content of 0.85 which is comparable to the value reported in literature (Fromm and Boor, 2004). Therefore, thermosonication along with heat did significantly improve the CN/TP content of milk at setting 1 as compared to heat alone.

To summarize, thermosonication for 11.9 s (with additional heating time of 15.9 s) along with heat in a laboratory-scale continuous system significantly reduced the microbial count after processing as compared to heat alone but did not affect the microbial counts after four weeks at setting 1, and three weeks at setting 2. Thermosonication also significantly improved the pH, FFA content, of milk during storage as compared to heat alone. This research helps establish the potential application of thermosonication for reducing microbial population in milk in future scale up studies. Also, pH, FFA content, and CN/TP content were significantly affected and can be used as indicators of milk quality in future studies. Previous research has shown applying sonication increases overall milk quality (Villamiel and De Jong, 2000; Bermúdez-Aguirre *et al.,* 2009b). These studies employed residence times ranging from 2 - 30 min, making it difficult for practical applications. Whereas, based on the results obtained from this study, implementation of thermosonication along with heat at a scale-up level could help improve milk quality during storage as compared to heat alone while keeping the residence times for thermosonication reasonable (10 - 15 s) for practical applications. Scale-up systems should involve use of HTST pasteurization conditions, to evaluate the effect of thermosonication in achieving higher milk quality, and greater microbial reduction.

Conclusion

Overall, the effect of thermosonication along with heat in a laboratory-scale continuous system on *G. stearothermophilus* cell reductions was not significant as compared to heat alone. Longer residence times showed higher *G. stearothermophilus* cell reductions, with log reductions ranging between 0.45 - 0.54 for treatments as compared to 0.25 - 0.37 for control. The log reductions observed were however not impactful enough to suggest scale-up applications. Thermosonication along with heat in a laboratory-scale continuous system significantly decreased the indigenous microbiota in milk as compared to heat alone at week 0. No significant differences were seen between log reduction for control and treatment samples after four weeks at setting 1, and three weeks at setting 2.

For all the samples, the pH and CN/TP content decreased while FFA content increased during storage. Thermosonication samples had significantly higher pH and CN/TP and lower FFA values as compared to control at setting 1 at week 4. Applying thermosonication for 11.9 s with a milk outlet temperature of 71°C helped reduce the indigenous milk microbiota when applied in a laboratory-scale system as compared to heat alone. Scale-up studies should use thermosonication along with pasteurization to help achieve greater microbial reduction. pH, FFA content, and CN/TP content can be used as indicators of milk quality. Future studies implemented in a scale-up system should focus on keeping the residence times for thermosonication suitable for industrial application and should include a sensory evaluation component.

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