Evaluation of high-intensity ultrasonication for the inactivation of endospores of 3 bacillus species in nonfat milk

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ABSTRACT

Endospores of *Bacillus licheniformis* [American Type Culture Collection (ATCC) 6634], *Bacillus coagulans* (ATCC 12245), and *Geobacillus stearothermophilus* (ATCC 15952) were spiked in sterile nonfat milk, and subjected to high intensity batch ultrasonication treatment at different amplitudes (80 or 100%) and durations (1 to 10 min). Increasing the amplitude from 80 to 100% did not result in enhanced inactivation of *G. stearothermophilus* endospores. However, an increase in the duration of ultrasonication from 1 to 10 min significantly increased the inactivation of endospores of all 3 species. About 48.96% of the *G. stearothermophilus* endospores were inactivated by ultrasonication alone, whereas ultrasonication and pasteurization combined increased the inactivation to 65.74%. Inactivation of endospores could be further enhanced to 75.32% by ultrasonication and higher heat (80°C/1 min) combination. Endospores of *B. licheniformis* and *B. coagulans* were inactivated to a lesser extent compared with *G. stearothermophilus* spores. Ultrasonicated *B. licheniformis* endospores germinated in higher numbers when compared with untreated endospores resulting in their greater inactivation during the combined treatment. During microstructure imaging of ultrasonicated endospores, although no structural damage was noticed, they showed irregular shrinkage and wrinkles with surface coarseness. This may also have contributed to their reduced thermal resistance, in addition to sporulation.

Key words: ultrasonication, sporeformers, nonfat milk, endospore

INTRODUCTION

Aerobic spore-forming bacilli are potential milk-spoilage organisms. Their endospores can survive milk pasteurization, germinate, and grow in the product,

thereby producing enzymes, acids, and other metabolites that can cause alteration in sensory properties of milk (Kalogridou-Vassiliadou, 1992; Berkeley, 2008). Most importantly, the psychrotrophic thermophiles, which can grow at refrigerated temperatures, have a much higher milk-spoilage potential than the obligate thermophiles or mesophiles (Burgess et al., 2010). Spores of these bacilli resist thermal treatment and germinate in products, whereas the vegetative cells grow further at refrigeration temperature, thereby continuing the product's spoilage. Such peculiar natures of spore-forming psychrotrophs highlight their important role in milk and dairy products spoilage (Meer et al., 1991). Shelf life is considered to be reduced when psychrotrophic counts in milk and dairy products rises to a level of 7 log cfu/mL or 1 g of the product (Berkeley, 2008; Heyndrickx and Scheldeman, 2008).

The resistance of bacterial endospores to different physical and biochemical treatments, dormancy, adhesiveness, and prompt germination under favorable conditions make them important contaminants in dairy systems (Atrih and Foster, 2002). *Bacillus* species are frequently isolated from various heat-treated milk products, such as pasteurized milk, UHT-processed milk, milk powder, cheese, and yogurt (Faille et al., 2001). Spores are generally unaffected by a variety of chemicals, including acids, bases, oxidizing agents, alkylating agents, aldehydes, and organic solvents (Russell, 1990; Setlow, 2006); as the spores are highly heat resistant, several other nonthermal methods for spore removal or inactivation have been investigated with varying degrees of success, such as bacto-fugation (Torres-Anjel and Hedrick, 1971; Lewis and Deeth, 2009), microfiltration (Kosikowski and Mistry, 1990; Villamiel et al., 2009; Mukhopadhyay et al., 2011), and high-pressure processing (Heinz and Knorr, 2002; Farkas and Hoover, 2008; Considine et al., 2008). High-intensity ultrasonication was effective in inactivating *Bacillus* spp. vegetative cells (Khanal et al., 2014) and, to a minor extent, endospores suspended in liquid medium (Burgos et al., 1972). Ultrasonication operating between 20 and 100 kHz, also referred to as power ultrasound, produces vibrations of the molecules in liquid medium, thereby

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generating disruptive physical effects such as cavitation (Bermúdez-Aguirre and Barbosa-Cánovas, 2011). During cavitation, temperature and pressure are assumed to increase inside the bubble up to 5,500°C and 50 MPa respectively, which causes microbial inactivation (Raso et al., 1998; Villamiel et al., 2009; Bermúdez-Aguirre and Barbosa-Cánovas, 2011). It has been reported that the sporicidal effect of ultrasonication depends on static pressure, amplitude of ultrasonic waves, and treatment temperature microbial inactivation (Raso et al., 1998; Cameron et al. (2009). The present study was conducted to evaluate the application of ultrasonication and the combination of ultrasonication and pasteurization on the inactivation of some of the highly heat-resistant spores of *Bacillus coagulans*, *Bacillus licheniformis*, and *Geobacillus stearothermophilus* in nonfat milk.

MATERIALS AND METHODS

Production of Endospores

Three different aerobic spore-forming bacterial species, namely *Bacillus coagulans*, *Bacillus licheniformis*, and *Geobacillus stearothermophilus*, were used in this work. Pure cultures of these strains were purchased from American Type Culture Collection (ATCC; Manassas, VA; Table 1). The original bacterial strains were grown in brain-heart infusion (BHI; BD Difco, Franklin Lakes, NJ) broth by incubating at their respective optimum growth temperatures (Table 1) as per the supplier instructions. The cultures were pelleted out and preserved at -80°C for future use in cryogenic vials (Cryobank, Copan Diagnostic Inc., Murrieta, CA) containing sterile beads and glycerol.

Endospores of different bacterial species were prepared by using methods suggested by Novak et al. (2005) and Martínez Viedma et al. (2010), with some modifications. Approximately 1 mL of actively growing broth culture of each of the spore-formers was spread-plated on the BHI agar plates (150 mm o.d. $\times$ 15 mm high). The plates were incubated at the appropriate incubation temperatures (Table 1) for up to 10 d. Spore staining of the culture was performed intermittently during the incubation period to monitor

the extent of sporulation. When approximately 90% of sporulation was attained, spores were harvested using a similar method described by Wang et al. (2009). Approximately 10 mL of sterile distilled water was flooded on the plate's agar surface and soaked for 2 to 3 min. The surface was scraped with sterile spreaders and the spore suspension was collected in 50-mL sterile centrifuge tubes. The tubes were centrifuged at $4,500 \times g$ for 30 min and the supernatants were discarded. The pellets were washed 2 times by suspending in 20 mL of distilled water and then centrifuged at $4,500 \times g$ for 30 min at 20°C . Finally, the washed pellets were suspended in 10 to 15 mL of distilled water and heated at 85°C for 10 min to kill all the remaining vegetative cells and cooled. The spore suspensions containing 10^7 to 10^9 spores/mL were stored at -18°C up to 1 mo.

Ultrasonication of Milk Samples

Nonfat milk collected from the dairy plant of South Dakota State University was autoclaved at 121°C for 15 min, cooled, and stored at 4°C before use. Spore suspensions were prepared in sterilized distilled water and inoculated in 250 mL of sterile cold milk at $\sim\log$ 5 to 6 cfu/mL. Spore-inoculated samples (20 mL) in sterilized glass vials (28 mm o.d. $\times$ 75 mm high) were subjected to ultrasonication treatments.

A 500-W, 20-kHz Vibra-Cell high intensity ultrasonic Processor VC 505 (Sonics & Materials Inc., Newtown, CT) with a stainless steel-coated 13-mm titanium solid probe was used for ultrasonication of milk. The equipment had provision to adjust the pulse continuity and the amplitudes (20 to 100%); it could be operated from 1 s to several hours of time. The equipment also had the ability to measure the ultrasonication power being generated during the process. The height of the probe from the bottom of the sample container was adjusted to 3 to 4 cm by moving the probe holder vertically. Before inserting in to the sample, the probe was washed thoroughly and disinfected by flooding with 70% ethanol. Any residual ethanol on the probe was washed off with sterile distilled water.

A 2 by 3 factorial experiment with 2 amplitudes and 3 treatment times was designed to obtain the most

Table 1. Organisms, sources, growth media, and optimum temperatures¹

Organism	Source	Growth media	Growth temperature ($^{\circ}\text{C}$)
<i>Bacillus coagulans</i> (ATCC 12245)	ATCC	BHI broth/agar	37
<i>Bacillus licheniformis</i> (ATCC 6634)	ATCC	BHI broth/agar	30
<i>Geobacillus stearothermophilus</i> (ATCC 15952)	ATCC	BHI broth/agar	55

¹ATCC = American Type Culture Collection; BHI = brain-heart infusion.

effective ultrasonication parameters. Samples were ultrasonicated at amplitude levels of 80 and 100% (100% corresponds to 114 μm) for time durations of 1, 5, and 10 min. Ultrasonication was carried out using a slight modification of the method as described by Cameron et al. (2009). Milk samples (20 mL) were ultrasonicated in 25 mL of borosilicate sterilized glass vials. The vials containing milk samples were immersed in an ice bath at 0°C to prevent any excessive temperature rise during ultrasonication. Ultrasonic waves were applied to the sample by maintaining 3-s on-off pulses without affecting the net ultrasonication time. After completion of sonication, temperatures of the sample were recorded. Vials were sealed, cooled to $\sim 4^\circ\text{C}$ by placing in an ice bath, and stored at the same temperature until plating. Ultrasonication amplitude was selected on the basis of maximum consistent power generated, and the percentage decline in *Geobacillus stearothermophilus* (ATCC 15952) spore count after ultrasonication.

Ultrasonication of milk inoculated with *B. licheniformis* (ATCC 6634) or *B. coagulans* (ATCC 12245) spores was carried out in a similar way at a selected (constant) amplitude for 1, 5, and 10 min. Samples were stored at $\sim 4^\circ\text{C}$ for maximum 6 h of ultrasonication and surviving spores counts were compared with that of corresponding untreated samples (controls).

Heat Treatment of Samples

Milk samples (treated or control) were subjected to heat treatment by similar methods. Exactly 2 mL of samples were transferred in sterile test tubes (10 $\times$ 75 mm) and heated by immersing in a temperature-controlled (63.5°C) water bath. The samples reached 63.5°C within 3 min and were allowed to remain at the same temperature for exactly 30 min. After 30 min, samples were transferred to an ice water bath and cooled to 3 to 5°C within 10 min before plating. For the high-heat treatment purpose, only the ultrasonicated samples with *G. stearothermophilus* spores were heated to 80°C for 1 min and cooled down to 3 to 5°C before agar plating. Spore counts before and after heat treatments were obtained and compared.

Germination of Ultrasonicated Endospores

Spore suspension (~ 6.5 log cfu/mL, with no live vegetative cells) of *B. licheniformis* (ATCC 6634) in nonfat milk was ultrasonicated at the 80% amplitude level for 10 min. Following ultrasonication, 2 mL of milk (in 5-mL sterile test tubes) was incubated at 30°C to allow spore germination up to 300 min. Control

samples (without ultrasonication) were also incubated parallel to the ultrasonicated samples. Control and ultrasonicated samples were taken out of the incubator at the interval of 60 min. To kill the vegetative cells formed after spore germination, the samples were heat treated at 63°C for 30 min, as described above. Survived spores were counted before and after heat treatment. As vegetative cells of same species are not resistant to the applied heat treatment (Khanal et al., 2014), any decline in numbers after heat treatment are the number of spores germinated in cells. The numbers of ultrasonicated spores that had undergone germination were compared with the untreated spores over the germination time of 0 to 300 min.

Enumeration of Endospores

Spores were enumerated in the milk samples by the aerobic spread plate technique on Tryptone Glucose Extract Agar (BD Difco) plates. Samples were serially diluted in sterile PBS solution at pH 7.4. Plates were incubated for 24 to 48 h and 25 to 250 colonies were counted. Colony-forming units per milliliter of samples were calculated using a method suggested by Wehr and Frank (2004).

Microscopic Examination of Endospores

Spores were observed under the scanning electron microscope for any posttreatment changes by using a similar method described by Bulla et al. (1969) and Khanal et al. (2014). The spores were suspended in sterile distilled water at ~ 5 log cfu/mL and the suspension was ultrasonicated and pasteurized. Exactly 15 μL each of the untreated, ultrasonicated, and ultrasonicated and pasteurized spore suspensions were placed on 10 $\times$ 15 mm clean and sterile glass slides and air dried. Smears were sputter coated with a 5-nm layer of gold using a CRC-150 sputtering system (Plasma Science Inc., Lorton, VA). Slides were observed at different magnifications under a Hitachi scanning electron microscope (model S-3400N, Hitachi SCI Systems Ltd., Tokyo, Japan) at a beam accelerating voltage of 30 kV and a working distance of 8.0 to 8.5 mm.

Statistical Analysis

The trials were repeated twice with 3 replications in each experiment. Statistical analyses were carried out using SAS software, version 9.2 (SAS Institute Inc. Cary, NC). Mean values ($n = 6$) were compared by *t*-test and ANOVA with least significance difference at $P < 0.05$.

Table 2. Effect of pasteurization treatment on inactivation of spores of bacilli in nonfat milk

Species ¹	Average count (log cfu/mL)		Log reduction
	Initial	After pasteurization ²	
<i>Bacillus coagulans</i> (ATCC 12245)	5.51 ^a	5.42 ^a	0.09
<i>Bacillus licheniformis</i> (ATCC 6634)	4.26 ^b	4.25 ^b	0.01
<i>Geobacillus stearothermophilus</i> (ATCC 15962)	5.48 ^a	5.39 ^a	0.09

^{a,b}Means within a row with different superscripts are significantly different ($P < 0.05$).

¹ATCC = American Type Culture Collection.

²Pasteurization by heating at 63°C for 30 min and cooling to 4°C.

RESULTS AND DISCUSSION

Sporulation of *Bacillus* Species

Adequate levels of sporulation of *B. coagulans* (ATCC 12245), *B. licheniformis* (ATCC 6634), and *G. stearothermophilus* (ATCC 15952) were achieved using BHI agar plates. After harvesting, endospore suspensions of up to 9.0 log cfu/mL were obtained for *B. licheniformis*; whereas, in the case of *G. stearothermophilus* and *B. coagulans*, suspensions with endospore concentrations up to 7.0 log cfu/mL could be obtained.

Thermal Resistance of *Bacillus* Endospores

The results on the effectiveness of pasteurization treatment on endospores of *B. coagulans* (ATCC 12245), *B. licheniformis* (ATCC 6634), and *G. stearothermophilus* (ATCC 15952) are shown in Table 2. No significant difference ($P > 0.05$) was noted between the log spore counts before and after pasteurization for all 3 types of endospores. Survivability of bacillus spores during pasteurization is not unusual, as these spores are not inactivated even by far more severe thermal treatments. The D-values of many of the bacillus spores at 100°C is more than 100 min in different media, including pH-neutral Ringer solution and milk (Scheldeman et al., 2006; Dogan et al., 2009). However, the heat resistance of spores in milk and milk products may vary on the

milk constituent's concentration as well as the individuality of the spore species (Behringer and Kessler, 1992).

Standardization of Ultrasonication Treatment Parameters

To select the most effective treatment, 6 treatment combinations, including 2 ultrasonication amplitude levels (100 and 80%) and 3 time durations (1, 5, and 10 min), were compared. Power generation during the treatment and the corresponding temperature rise were observed. Effectiveness of these treatments on the inactivation of *G. stearothermophilus* endospores in milk was also compared, and the most effective treatment combination was selected as explained herein.

Ultrasonic Power and Temperature Increase. Results of the effect of amplitude and time on ultrasonic power generation during the treatment are presented in Table 3. In 20-mL samples treated at 80% amplitude, mean power generation after 1 and 5 min was 63.45 and 63.55 W, respectively, and was not significantly different ($P > 0.05$). A significantly higher level of ultrasonic power generation (73.58 W) was observed for 10 min of sonication time. In comparison, the power generated at 100% amplitude was 94.18, 83.66, and 72.68 W for 1, 5, and 10 min of ultrasonication time, respectively (Table 3).

Temperature increases during ultrasonication at different treatments are presented in Table 3. When 20

Table 3. Temperature increase and power generation during ultrasonication treatment of nonfat milk

Amplitude ¹ (%)	Time (min)	Temperature increase ± SEM (°C)	Ultrasonic power generated ± SEM (W; n = 6)
80	1	22.7 ± 0.31 ^c	63.45 ± 0.36 ^d
	5	28.2 ± 0.39 ^b	63.55 ± 0.55 ^d
	10	33.7 ± 0.46 ^a	73.58 ± 0.48 ^c
100	1	29.3 ± 0.57 ^b	94.18 ± 1.11 ^a
	5	32.7 ± 0.51 ^a	83.66 ± 1.72 ^b
	10	33.9 ± 0.57 ^a	72.68 ± 0.60 ^c

^{a-d}Means within the column with different superscripts are significantly different ($P < 0.05$).

¹100 and 80% of amplitude correspond to 114 and 91.2 µm, respectively.

Table 4. Standardization of the ultrasonication parameters using *Geobacillus stearothermophilus* spores in nonfat milk¹

Amplitude (%)	Time (min)	Average initial count (cfu/mL)	Average final count (cfu/mL)	Reduction of spores (%) $\pm$ SEM (n = 6)
80	1	9.29×10^5	7.95×10^5	14.46 ± 4.25^c
	5	7.77×10^5	6.47×10^5	16.70 ± 3.49^c
	10	8.96×10^5	4.57×10^5	48.96 ± 3.17^a
100	1	8.70×10^5	7.66×10^5	11.96 ± 4.79^c
	5	8.45×10^5	6.65×10^5	21.26 ± 4.69^{bc}
	10	8.09×10^5	5.50×10^5	32.01 ± 4.16^b

^{a-c}Means within a column with different superscripts are significantly different ($P < 0.05$).

mL of sample was ultrasonicated at the 80% amplitude level, the average increase in temperature during 1, 5, and 10 min was 22.71, 28.16, and 33.71°C, respectively. At 100% amplitude, the temperature increased by 29.35, 32.66, and 33.91°C, respectively, for the 3 treatment times. Values of power generated and temperature increase obtained by varying amplitude and treatment time were analyzed by 2-way ANOVA. It was observed that the effect of amplitude, time, and interaction of amplitude and time on power generation was significant ($P < 0.0001$). Similarly, a significant effect ($P < 0.0001$) was observed for amplitude, time, and interaction of amplitude and time in temperature increase.

The results of the experiments revealed that 63 to 73.58 W of power was generated at 80% amplitude and 10 min ultrasonication time. In addition, the power generated was more consistent during the extended ultrasonication time of 10 min. At 100% amplitude a higher value of power was generated (72.68 to 94.18 W), but unlike at 80% amplitude, power dropped during longer ultrasonication time of 10 min. Sonication at 80% amplitude did not cause any churning, as the ultrasonic waves were apparently absorbed in to the sample itself. Conversely, sonication at 100% amplitude produced rigorous agitation of milk. As similar ultrasonication treatments produced markedly lesser power generation in the air medium, it may be assumed that the excessive churning and splashing might have reduced the contact surface between sonotrode and milk medium, thereby causing the reduced power generation.

Effectiveness of Amplitude and Ultrasonication Time on Endospore Inactivation. *Geobacillus stearothermophilus* endospores were chosen as the test organism because its spores are considered the most heat resistant and are used in testing sterility of the autoclaving process (Rutala et al., 1982). A nonfat milk sample spiked with spores was ultrasonicated at 80 and 100% amplitude levels for 1, 5, and 10 min. The effect of ultrasonication amplitude and time on *G. stearothermophilus* is shown in Table 4. The mean percent reductions in spore counts in samples treated

at 80% amplitude for 1, 5, and 10 min were 14.46, 16.67, and 48.96%, respectively. When similar samples were ultrasonicated at 100% amplitude for 1, 5, and 10 min, 11.96, 21.26, and 32.01% of spore inactivation was achieved, respectively (Table 4).

The increased amplitude from 80 to 100% did not have a significant ($P = 0.1524$) effect on the inactivation of *G. stearothermophilus* spores. Conversely, increasing ultrasonication time from 1 to 10 min significantly increased ($P < 0.0001$) spore inactivation. The interaction effect of amplitude and time was significant ($P < 0.0426$); however, the main effect of amplitude was not significant. This means that the increase in amplitude from 80 to 100% did not result in a significant increase in spore inactivation. A comparison of means of percent spore reductions corresponding to the individual treatments with least significant difference (Table 4) showed the 80% amplitude level for 10 min resulted in the highest inactivation of spores. Based on the analysis of temperature rise, power generation, and spore inactivation, 80% amplitude was selected as optimum to be used in further studies. Even though 10 min of sonication time resulted in maximum inactivation, treatment times of 1, 5, and 10 min were tested in other spores, assuming that different spores may have differences in resistance to ultrasonication.

The effectiveness of ultrasonication on liquid medium depends on different factors, including frequency, power, amplitude, and treatment time. In addition, pressure, temperature, viscosity, and concentration of solids also affect the overall ultrasonication effectiveness in many ways (Mason et al., 1996; Patist and Bates, 2008). The intensity of ultrasonication was reported to be proportional to the amplitude of vibration of the sonotrode, and, in general, the increase in amplitude was also found to increase the overall effectiveness of ultrasonication (Santos et al., 2009; Kentish and Ashokkumar, 2011). Conversely, a minimum intensity is required to achieve the cavitation threshold for a particular liquid medium while passing ultrasonication waves in it. The amplitudes higher than the threshold may not always produce favorable results during extraction or inactiva-

tion processes. Moreover, higher amplitude may also cause a rapid fall of the ultrasonic transducer that may result in vigorous liquid agitation, thereby causing poor cavitation (Santos et al., 2009). Temperature of the medium is another important factor that governs the effectiveness of ultrasonication. More intense ultrasonic cavitation can be attained at lower temperatures when other ultrasonic parameters are constant (Santos et al., 2009). Piyasena et al. (2003) described the temperature to be an extremely important factor that affects ultrasonication effectiveness. Temperature is reported to be inversely related to the cavitation threshold. At higher temperatures, the vapor pressure of the solvent rises and the vapor fills the cavitation bubbles, thereby causing them to collapse less violently. This leads to the overall decrease in cavitation and the effect produced is lesser than expected (Santos et al., 2009). One way to extend the effect of cavitation is to increase the ultrasonication time. Extended ultrasonication time from 1 to 10 min at constant amplitude was found to be effective in inactivating additional 3 logs of *Anoxybacillus flavithermus* cells in skim milk, as reported in our previous study (Khanal et al., 2014). Intense cavitation can also be produced by ultrasonication of increased volume of the sample, but this may not always support the inactivation of cells or spores possibly because the net ultrasonication energy per unit volume may not change (Joyce et al., 2003a,b).

Effectiveness of Ultrasonication Treatment on Bacterial Endospores

Durations of 1, 5, and 10 min of ultrasonication inactivated 0.52, 14.9, and 33.2% of *B. licheniformis* spores, respectively. A combined effect of ultrasonication and

pasteurization resulted in 13.1, 29.5, and 37.9% of spore inactivation for all 3 treatment times, respectively (Table 5). For *B. coagulans*, ultrasonication alone for 1, 5, and 10 min resulted in 12.95, 11.63, and 35.63% of spore reduction. When ultrasonicated spores were subjected to pasteurization, the inactivation levels increased to 24.55, 28.07, and 50.39%, respectively (Table 5). The inactivation levels achieved in the case of *G. stearothermophilus* endospores were 14.46, 16.90, and 48.96% when subjected to ultrasonication alone for 1, 5, and 10 min; whereas, ultrasonication and pasteurization combined resulted in increased levels of 18.90, 21.47, and 65.74% of spore inactivation. Ultrasonication for 10 min and heating at a higher temperature of 80°C for 1 min further increased inactivation of *G. stearothermophilus* endospores to 75.3% (Table 5). In comparison to ultrasonication alone, significant increases ($P < 0.05$) were observed for inactivation of all 3 spore types when ultrasonicated samples were pasteurized (Table 5, Figure 1). The results obtained in the present study showed that when treatment time increased to 10 min, percent reduction in *B. coagulans* and *G. stearothermophilus* endospores significantly increased to 35.63 and 48.96%, respectively (Figure 1). The overall effect of ultrasonication time was observed to be significant for inactivation of all tested spores.

Previous reports indicate that longer ultrasonication times are required to achieve inactivation of spores beyond 1 log. Short treatment times, up to 12 min, did not cause the spore inactivation as such, but reduced their resistance to thermal treatments (Burgos et al., 1972). Betts and) Williams (1999) have reported heating in combination with ultrasonication as one of the most commonly applied inactivation techniques. They have reported a greater inactivation of bacteria and

Table 5. Effect of ultrasonication and ultrasonication plus pasteurization on bacilli spores in nonfat milk

Spore species ¹	Time (min)	Initial count (cfu/mL)	Reduction in count ² (%) ± SEM		
			Ultrasonication ³ alone	Ultrasonication and pasteurization ⁴	Ultrasonication and high heat ³
<i>Bacillus licheniformis</i> (ATCC 6634)	1	2.83×10^4	0.52 ± 2.68^k	13.1 ± 3.02^j	
	5	2.37×10^4	14.91 ± 5.21^{ij}	29.45 ± 3.68^{ef}	
	10	2.43×10^4	33.23 ± 3.15^{de}	37.88 ± 3.67^d	—
<i>Bacillus coagulans</i> (ATCC 12245)	1	7.75×10^5	12.95 ± 4.25^j	24.55 ± 5.94^{fg}	
	5	7.68×10^5	11.63 ± 3.49^j	28.07 ± 0.93^{ef}	
	10	7.85×10^5	35.63 ± 3.17^d	50.39 ± 5.31^c	—
<i>Geobacillus stearothermophilus</i> ATCC (15952)	1	8.57×10^5	14.46 ± 4.25^j	18.90 ± 3.59^{hi}	
	5	8.79×10^5	16.70 ± 3.49^{ij}	21.47 ± 5.6^{gh}	
	10	8.87×10^5	48.96 ± 3.17^c	65.74 ± 5.40^b	75.32 ± 3.19^a

^{a-k}Means with different superscripts are significantly different ($P < 0.05$).

¹ATCC = American Type Culture Collection.

²80°C for 1 min.

³Ultrasonication at 80% amplitude.

⁴Pasteurization at 63°C for 30 min.

Figure 1. Effect of ultrasonication and ultrasonication plus pasteurization on inactivation of bacilli spores in nonfat milk. An open circle (○) represents *Bacillus licheniformis* [American Type Culture Collection (ATCC) 6634] spores; a diamond (◆) represents *Bacillus coagulans* (ATCC 12245); and a filled circle (●) represents *Geobacillus stearothermophilus* (ATCC 15952) spores. Dotted lines (···) represent ultrasonication at 80% amplitude (91.2 μ m) for 10 min. Solid lines (—) represent ultrasonication at 80% amplitude (91.2 μ m) for 10 min followed by pasteurization at 63°C for 30 min. (n = 6; error bar denotes SEM).

spores by heat when preceded by ultrasonication. In another report, ultrasonication resulted in a decrease of the thermal resistance of bacterial spores up to 43% (Betts and Williams, 1999). Similarly, a decline in D-value of ultrasonication-treated *B. licheniformis* spores in ringer solution has also been reported by Burgos et al. (1972). Those authors also reported that the D-value of *Bacillus cereus* spores at 110°C decreased from 11.5 to approximately 1.5 min as a result of ultrasonication treatment. Ultrasonication in combination with thermal treatment (thermosonication) led to more than 99.9% of *B. subtilis* spore inactivation (Raso et al., 1998). The overall results from the present study indicate reduction in thermal resistance of bacterial endospores already subjected to ultrasonication in skim milk. This effect is more likely due to the germination of endospores, as evident by the results obtained during the study conducted with *B. licheniformis* endospores.

Effect of Ultrasonication on Endospore Germination in Nonfat Milk

We hypothesized that the ultrasonication would induce endospore germination, which was verified by allowing the ultrasonicated endospores to germinate (by incubation). These germinated endospores (in the

form of vegetative cells) were more susceptible to heat treatments, such as pasteurization, compared with nonultrasonicated endospores. The survival counts for *B. licheniformis* endospores subjected to germination (with or without ultrasonication) followed by heat treatment are presented in Table 6. The results revealed that a greater number of endospores germinated in the ultrasonicated samples as compared with the untreated samples. Heat treatment (63°C for 30 min) alone did not result in any reduction in endospore counts in nonultrasonicated sample. Conversely, mean counts of 6.02 log cfu/mL were reduced by 0.48 logs when ultrasonicated (80% amplitude for 10 min) milk samples were subjected to heat treatment. Endospore survival continued to decrease gradually as the ultrasonicated samples were incubated for germination before the heat treatment (Table 6, Figure 2).

After 300 min of incubation the endospore counts were 5.35 log cfu/mL in treated samples, as compared with the nonultrasonicated samples that showed a higher endospore survival of 5.85 log cfu/mL. Thus, the results indirectly indicated a greater reduction of endospores by ~ 0.50 log cfu/mL due to complete or partial germination of the ultrasonicated endospores during incubation. Overall, 68% or more ultrasonicated endospores were heat inactivated in comparison to the nonultrasonicated endospores. The difference in thermally resistant endospore counts between ultrasonicated and nonultrasonicated samples was consistent

Figure 2. Effect of ultrasonication on germination of *Bacillus licheniformis* (American Type Culture Collection 6634) spores in nonfat milk at 30°C. A diamond (◆) indicates the spore count without any treatment; a closed circle (●) indicates spore count after pasteurization at 63°C for 30 min; and an open circle (○) represents spores ultrasonicated at 80% amplitude (91.2 μ m) for 10 min and heat treated at 63°C for 30 min. (n = 6; error bar denotes SEM).

Table 6. Effect of ultrasonication on germination of *Bacillus licheniformis* spores in nonfat milk

Incubation ¹ time (min)	Count (log cfu/mL $\pm$ SEM)			
	Incubated without treatment	Incubated without treatment and pasteurized	Ultrasonicated ² , incubated and pasteurized ³	Δ Log ⁴
0	6.50 $\pm$ 0.03 ^a	6.50 $\pm$ 0.03 ^a	6.45 $\pm$ 0.48 ^a	0.48
60	6.45 $\pm$ 0.01 ^a	6.30 $\pm$ 0.08 ^a	5.90 $\pm$ 0.60 ^{bc}	0.60
120	6.44 $\pm$ 0.01 ^a	6.20 $\pm$ 0.14 ^{ab}	5.72 $\pm$ 0.78 ^{cd}	0.78
180	6.49 $\pm$ 0.01 ^a	6.04 $\pm$ 0.11 ^{bc}	5.65 $\pm$ 0.85 ^{de}	0.85
240	6.48 $\pm$ 0.01 ^a	5.97 $\pm$ 0.13 ^{bc}	5.55 $\pm$ 0.95 ^{de}	0.95
300	6.49 $\pm$ 0.02 ^a	5.80 $\pm$ 0.16 ^{bc}	5.35 $\pm$ 1.15 ^e	1.15

^{a-e}Means with different superscripts are significantly different ($P < 0.05$).

¹Incubated at 30°C.

²Ultrasonicated at 80% amplitude for 10 min.

³At 63°C for 30 min.

⁴Difference between Log counts of untreated spores after heat treatment and ultrasonicated spore after heat treatment.

during the germination time from 60 to 300 min (Figure 2). When spores, suspended in milk, are incubated, some spores start germinating into vegetative cells. As their vegetative counterparts (cells) are not thermally resistant, they can be inactivated after the pasteurization treatment that results in a reduced survival count.

In cases of ultrasonicated samples, a relatively higher number of spores might have germinated, resulting in a higher percentage reduction in spore counts in comparison to the control (untreated sample). It is obvious that all the spores do not germinate at once, the extended incubation time allowed a higher number of spores

Figure 3. Scanning electron microscopic images of *Bacillus licheniformis* (American Type Culture Collection 6634) spores. (A) Before ultrasonication, viewed at lower magnification (5,000 $\times$). (B) After ultrasonication at 91.2 μ m amplitude for 10 min, viewed at lower magnification (5,000 $\times$). (C) Before ultrasonication, viewed at higher magnification (25,000 $\times$). (D) After ultrasonication at 91.2 μ m, viewed at higher magnification (25,000 $\times$). (E) After ultrasonication at 91.2 μ m and pasteurization (63°C, 30 min), viewed at higher magnification (25,000 $\times$).

Figure 4. Scanning electron microscopic images of *Bacillus coagulans* (American Type Culture Collection 12245) spores. (A) Before ultrasonication, viewed at lower magnification (3,200×). (B) After ultrasonication at 91.2 μm amplitude for 10 min, viewed at lower magnification (3,500×). (C) Before ultrasonication, viewed at higher magnification (25,000×). (D) After ultrasonication at 91.2 μm , viewed at higher magnification (25,000×). (E) After ultrasonication at 91.2 μm and pasteurization (63°C, 30 min), viewed at higher magnification (25,000×).

to germinate, leading to higher reductions in counts after heating. Information on effectiveness of ultrasonication in induction of spore germination is scarcely available; however, some reports have suggested that spores exposed to a sublethal dose of any treatment may germinate faster. Atrih and Foster (2002) reported activation of spore germination by thermal treatment that was insufficient to kill them. They also observed extraction of some of the coat proteins by a sublethal NaOH treatment that eventually speeded up germination. Germination of spores in presence of nutritional medium was also reported to be induced by a prior sublethal heat treatment termed as heat induction (Setlow, 2003; Wei et al., 2010). Induction of spore germination by a treatment insufficient to kill the spore has also been described in the case of high-pressure processing of cheese (López-Pedemonte et al., 2003). Spore germination is essentially a degradative biophysical process, which commences with increase in fluidity of the spore's inner membrane and initiation of ion fluxes, thereby allowing movement of monovalent cations (potassium and sodium) across the spore membrane (Setlow, 2003). The increased induction of endospore germination by ultrasonication can be explained on the

basis of the assumption that the Ca^{2+} -DPA (dipicolinic acid) released from any 1 endospore, due to any reason, may excite the neighboring endospores to germinate (Setlow, 2003).

Effect of Ultrasonication on Morphology of Endospores

Endospores of *B. coagulans*, *G. stearothermophilus*, and *B. licheniformis* were visualized under the scanning electron microscope. The images of endospores after ultrasonication at 80% amplitude for 10 min, as well as a combined ultrasonication and pasteurization treatment (63°C for 30 min), were taken at different magnifications for comparison purposes. The images were observed for physical damages such as cracks, fissures, or leakage of core materials.

The endospores of *B. licheniformis* before and after subjecting to ultrasonication and combined ultrasonication-pasteurization treatments are shown in Figure 3. These spores were 1.2 to 1.5 μm in length and 0.72 to 0.82 μm in width (data not shown). In the images taken at a lower magnification of 3,000 to 5,000×, the vegetative cells and spores were clearly distinguished.

The spores were observed as shorter, denser, and rice grain-like structures; whereas, vegetative cells were rod shaped and closely gelled together. After ultrasonication, spores structures were almost similar to the untreated spores; whereas, the vegetative cells were disintegrated in to pieces (Figure 3). The images of *B. coagulans* endospores at 25,000 $\times$ magnification gave a more clear view on morphology. The spore surface before ultrasonication was smooth and shiny, which turned into a rough or coarse surface after ultrasonication. The spore structure remained unchanged upon thermal treatment of the ultrasonicated spores (Figure 4).

A similar effect of ultrasonication was observed on the endospores of *G. stearothermophilus* (Figure 5). The shape and surface smoothness of the *G. stearothermophilus* spores was almost similar to the *B. coagulans* endospores. Conversely, *B. licheniformis* endospore surface was observed as irregular, with shrinkages, wrinkles, and ridges (Figure 4). Similar to *B. coagulans*, no changes were observed in *G. stearothermophilus* and *B. licheniformis* endospores' morphology after ultrasonication or combined ultrasonication and pasteurization treatment. The spore surfaces of all the observed species were clean and free of matrices, indicating that

ultrasonication cleaned the adhering materials surrounding the endospores.

Bradley and Williams (1957) studied different types of *Bacillus* spores, including *B. licheniformis*, under transmission electron microscope, and reported structures similar to the ones obtained in our study. Their transmission electron microscope images of *B. licheniformis* spores, obtained as carbon replica, also showed irregular shrinkages and wrinkles in the spore surface. Laroussi (2002) used scanning electron microscopy to obtain images of *B. subtilis* spores followed by exposure to nonthermal atmospheric plasma and observed the damaged surfaces of spores subjected to the treatments. Lerouge et al. (2001) also used scanning electron microscopy to evaluate the effect of nonthermal plasma discharge on unknown *Bacillus* spp. spores and reported some distinct differences in spore surfaces after the treatment. Chandler et al. (2001) found the spore surface to be smooth and regular before treatments, whereas ultrasonicated spores showed evidence of breakages, surface damage, and emergence of pustules on the surface due to leakage of spore contents through the broken layers. We did not observe any evidence of major structural changes in the spores in

Figure 5. Scanning electron microscopic images of *Geobacillus stearothermophilus* (American Type Culture Collection 15952) spores. (A) Before ultrasonication, viewed at lower magnification (3,200 $\times$). (B) After ultrasonication at 91.2 μ m amplitude for 10 min, viewed at lower magnification (3,500 $\times$). (C) Before ultrasonication, viewed at higher magnification (25,000 $\times$). (D) After ultrasonication at 91.2 μ m, viewed at higher magnification (25,000 $\times$). (E) After ultrasonication at 91.2 μ m and pasteurization (63°C, 30 min), viewed at higher magnification (25,000 $\times$).

this research. Lesser damage to the spore's surfaces by ultrasonication was probably due to their robust spore coat, relatively oval-shaped structure, and minimal size. In comparison to the long rod-shaped vegetative counterparts, oval-shaped smaller spores might have lesser inertia to sustain such damage. Moreover, the spore membranes are expected to be relatively brittle at lower temperatures, and the violent shaking during ultrasonication in our research might have ruptured the inner membranes of the spore. Such internal damages might have caused leakage of the materials from the core making them unable to germinate.

CONCLUSIONS

Combination of ultrasonication and pasteurization resulted in an overall endospore inactivation up to 65.74%. When ultrasonication was followed by a higher heat treatment (80°C for 1 min), the endospore inactivation was further enhanced. This shows that ultrasonication reduced the resistance of endospores to thermal treatment such as pasteurization. Increased ultrasonication amplitude did not result in any increase in the endospore inactivation levels. It was the increase in ultrasonication time that had a significant influence on the endospore inactivation. Ultrasonication induced a greater number of spores to germinate, resulting in a higher reduction in endospore counts after pasteurization. Microscopic observation did not reveal any severe damage to the structure of endospores at these levels of treatment. The results from our study thus provide preliminary evidence on the possible application of ultrasonication and pasteurization combined for the inactivation of bacterial endospores in skim milk. The findings of our study may be useful in further research in scaled-up capacities so as to understand the effectiveness of high-throughput continuous flow ultrasonication as an aid to spore inactivation along with continuous pasteurization of milk.

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