Influence of thermosonication on *Geobacillus stearothermophilus* inactivation in skim milk

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Abstract

This study explored the influence of high intensity ultrasound (HIU) coupled with thermoprocessing (thermosonication) on the inactivation of *Geobacillus stearothermophilus* vegetative cells and spores in skim milk powder using response surface methodology (RSM) and two polynomial models were developed. Optimization of cell reduction (4.8 log) was found to be at 19.75% total solids (TS), 45°C, and 30 sec, while optimization of spore reduction (0.45 log) was found to be at 31.5% TS, 67.5°C, and 17.5 sec. Model verification experiments were performed using common milk powder processing conditions. Results showed the inactivation of cells and spores to be most effective before (9.2% TS, 75°C, and 10 sec) and after (50% TS, 60°C, and 10 sec) the evaporator during milk powder processing and may produce an additive effect in microbial reduction when the two locations are combined, resulting in a 5.8 log reduction for vegetative cells and 0.51 log reduction for spores.

25

26 **1. Introduction**

27 Generally, high temperature short time (HTST; 72°C for 15 sec) pasteurization
28 conditions are used in the processing of fluid milks and milk products. These conditions allow
29 for the destruction of most pathogenic and spoilage-causing microorganisms without
30 significantly affecting the physical and chemical composition of the final product (Walstra,
31 Geurts, Noomen, Jellema, & van Boekel, 1999). However, pasteurization is not always effective
32 at producing the desired log reduction of mesophilic and thermophilic spore-forming bacteria,
33 which are responsible for spoilage and decreased quality in milk products (Cameron, McMaster,
34 & Britz, 2009). Compared to pasteurization, ultra high temperature (UHT; 135-150°C for 4 to 15
35 sec) and retort sterilization (116 °C for 20 min) processing conditions destroy higher numbers of
36 mesophilic and thermophilic spore-formers. However, higher heat treatments tend to produce
37 sulfide-like cooked flavors, often described as burnt, scalded or caramel, that consumers find
38 undesirable (Piyasena, Mohareb, & McKellar, 2003; Alvarez, 2009; Bermúdez-Aguirre,
39 Mawson, Versteeg, & Barbosa-Cánovas, 2009).

40 *Bacillus* (and related) spp. are of particular concern to the dairy industry, specifically in
41 milk powder manufacturing, due to their ability to form spores and contribute to biofilm
42 formation (Scott, Brooks, Rakonjac, Walker, & Flint, 2007; Lücking, Stoeckel, Atamer,
43 Hinrichs, & Ehling-Schulz, 2013; Watterson, Kent, Boor, Wiedmann, & Martin, 2014).
44 Although the initial concentration of thermophiles and spores in raw milk entering a dairy
45 processing facility are estimated to be <10 cfu mL^{-1}, they are able to survive pasteurization and
46 grow in biofilms (Burgess, Lindsay, & Flint, 2010; Scott et al., 2007). Spores, residing in
47 biofilms, contaminate the product as it flows through the processing line. A recent study by
48 Beuhner (Buehner, Anand, & Djira, 2015) showed levels of spores and thermoduric bacteria in

49 milk powders processed in the Midwestern USA were 3.6 log cfu g^{-1} and 3.5 log cfu g^{-1}
50 respectively with the predominant organisms identified as *Bacullus lichenformis, Bacillus*
51 *pumilus, Bacullus sonorensis,* and *Geobacillus stearothermophilus* (Buehner et al., 2015),
52 suggesting issues of contamination during processing. Once introduced to a more favorable
53 environment during reconstitution of dry milk powder, the spores can germinate, grow, and
54 produce proteases and lipases, resulting in off-flavor development and spoilage in the products
55 containing milk powders (Scott et al., 2007; Lücking et al., 2013).
56 In recent years, the application of thermosonication, or high intensity ultrasound (HIU),
57 has been explored as a means to increase the inactivation of vegetative and spore-forming
58 bacterial populations when coupled with standard thermal processing conditions (Villamiel &
59 Jong 2000; Awad, Moharram, Shaltout, Asker, & Youssef, 2012; Herceg, Jambrak, Lelas, &
60 Thagard, 2012). Such treatments could potentially increase dairy product shelf life and quality
61 without imparting undesirable cooked flavors that often occur in milk products treated at higher
62 processing temperatures, such as UHT and retort sterilization (Bermúdez-Aguirre et al., 2009).
63 It has been proposed that the cavitational effects of HIU damage bacterial cell walls and cellular
64 structural and functional components such as DNA (Chandrapala, Oliver, Kentish, &
65 Muthupandian, 2012a; Chandrapala, Oliver, Kentish, & Ashokkumar, 2012b) leading to cell
66 death. Factors contributing to microbial inactivation include bacterial strain (Gram-positive vs.
67 Gram-negative), bacterial growth phase, temperature, time, medium, solids concentration and
68 acoustic power (Piyasena et al., 2003; Milly, Toledo, Harrison, & Armstead, 2007; Cameron et
69 al., 2009).
70 Herceg et al. (2012) investigated the influence of HIU on the reduction of *Staphylococcus*
71 *aureus* and *Escherichia coli* in fluid milk containing 4% milk fat. Data analysis was performed
72 using response surface methodology (RSM) in order to study the effect of 3 variables: HIU time,

temperature, and amplitude. Ultrasound was observed to have a greater effect on *E. coli* (1.34 to 3.07 log reduction) than *S. aureus* (0.22 to 1.49 log reduction). The results showed amplitude, treatment time, and treatment temperature to be the parameters significantly affecting the inactivation of both *S. aureus* and *E. coli* in fluid milk.

Compared to Herceg et al. (2012), Cameron et al. (2009) performed HIU treatments on *E. coli, Pseudomonas fluorescens,* and *Listeria monocytogenes* in fluid milk held at 24 to 26°C for 2.5 to 10 min. Observed log reductions ranged from 3.26 to 5.64, respectively, resulting in a 99-100% inactivation for all organisms. Ganesan (Ganesan, Martini, Solorio, & Walsh, 2015) showed that applying HIU for 10.2 sec at 72°C lead to 5, 1.6 and 6.6 log reductions in indigenous bacteria in pasteurized milk, *Bacillus atrophaeus* spores inoculated into sterile milk and *Saccharomyces cerevisiae* inoculated into sterile orange juice respectively.

Previous work by Evelyn & Silva (2015) cited log reductions of less than 0.5 when exploring the microbial inactivation of *Bacillus cereus* spores in skim milk when HIU was applied for 1.5 min at 70°C. In addition to skim milk, Evelyn & Silva (2015) investigated the effects of applying HIU (1.5 min, 70°C) to beef slurry, cheese slurry, and rice porridge inoculated with *B. cereus* spores. The observed log reductions in these experiments were greater than 3.2, suggesting that foods with higher solids concentration influence the effectiveness of HIU. However, no explanation was offered as to why this effect was observed. Recently, Ferrario (Ferrario, Alzamora, & Guerroro, 2015) observed minimal to zero inactivation of *Alicyclobacillus acidoterrestris* spores in apple juice when treated with HIU for 30 min at 30°C and 44°C. In contrast, a 2.5 log reduction was shown with *S. cerevisiae* when exposed to the same conditions, confirming a difference in microbes to be an important factor in the effectiveness of HIU.

96 While there has been previous research investigating HIU as a means to reduce microbial
97 populations in milk and beverages, little research has been conducted regarding the application
98 of this technology in concentrated skim milk with thermophilic bacteria and spores. Since
99 thermophilic bacteria form biofilms, reduction of these microbes may reduce biofilm formation.
100 Additionally, with a reduction in microbes, less microbial proteases and lipases would be
101 produced with may reduce off-flavors in the dry milk products.
102 The objective of this research was to explore the influence and significance of three
103 variables (solids concentration, temperature, and treatment time) on the inactivation of
104 *Geobacillus stearothermophilus* vegetative cells and spores in reconstituted skim milk powder
105 (rSMP) in order to develop a model capable of predicting levels of microbial inactivation under
106 SMP production conditions in which the total solids varies from approximately 9 to 50% and the
107 temperature ranges from approximately 55 to 75 °C. D-values at 73 °C were also calculated to
108 compare the effectiveness of HIU to that of thermal processing alone.
109
110 **2. Materials and Methods**
111 *2.1 Experimental design*
112 To explore the effects of independent variables on the response a RSM design (Box-
113 Behnken, SAS 9.4, The SAS Institute Cary, NC, USA) with three variables, rSMP total solids
114 (TS) (range from 8 to 55%), temperature (range from 45 to 75°C), and sonication time (range
115 from 5 to 17.5 sec) was performed at an amplitude of 240 μm. The response variable was $\log_{10}$
116 reduction (Y_1) of microbes. The design consisted of 13 experimental points (Table 1) that were
117 conducted in duplicate, except for the center point that was conducted in duplicate and
118 replicated. The coded values were low (-1), central (0) and high (1). In addition, analysis
119 comparing the effect of HIU with that of thermal treatment alone was done using a two-tail t-test.

120 Significance was declared at $p \leq 0.05$. D-values (the amount of time required to destroy 90% of
121 the initial microbial population) were determined for *G. stearothermophilus* vegetative cells at
122 73°C with and without HIU in tryptic soy broth (TSB: VWR, Atlanta GA, USA) at an amplitude
123 of 240 μm.
124
125 *2.2 Response surface analysis*
126 The response surface regression (RSREG) procedure of statistical analysis was used to
127 analyze the experimental data (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008; Herceg et
128 al., 2012; Ganesan et al., 2015). Experimental data were fitted to a second order polynomial
129 model and regression coefficients were obtained. Validity of the polynomial model was tested
130 with analysis of variance (ANOVA). The significances of all terms in the polynomial were
131 judged statistically by computing the F-value at $p = 0.05$. The lack-of-fit significance, as well as
132 R^2 and adjusted R^2 were evaluated for model accuracy. The design software was used to
133 generate response surface plots while holding a variable constant in the second-order polynomial
134 model and maximizing Y_1. Numerical optimization was done to find the variable conditions
135 resulting in maximum Y_1. Canonical analysis was conducted to determine the overall shape of
136 the curve and to determine which variables(s) were the most influential. The predicted models
137 generated were verified by selecting variable conditions and using the response calculator to
138 generate the Y_1 response. Experimental runs were conducted with the same variable conditions
139 and compared to the predicted Y_1 values.
140
141 *2.3 Growth of Geobacillus stearothermophilus*
142 *G. stearothermophilus* spores were germinated using 0.1 mL of stock solution (NAMSA
143 *G. stearothermophilus* 2.4 x 10^6 in 0.1 mL, VWR, Atlanta GA, USA) inoculated into 10 mL of

144 sterile water. The sample was incubated for 10 min in an 80°C water bath to germinate the
145 spores. Twenty-five milliliters of tryptic soy broth (TSB) was inoculated with 1 mL of
146 germinated bacteria in a sterile 250 mL Erlenmeyer flask covered with sterile foil and incubated
147 at 55°C aerobically for 24 hrs in a shaker at 100 rpm. The OD 600 nm was measured to be
148 approximately 0.566 after 24 hrs, which corresponded to 10^7 cfu mL^{-1} as determined by plating
149 on tryptic soy agar (TSA: VWR, Atlanta GA, USA). A subculture was grown by inoculating 25
150 mL of TSB with 0.1 mL of culture grown from germinated cells in a sterile 250 mL Erlenmeyer
151 flask covered with sterile foil. Cells were grown aerobically at 55°C in a shaker at 100 rpm for
152 16-18 hrs (Kotzekidou, 2014).
153 Freezer stocks were made by inoculating 20 mL of TSB containing 30% w/v glycerol
154 with 2 mL of subculture and aliquoted and stored in 2 mL cryo-vials at -20°C. Cultures for
155 experiments were grown by inoculating 25 mL TSB with 0.1 mL of freezer stock and incubated
156 at 55°C in a shaker at 100 rpm for 16 to 18 hrs. For spore samples*, G. stearothermophilus* spores
157 were obtained directly from the commercial stock solution.
158
159 *2.4 Preparation of skim milk powder*
160 Skim milk powder (Extra Grade Spray Process, Darigold, Inc., Seattle, WA, USA) was
161 reconstituted to between 8 and 55% TS. The powder was weighed and mixed with 60°C sterile
162 water for 3 min using a hand-held high-speed blender, followed by a solids test performed using
163 the oven drying method. Briefly, the total solids was determined by the weight difference of 3.5
164 mL of sample before and after drying at 80°C for 12 hrs. The rSMP was then heated at 80°C for
165 20 min to destroy any existing bacteria that might cause potential contamination during
166 experiments. The 8 and 31.5% TS rSMP were stored at 4°C for up to 1 week before a new
167 sample was made. The 55% TS RSMP was remade prior to each experiment due to solidification

168 at temperatures below 30°C. During treatments, rSMP was held at 60°C to ensure fluidity and
169 easy pouring for experiments, then adjusted to the experimental temperature.
170
171 *2.5 Thermosoication conditions*
172 Treatments with HIU were performed in batch using a 10 mL double-walled glass
173 cylinder (diameter: 2.8 cm outside, 1.7 cm inside; height: 6.3 cm outside, 5.3 cm inside)
174 containing 6 mL of sample. A water bath was used to control the temperature and to bring the
175 rSMP up to the appropriate temperatures prior to inoculation. Experiments were performed using
176 a 20 kHz Misonix Sonicator® 3000 (QSonica, LLC, Newtown, CT, USA) with a 0.32 cm
177 (diameter) stainless steel tapered sonicator microtip (ID: 4418, QSonica, LLC, Newtown, CT,
178 USA) with an amplitude of 240 μm at a dial setting of 10. All materials were rinsed with 10%
179 w/v bleach solution, followed by sterile water before and after each treatment to avoid cross-
180 contamination. Treatments not involving HIU (heat only) were done in a water bath using 15
181 mL sterile tubes containing 6 mL of sample. The rSMP was brought to temperature in the tubes,
182 followed by inoculation of the microorganism.
183 For vegetative cells, 6 mL of rSMP was brought to the specified treatment temperature in
184 either the 10 mL glass cylinder (HIU treatments) or 15 mL sterile tube (non-HIU treatments)
185 using the water bath. Once brought to the appropriate temperature, the sample was inoculated
186 with 1 ml (10^8 cfu mL^{-1}) of *G. stearothermophilus* culture. After inoculation, 0.5 mL of sample
187 was collected and placed on ice until ready to plate. The remaining sample was then treated with
188 HIU or thermal processing. After treatment, the sample was poured into a 15 mL sterile tube and
189 kept on ice until ready to plate. This entire procedure was performed each time for each
190 experiment and its duplicate. Dilutions of samples were made in sterile water and plated on TSA
191 and incubated for 24 to 48 hrs in a humidified incubator at 55°C to determine log reductions.

192 Sores were thermosonicated or heated without sonication as described above for
193 vegetative cells. Samples were inoculated with 0.1 mL of *G. stearothermophilus* spores (10^6
194 spore mL^{-1}). After treatment, dilutions were made in sterile water and germinated at 80°C for 10
195 min. Germinated samples were plated on TSA and incubated for 24 to 48 hrs in a humidified
196 incubator at 55°C to determine log reductions.
197 D-values were determined for *G. stearothermophilus* vegetative cells at 73°C with and
198 without HIU in TSB. D-values were determined at 73°C since many milk processors use
199 temperatures higher than 72°C for pasteurization and 73° is within the processing range for
200 HTST pasteurization. D-values (termed D73) were determined from the negative reciprocal of
201 the slope of the regression line (log_{10} cfu mL^{-1} versus treatment time) and calculated using the
202 equation D = t/(logN0 - logNf), where D = decimal reduction time, t = duration of treatment, N0
203 = initial bacterial population, and Nf = surviving bacterial population after treatment (Mazzola,
204 Penna, & da S Martins, 2003).
205
206 *2.6 Acoustic power calculations*
207 Acoustic power delivered to the samples during HIU was calculated using P = M x Cp x
208 (dT dt^{-1}) where P is the acoustic power (W), M is the mass of the HIU sample (g), Cp is the
209 specific heat capacity of medium at constant pressure (J g^{-1} $°C^{-1}$), and dT $dt^{-1}$is the increase in
210 temperature (°C s^{-1}) during HIU (Jambrak et al., 2011; Ganesan et al., 2015). Increase in
211 temperature during HIU was measured using a thermocouple (Traceable® Total-Range
212 Thermometer, VWR, Atlanta GA, USA) and plotted as a linear graph to determine precision
213 among replicates. Specific heat capacity was determined using a differential scanning
214 calorimeter (DSC, Auto Q20 2910, TA Instruments, New Castle, DE, USA) for 8%, 31.5%, and
215 55% TS rSMP in duplicate. A baseline control was run from 25 to 80°C with a 5 min holding

period at 25°C and 80°C and a ramp rate of 5°C min^{-1}. Five to 15 mg of rSMP sample (8%, 31.5%, 55% TS) was placed in an aluminum pan for DSC analysis. The sample was heated to 80°C with a ramp rate of 5°C min^{-1} to determine the specific heat capacity at 45°C, 60°C, and 75°C. Each sample was run in duplicate.

3.0 Results and discussion

3.1 Geobacillus stearothermophilus vegetative cells

In general, log reductions of *G. stearothermophilus* vegetative cells with HIU were significantly greater than log reductions from thermal processing treatments alone as shown in Table 1. Log reductions with HIU ranged from 0.77 ± 0.29 to 5.0 ± 0.38 while heat treatments without HIU yielded less than 1.5 log reductions. The D73-value for *G. stearothermophilus* vegetative cells treated without HIU was 2.1 min while the D73-value for cells treated with HIU was 5.3 sec. This strongly shows that HIU was effective for a synergistic inactivation of cells.

Higher log reductions were observed in samples treated with HIU for longer amounts of time, regardless of TS content or temperature. For example, rSMP at 31.5% TS treated with HIU for 30 sec at 45°C yielded a 5 log reduction while with the same solids content treated with HIU for 5 sec at 45°C resulted in a 1.1 log reduction. This trend is similar among samples with the same solids content throughout the table, implying that higher log reductions are achieved with longer HIU treatment times.

Another interesting aspect shown in the data is the influence of solids content. rSMP samples with 8% TS treated with HIU for 17.5 sec (45°C) (1.8 log reduction) and 5 sec (60°C) (0.77 log reduction) resulted in lower log reductions than 55% TS rSMP treated with the same conditions (2.5 and 2.4 log reductions respectively). However, 8% TS rSMP treated with HIU at 60°C for 30 sec (3.5 log reduction) and 75°C for 17.5 sec (3.8 log reduction) yielded higher log

reductions than 55% TS rSMP treated under the same conditions (2.9 and 2.8 log reductions respectively). Higher solids concentration may, therefore, contribute to a greater bactericidal effect at lower temperatures coupled with shorter treatment times since it results in a higher amount of energy, or acoustic power being transferred into the media. The highest acoustic power values were observed at 55% TS (39.1 W) followed by 31.5% TS (39.7 W). The increase in acoustic power translates to greater acoustic cavitation and more direct damage to the cell for increased cell death. However, greater acoustic power generated within the system did not always directly correlate with a higher log reduction. As such, log reductions induced by HIU must be a result of a combination of many factors as described by Chandrapala et al., (2012a).

ANOVA (Table 2) determined the significant variables in the predictive model, in order with the largest effect first were time, temp*time, solids*time, time*time, and temp. The master and predictive models were both significant with linear, cross product and quadratic regressions contributing to the models. The coefficient of determination (R^2) for the master and predictive models were 0.92 and 0.82 respectively. The ANOVA of the master model explains the total variance of the model and treatments. Generally, insignificant treatments are eliminated building the predictive model, unless removing the treatments reduces the R^2. According to the RMS model, the log reduction of *G. stearothermophilus* vegetative cells achieved by thermosonication can be described with the polynomial equation:

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

where S is solids concentration (%), T is temperature (°C), and TT is treatment time (sec).

263 The response surface plots shown in Figure 1 describe the predicted log reductions of *G.*
264 *stearothermophilus* vegetative cells in rSMP treated with HIU. Each of the plots (A-C) indicates
265 a linear association between each of the variables (temperature, solids, and time) and log
266 reduction. In Figure 1A and B, there is an increase in log reduction with an increase in TS vs.
267 time and temperature respectively. As stated previously, the highest acoustic power values were
268 obtained at 55 and 31.5% TS and results in more caviatation. This would translate to a higher
269 degree of damage to cells, resulting in increased microbial destruction.
270 In Figure 1B, there is an observed increase in log reduction at a high solids concentration
271 over an increase in treatment time when temperature is held constant. This effect supports the
272 equation generated for the predictive model where treatment time is the most significant
273 predictor. A longer treatment time allows for longer exposure to elevated temperatures and
274 cavitation, resulting in a greater bactericidal effect. The second most significant predictor in the
275 model is the interaction between temperature and time, which can be seen in Figure 1C. At low
276 temperatures, log reductions were highest at longer treatment times. However, there is an
277 observed decline in log reduction at higher temperatures and longer treatment times. The
278 interaction between temperature and treatment time shows a linear relationship with that of log
279 reduction. Numerical optimization results based on the conditions defined in the experimental
280 parameters predicted the largest log reduction (4.8) to occur when HIU is applied to 19.75%
281 rSMP at 45°C for 30 sec. A maximum optimum, however, was not observed in this model using
282 the defined experimental parameters, which fall outside of common conditions utilized in milk
283 powder processing facilities (e.g. longer treatment times and/or higher temperatures).
284 In comparison to the data obtained in this experiment, log reductions from HIU were
285 greater than those observed by Herceg et al., (2012) when HIU was applied to *S. aureus* and *E.*
286 *coli* in fluid milk using ultrasound for 6 to 12 min at amplitudes of 60 to 120 μm. However,

287 microbial inactivation was similar to the results Cameron et al., (2009) observed for *E. coli, P.*
288 *fluorescens,* and *L. monocytogenes* in fluid milk using HIU for 6 to 10 min at an amplitude of
289 124 μm. The significance of treatment time and the effects of temperature and time together are
290 similar to the model generated by Herceg et al., (2012). The influence of temperature may be due
291 to the instability of microbes as temperatures exceed their growth range, which is 75°C for *G.*
292 *stearothermophilus* (Burgess, Lindsay, & Flint, 2010).
293
294 *3.2 Verification of G. stearothermophilus vegetative cell model*
295 The response surface plots in Figure 1 show that there was an increase in microbial
296 destruction with an increase in both temperature, TS, and time, but at maximum values of each,
297 there was a dip in the surface. To confirm the accuracy of the model, experimental runs were
298 done (Table 3). When only the time is changed from 20 to 30 sec with conditions of 55% TS
299 and 72°C, we see the log reductions are higher at 20 sec, confirming the dip in the surface with
300 longer times. Additional verification runs were performed at low (10%), medium (30%) and
301 high (55%) TS content at the same treatment time (10 sec) and temperatures (45°C) (Table 3).
302 The experimental log reductions fall within the range of the predicted, confirming accuracy of
303 the model, and showing the highest inactivation at 55% TS.
304 During SMP production, skim milk is preheated to approximately 55°C before
305 pasteurization at temperatures of approximately 75°C (containing 9.2% TS) followed by
306 evaporation at 60°C. Evaporation at 60°C is done until the solids content reaches 50% and this
307 is followed by spray drying. We wanted to determine the location in a SMP processing plant
308 where *G. stearothermophilus* bacterial cell destruction would be maximized at low treatment
309 times of 10 sec (to simulate a flow-through sonicator) by incorporation of HIU. Table 3 shows
310 that the highest microbial inactivation (2.99 log) occurs at 9.2% TS which is after pasteurization

311 and before evaporation. The second highest inactivation (2.76 log) was seen at 50% TS at 60°C
312 which is after evaporation.
313 Although the level of thermophilic bacteria entering a dairy processing plant is generally
314 low, <10 cfu mL^{-1}, they are found at levels of 3.5 log cfu g^{-1} in the dried milk powders (Buehner
315 et al., 2015). They can form biofilms on plate heat exchangers with the predominant sites of
316 bacterial biofilms present in the preheater plate heat exchanger (before the pasteurizer) and in the
317 evaporator (temperatures at 40 to 60°C) (Burgess et al., 2010; Scott et al., 2007). Biofilms
318 provide a constant inoculum to the milk stream yielding higher levels of microbes in the final
319 product than the incoming milk. Our verification results suggest that placing a sonicator after a
320 plate heat exchanger, before the pasteurizer (55 °C) would result in minimal inactivation (1.44
321 log) compared to the higher temperatures after the pasteurizer.
322
323 *3.3 Geobacillus stearothermophilus spores*
324 Seven of the 13 treatments showed log reductions for *G. stearothermophilus* spores
325 treated with thermosonication compared to without HIU (Table 4) to be significantly higher.
326 Compared to vegetative cells, the log reductions observed for spores treated with HIU were less,
327 ranging from 0.06 ± 0.04 to 0.44 ± 0.13. The decrease in microbial inactivation in spores
328 compared to vegetative cells was expected due to increased resistance of spores in adverse
329 environmental conditions (Burgess et al., 2010; Hill & Smythe, 2004; Kotzekidou, 2014). D-
330 values were not determined since the destruction of 90% of the initial spore population at 73°C
331 with HIU would require a time frame outside feasible processing conditions.
332 Referring to Table 4, it is difficult to relate the influence of treatment time with %TS.
333 HIU treatments on 55% TS rSMP at 60°C for 30 sec resulted in log reductions at twice the level
334 observed in 8% TS rSMP treated under the same conditions. Conversely, HIU on 55% TS rSMP

335 at 75°C for 17.5 sec yielded a lower reduction than in the 8% TS rSMP treated with the same
336 temperature and time. In both cases, the higher log reductions corresponded to higher levels of
337 acoustic power and differences in solids concentration. However, this relationship between log
338 reduction and acoustic power does not follow through with all of the experiments concerning
339 spores. Overall, log reductions were fairly similar among samples with 8% and 55% TS and
340 increased in samples with 31.5% TS. The largest reductions were observed in 31.5% TS at the
341 high and mid temperatures with shorter treatment times (Table 4) as opposed to longer treatment
342 times.
343 ANOVA (Table 5) determined the significant variables in the predictive model, in order
344 with the larges effect first were solids*solids, time*time, temp, and temp*temp. For the spores
345 model, removing the treatments of solids and time reduced the $R^{2,}$ so the treatments were kept in
346 the predictive model. The master and predictive models were both significant with quadratic and
347 linear regressions contributing to the models. The R^2 for both the master and predictive models
348 were 0.82 and 0.81, respectively. The lower R^2 for spores may be due to less treatment
349 combinations showing significantly less spore reduction with HIU compared to heat alone (Table
350 4). According to the RSM model, the log reduction of *G. stearothermophilus* spores achieved by
351 thermosonication can be described with the polynomial equation:
352
353 $Y1 = 0.658961 + 0.0015*S + 0.065579*T - 0.023711*TT - 0.160592*S^2 - 0.08469*T^2 -$
354 $0.065341*T*TT - 0.159631*TT^2$
355
356 where S is solids concentration (%), T is temperature (°C), and TT is treatment time (sec).
357 Canonical analysis in RSM determined that based on the shape of the plots a maximum log
358 reduction can be obtained from the predictive model. The maximum log reduction predicted by

numerical optimization of the model was determined to be 0.45 log at 31.5% TS sonicated at 67.5°C for 17.5 sec.

In Figure 2, low log reductions are shown at both high and low solids as well as at long and short times whereas the effect of temperature is linear. Figure 2B displays a similar trend seen in Figure 2A for the effect of solids vs. temperature and time. Unlike the *G. stearothermophilus* vegetative cells, an increase in time does not correspond to an increase in log reduction at low or high solids concentrations. Instead, the largest log reductions are observed at median treatment times and solids concentrations. In Figure 2C, increases in temperature with a slight increase in treatment time resulted in a maximum log reduction of spores; however, the level of reduction plateaus shortly before the highest experimental temperature of 75°C, which is similar to the shape of the graph in Figure 2A. The plateaued effect observed in both Figures 2A and 2C show that temperature as a single predictor does not heavily influence the degree of microbial inactivation compared to solids and time. Solids concentration and treatment time prove more influential in dictating which way the plateau falls along the plane of the plot, which supports the level of significance of these two predictors and their degree of interaction within the model.

Compared to vegetative cells, the log reductions observed for spores treated with thermosonicaton were similar to results obtained by Evelyn & Silva (2015) for *B. cereus* spores. However, log reductions observed in this study were greater than those observed for *A. acidoterrestris* spores (Ferrario et al., 2015). The influence of solids and time support the conclusions reached in previous studies concerning the effects of higher solids concentrations and increased time as being influential factors in increasing microbial inactivation via HIU (Cameron et al., 2009; Herceg et al., 2012; Evelyn & Silva, 2015). The conclusions from this study, however, differ from the experimental data collected for vegetative cells in that the

383 inactivation of spores is not as dependent upon HIU temperature as vegetative cells. Instead,
384 spore destruction is more heavily dependent on solids concentration, which was not a variable
385 that was shown to substantially affect vegetative cell inactivation. In the case of both vegetative
386 cells and spores, however, the length of exposure to HIU has proven to be a common significant
387 factor.
388
389 *3.4 Verification of G. stearothermophilus spores model*
390 Experiments were performed to verify the RSM predictive spore model at high (50%),
391 middle (32%) and low (8%) TS (Table 3). All observed log reductions were within the
392 predictive range, validating the model even though not all conditions in Table 4 showed a
393 significant reduction in spores with HIU treatment. Verification experiments were then run using
394 common SMP processing conditions. Results show that incorporation of a flow-through
395 sonicator would be most efficient at conditions of 50% TS, 60°C and 10 sec resulting in a 0.27
396 log reduction of *G. stearothermophilus* spores. Therefore, the most effective location of a flow-
397 through sonicator would be before spray drying, directly after evaporation. The second best
398 location would be at 9.2% TS, 75°C and 10 sec which is just after the pasteurizer, before
399 evaporation.
400 Although this research showed less than one log reduction in spores was achieved under
401 all conditions tested, this still may be beneficial to a SMP processing facility. If flow-through
402 sonicators are placed before the evaporator specifically for vegetative cell inactivation and after
403 the evaporator for spore inactivation, the total combined reduction would be 5.75 log vegetative
404 cells and 0.51 spores. Obviously a 5.75 log reduction in thermophilic vegetative cells may not
405 be necessary, but since these cells can survive pasteurization conditions and contribute to biofilm
406 formation, vegetative cell reduction in general may reduce overall biofilm formation.

3.4 Conclusion

Thermophilic bacteria and spores are difficult to eliminate from a dairy manufacturing process because thermophilic bacteria and spores can survive pasteurization and form heat-resistant biofilms on plate heat exchangers. The predominant sites of bacterial biofilms are in the preheater plate heat exchanger (before the pasteurizer) and in the evaporator. Results show that thermosonication proved to be more effective than heat treatment alone in reducing the microbial population of *G. stearothermophilus*. For vegetative cells, D73-values improved when HIU was applied as compared to D73-values observed for heat treatment without HIU. Based on the observed log reductions, predictive models were generated for *G. stearothermophilus* vegetative cells and spores in rSMP at various TS, temperatures and times. These models were validated and used to determine effective locations for implementing HIU treatments during milk powder processing. Treatments applied directly before and after the evaporator would theoretically produce an additive effect that would result in higher levels of microbial inactivation for vegetative cells and spores, respectively. Further research is necessary, however, to determine optimum conditions on a pilot-scale model with thermophilic spore-formers commonly found in milk.

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Table 1 Average log reductions of *G. stearothermophilus* vegetative cells observed in rSMP with and without HIU

rSMP Total Solids (%)	Treatment Temperature (°C)	Treatment Time (s)	Acoustic Power[a] (W)	Log Reduction with HIU	Log Reduction without HIU	*P*-value[b]
8	45	17.5	28.36	1.8 ± 0.53	0.27 ± 0.06	0.134
8	60	5	21.81	0.77 ± 0.29	0.23 ± 0.31	0.427
8	60	30	19.27	3.5 ± 0.29	0.10 ± 0.07	0.029
8	75	17.5	20.05	3.8 ± 0.11	1.0 ± 0.08	0.004
31.5	45	5	34.79	1.1 ± 0.03	0.24 ± 0.05	0.044
31.5	45	30	28.75	5.0 ± 0.38	0.18 ± 0.10	0.026
31.5	60	17.5	34.72	3.7 ± 0.35	0.27 ± 0.12	3.82E-6[c]
31.5	75	5	27.00	2.8 ± 0.05	0.80 ± 0.01	0.015
31.5	75	30	17.90	3.1 ± 0.01	0.94 ± 0.03	0.004
55	45	17.5	39.10	2.5 ± 0.08	0.09 ± 0.02	0.012
55	60	5	31.25	2.4 ± 0.10	0.16 ± 0.05	0.029
55	60	30	21.07	2.9 ± 0.23	0.51 ± 0.11	0.054
55	75	17.5	17.54	2.8 ± 0.05	1.4 ± 0.01	0.013

rSMP = reconstituted skim milk powder
HUI = high intensity ultrasound
[a]Acoustic power was only calculated for treatments where HIU was applied.
[b]Significance declared at $P \leq 0.05$ to determine whether HIU with temperature was significantly different than temperature alone
[c]Midpoint of experimental design – performed in duplicate 3 times.

Table 2. ANOVA analyzing the influence of solids content, temperature, and time on the log reduction of *G. stearothermophilus* vegetative cells in rSMP treated with HIU

	Master Model		Predictive Model	
	F	Pr > F[a]	F	Pr > F[a]
Solids	4.592	0.0446	2.176	0.1543
Temp	13.566	0.0015	6.430	0.0188
Time	106.080	< .0001	50.278	< .0001
Solids*Solids	25.900	< .0561		
Solids*Temp	9.711	0.0054	4.602	0.0532
Solids*Time	24.510	< .0001	11.617	0.0025
Temp*Temp	1.227	0.2811		
Temp*Time	41.570	< .0001	19.703	0.0002
Time*Time	19.982	0.0002	7.773	0.0107
Model	26.98345	< .0001	14.654	< .0001
Linear	41.41335	< .0001		
Quadratic	14.27277	< .0001		
Cross Product	25.26425	< .0001		

ANOVA = analysis of variance
rSMP = reconstituted skim milk powder
HUI = high intensity ultrasound
[a]Significance declared at $P \leq 0.05$.

Table 4. Average log reductions of *G. stearothermophilus* spores in rSMP with and without HIU

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

rSMP = reconstituted skim milk powder
HUI = high intensity ultrasound
[a]Acoustic power was only calculated for treatments where HIU was applied.
[b]Significance declared at $P \leq 0.05$ to determine whether HIU with temperature was significantly different than temperature alone
[c]Midpoint of experimental design – performed in duplicate 3 times.

Table 3. Validation of RSM predictive models for *G. stearothermophilus* vegetative cells and spores and under milk powder processing conditions

Cell Type	rSMP Total Solids (%)	Treatment Temperature (°C)	Treatment Time (s)	Predicted Log Reduction from Model	Observed Log Reduction
Model Verification					
Vegetative	10	45	10	0.89 (0.76, 1.04)	0.88 ± 0.02
Vegetative	30	45	10	1.49 (1.36, 1.62)	1.6 ± 0.40
Vegetative	55	45	10	2.44 (2.31, 2.56)	2.5 ± 0.25
Vegetative	55	72	20	3.16 (3.06, 3.26)	3.1 ± 0.11
Vegetative	55	72	30	2.29 (2.19, 2.39)	2.4 ± 0.02
Spores	8	60	10	0.21 (0.06, 0.36)	0.24 ± 0.09
Spores	32	60	17	0.44 (0.35, 0.52)	0.43 ± 0.21
Spores	50	60	10	0.27 (0.21, 0.32)	0.31 ± 0.05
Processing Verification					
Vegetative	9.2	75	10	2.99 (2.90, 3.09)	3.0 ± 0.03
Vegetative	9.2	55	10	1.44 (1.35, 1.64)	1.6 ± 0.60
Vegetative	12.5	55	10	1.52 (1.44, 1.61)	1.6 ± 0.10
Vegetative	50	60	10	2.76 (2.68, 2.85)	2.8 ± 0.08
Spores	9.2	75	10	0.24 (0.17, 0.32)	0.17 ± 0.12
Spores	9.2	55	10	0.18 (0.11, 0.25)	0.21 ± 0.01
Spores	12.5	55	10	0.22 (0.16, 0.28)	0.20 ± 0.04
Spores	50	60	10	0.27 (0.21, 0.32)	0.31 ± 0.05

rSMP = reconstituted skim milk powder
Parenthesis give the predicted log reduction range

Table 5. ANOVA analyzing the influence of solids content, temperature, and time on the log reduction of *G. stearothermophilus* spores in rSMP treated with HIU

	Master Model		Predictive Model	
	F	Pr > F[a]	F	Pr > F[a]
Solids	0.007	0.9344	0.006	0.9412
Temp	13.258	0.0016	10.630	0.0034
Time	1.733	0.2029	1.389	0.2505
Solids*Solids	36.695	< .0001	29.422	< .0001
Solids*Temp	0.788	0.3851		
Solids*Time	1.316	0.2648		
Temp*Temp	10.205	0.0046	8.182	0.0088
Temp*Time	6.581	0.1850		
Time*Time	36.258	< .0001	29.071	< .0001
Model	10.76955	< .0001	11.791	< .0001
Linear	4.999612	0.0095		
Quadratic	24.41365	< .0001		
Cross Product	2.895382	0.0606		

ANOVA = analysis of variance
rSMP = reconstituted skim milk powder
HUI = high intensity ultrasound
[a]Significance declared at $P \leq 0.05$.

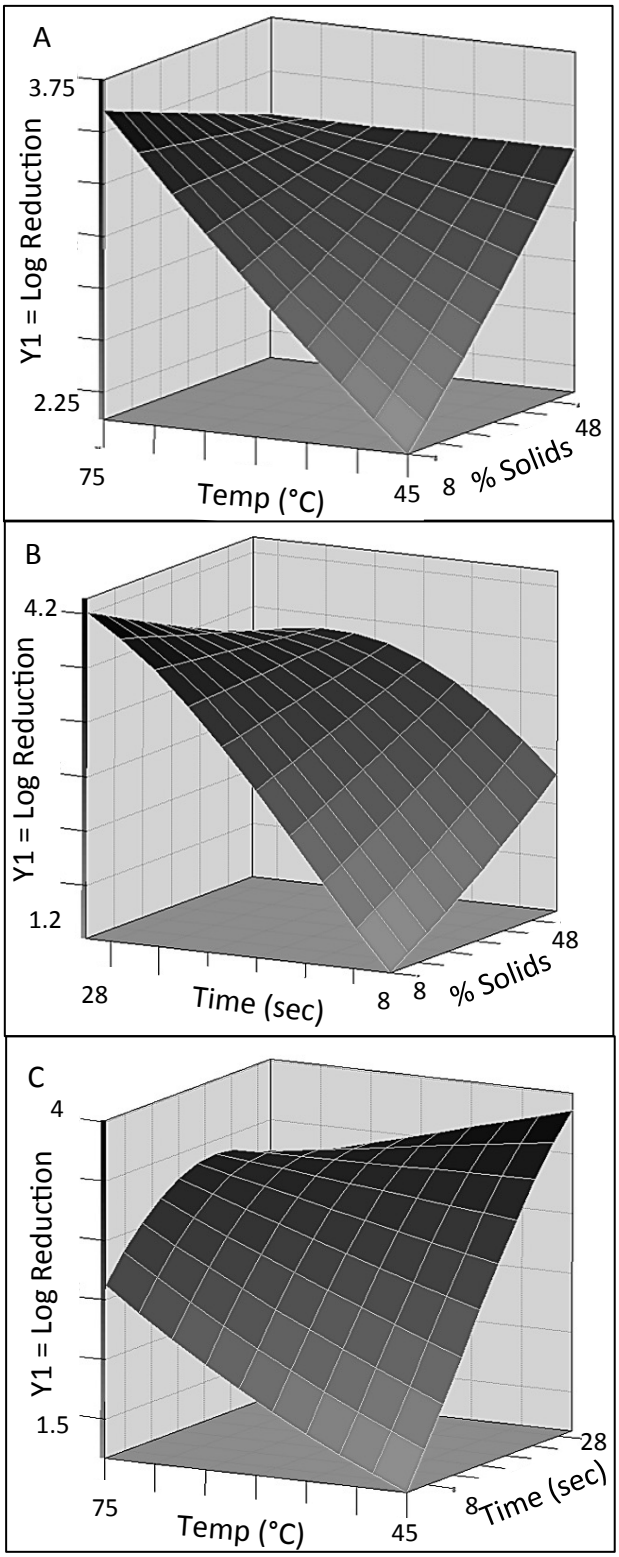

Figure 1. Response surface plots showing the optimization of sonication time, temperature, and solids content on the log reductions of *Geobacillus stearothermophilus* vegetative cells in skim milk. A, fixed time of 17.5 sec; B, fixed temperature of 60°C; fixed solids level at 31.5%.

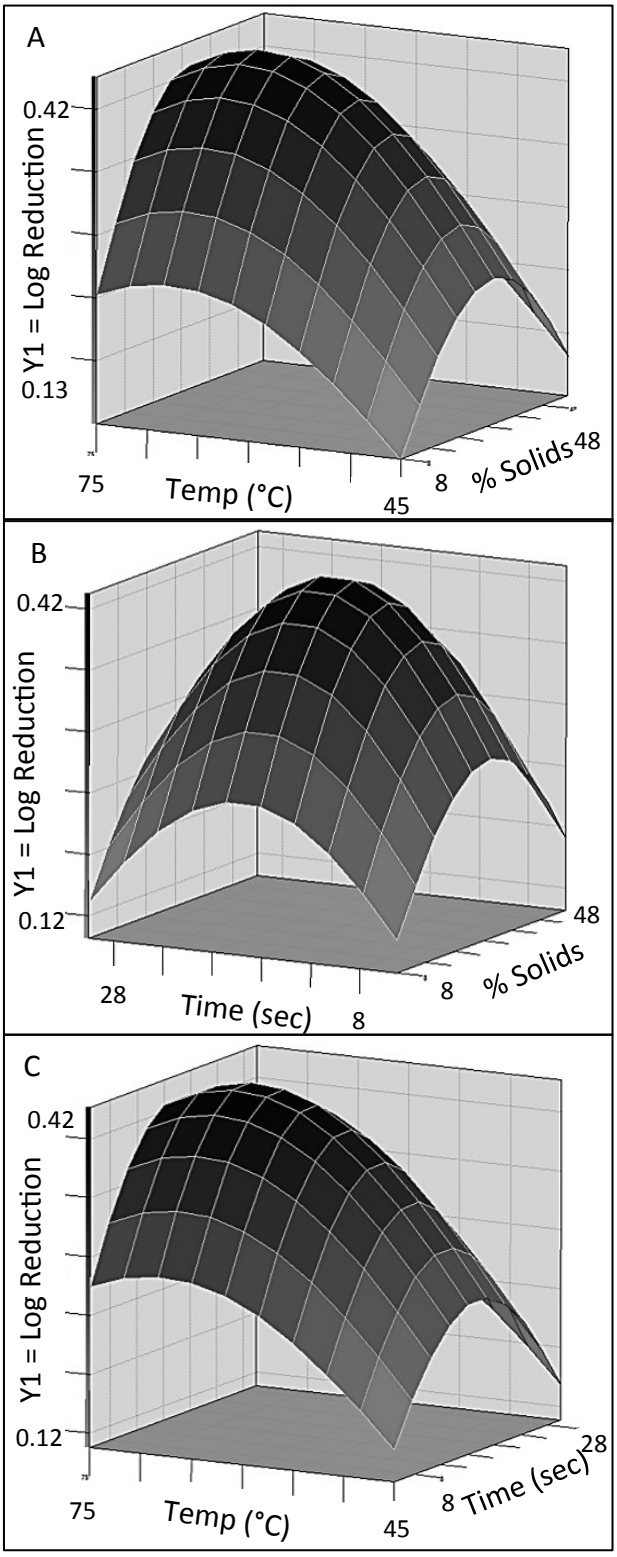

Figure 2. Response surface plots showing the optimization of sonication time, temperature, and solids content on the log reductions of *Geobacillus stearothermophilus* spores in skim milk. A, fixed time of 17.5 sec; B, fixed temperature of 60°C; fixed solids level at 31.5%.