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Effect of Mild Sonication Conditions on the Attributes of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12

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ABSTRACT

Lactobacillus delbrueckii ssp. *bulgaricus* is a widely used bacterium for the production of some fermented dairy products. Mild sonication intensity condition is a non-destructive technique that uses sound waves to cause cavitation in aqueous solutions and may improve the permeability of membranes, speed up the transfer of substrates and promote cellular growth and propagation. The objective was to determine the effect of mild sonication intensities at different temperatures on growth, bile tolerance and protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12. The treatments were four sonication intensities (8.07, 14.68, 19.83 and 23.55 W/cm^2) randomized at three different temperatures (4˚C, 22˚C and 40˚C). The energy input (1500 J) was kept constant in all treatments. Control samples did not receive any sonication treatment. Growth and bile tolerance were determined every 2 h for 12 h of incubation.Protease activity was determined at 0, 12 and 24 h. Mild sonication conditions included 1) mild sonication intensities, 2) temperatures and 3) times, all three of which played a role in influencing the desirable attributes of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12. Of all the mild sonication intensities studied, 14.68 W/cm^2 had the best overall influence at certain time points forimproving the bile tolerance and growth at 4˚C and protease activity at 40˚C. Mild sonication intensity of 23.55 W/cm^2 had the best overall influence at certain time points for protease activity of at 22˚C. Some mild sonication conditions could be recommended for improvement of some characteristics of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12.

Keywords: Sonication; Starter Culture; *Lactobacillus bulgaricus*

1. Introduction

The legal description of yogurt indicates that *Lactobacillus bulgaricus* and *Streptococcus thermophilus* are required in a 1:1 ratio in yogurt production [1]. Yogurt is one of the most consumed dairy products. Yogurt sales and consumption increased by 5.9% between 2008 and 2009 was 5.9% [2]. The global projection predicted by Global Industry Analysts Inc. [3] reported that for 2015 yogurt consumption will reach 20.6 million tons, equaling $67 billion in sales. The consumption of yogurt was enhanced mainly because of its nutritional value and the beneficial health effects of yogurt culture bacteria [4].

Low sonication intensity is a non-destructive technique that consists of the application of low energy, high frequency (1 - 10 MHz) and power intensities below 1 W/cm^2 [5]. Some microorganisms are resistant to the effects of sonication, thus extended periods of sonication would be required to render a product safe. The cells may grow in the presence of low sonication (<2 W/cm^2) due to ability of ultrasound to increase the transport of small molecules (amino acid, peptide, carbon dioxide and water) in solution and the inability of ultrasound to completely remove cells (or even non-living particles) from surfaces [6].

One of the most important characteristics of probiotic microorganisms is their ability to survive acid in the human stomach and bile in the intestine before they can establish in the lower gastrointestinal tract and confer the health benefit upon the host. Lactic acid bacteria (LAB) have the highest lactase activity due to its content of lactase or β-D-galactosidase which is an intracellular enzyme [7]. The LAB cells exhibit very little extracellular lactase activity, and it can be increased several times by bacterial cell lysis induced by sonication. It could also hydrolyze a portion of lactose in milk and the products of lactose hydrolysis, glucose, and galactose could be used by slow growing organisms such as *Lactobacillus acidophilus* and *Bifidobacterium* ssp. In addition, *Lactobacillus delbrueckii* ssp. *bulgaricus* B-5b when sonicated using a sonicator 300 dismembrator at a frequency of 16

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kHz showed the highest amount of β-galactosidase released by sonication-fermentation after 4 h of the culture incubation in milk fermentation [8].

The factors that affect the microbial inactivation with sonication conditions depend on the process (amplitude, time, temperature, and frequency) and microbial entity (type and growth stage of microorganism). In the production of fermented milk products, the use of sonication improves the acidifying activity of lactobacilli, therefore reducing production time and while accelerating lactose hydrolysis [9,10]. Preliminary work in our lab indicated that sonication intensities from 8.07 up to 23.55 W/cm^2 had favorable influences on protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12. It is not known if mild sonication intensities between 8.07 and 23.55 W/cm^2 may stimulate yogurt culture bacterium *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 to enhance its desirable characteristics. The aim of this study was to elucidate the influence of mild sonication intensities (0, 8.07, 14. 68, 19.83 and 23.55 W/cm^2) on the growth, bile tolerance, and protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 at refrigeration (4˚C), room (22˚C) and incubation (40˚C) temperatures.

2. Materials and Methods

2.1. Experimental Design

Freshly thawed *Lactobacillus bulgaricus*LB-12 (F-DVS, Chr. Hansen's Laboratory, Milwaukee, WI) culture was suspended in 0.1% sterilized peptone water and 18 ml of sample was sonicated using a 13 mm diameter probe set at a maximum acoustic power output of 750 W, frequency 24 kHz. Before sonication, the inoculated samples were set at three different temperatures (4˚C, 22˚C and 40˚C). Four sonication treatments with intensities of 8.07, 14.68, 19.83 and 23.55 Watts/cm^2 were performed in a random manner at the three different temperatures mentioned above. The control was the sample that did not receive any sonication treatment at each respective temperature. The control and mild sonicated samples were tested for growth, bile tolerance, and protease activity. Growth and bile tolerance were determined by plating the control and mild sonicated samples every 2 h for 12 h of incubation. Protease activity of the control and the mild sonicated samples was determined by measuring optical density (absorbance units) at 0, 12 and 24 h of incubation of the samples. The experimental design was a completely randomized design (CRD). Three replications were conducted for each experimental condition.

2.2. Sample Preparation

Control and mild sonicated samples for the growth, bile tolerance, and protease activity analyses were prepared by inoculating 5 ml of freshly thawed pure frozen concentrated stock culture of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 (Chr. Hansen's Laboratory, Milwaukee, WI) into 495 ml of sterile 0.1% peptone water at certain temperatures (4˚C, 22˚C, 40˚C) to make it 1% (v/v) and treated in a Sonicator (750 VCX Sonics, Vibracell). For the analysis of protease activity, *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 in control and mild sonicated samples were inoculated at 10% (v/v) into sterile skim milk (sterilized at 121˚C for 15 min).

2.3. Mild Sonication Treatments

The mild sonication treatment conditions consisted in four sonication intensities of 8.07, 14.68, 19.83 and 23.55 Watts/cm^2 using constant frequency (20 kHz) and constant energy (1500 J), randomized at three different temperatures (4˚C, 22˚C and 40˚C) of the peptone water with the culture before it was sonicated.

2.4. Preparation of Media

2.4.1. Lactobacilli MRS Agar

MRS agar was prepared according to the manufacturer instructions (Difco^{TM}, Dickinson and Company, Sparks, MD).

2.4.2. pH Modified MRS Agar (pH 5.2)

The pH of the MRS agar (Difco^{TM}, Dickinson and Company, Sparks, MD) was adjusted to a pH of 5.2 using 1 N HCl [11].

2.5. Analytical Procedures

2.5.1. Growth

Growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 was determined by the method proposed by Dave and Shah [11] with slight modification. Control and sonicated samples were inoculated (10% [v/v]) into MRS broth (Difco^{TM}, Dickinson and Company, Sparks, MD) which was previously autoclaved at 121˚C for 15 min at pH 6.5 ± 0.2. Growth of the cultures was determined every 2 h for 12 h of incubation at 43˚C for *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12. The inoculated broth (1 mL) was serially diluted in peptone water (0.1% wt/v) and pour plated. The culture, *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12, was enumerated using pH modified (5.2) Lactobacilli MRS agar [12]. During the incubation period for *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12, plates were kept at 43˚C anaerobically for 72 h [13]. The colonies were then counted.

2.5.2. Bile Tolerance

The bile tolerance was determined according to the method of Pereira and Gibson [14] with slight modifications.

The bile tolerance of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 was analyzed in MRS-THIO broth [MRS broth (Difco™, Becton, Dickinson and Company, Sparks, MD)] supplemented with 0.3% (wt/v) oxgall (bovine bile) (US Biological, Swampscott, MA) and 0.2% (wt/v) sodium thioglycolate (Acros Organics, Fair Lawn, NJ). Oxgall was added to test bile tolerance of the bacteria and sodium thioglycolate was used in the broth as an oxygen scavenger. Control and sonicated cultures were inoculated at 10% (v/v) separately into MRS-THIO broth and incubated at 43˚C for 12 h. The inoculated broth (1 mL) was serially diluted in peptone water (0.1% wt/v) and pour plated every 2 h for 12 h. The culture *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 was enumerated using pH modified Lactobacilli MRS agar [14]. The petri plates were incubated anaerobically at 43˚C for 72 h. The colonies were then counted.

2.5.3. Protease Activity

The extracellular protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 was determined using the *o*-phthaldialdehyde (OPA) spectrophotometric method proposed by Oberg *et al.* [15] with slight modifications. The control and sonicated samples were inoculated (10% [v/v]) into sterile skim milk (autoclaved at 121˚C for 15 min), and incubated at 40˚C for 0, 12 and 24 h. After incubation, 2.5 ml from each sample were mixed with 1 ml distilled water and transferred into test tubes containing 5 ml of 0.75 Ntrichloroacetic acid (TCA) (Fisher Scientific) and the test tubes were immediately vortexed. After holding at room temperature for 10 min, the acidified samples were filtered through a Whatman Number 2 filter paper (Clifton, NJ). Duplicate aliquots from each TCA filtrate were analyzed by OPA using a spectrophotometer (Nicolet Evolution 100, Thermo Scientific; Madison, WI, USA). The OPA solution was prepared by combining 25 ml of 100 mM sodium borate (Fisher Scientific), 2.5 ml of 20% (wt/wt) SDS (Fisher Scientific), 40 mg of OPA (Alfa Aesar, Ward Hill, MA) dissolved in 1 ml methanol (Sigma), and 100 µl of *β*-mercaptoethanol (Sigma) and diluting to a final volume of 50 ml with distilled water. Each TCA filtrate (150 µL) was mixed with 3 ml of OPA reagent in a 3 ml cuvette, and the absorbance at 340 nm was read. Absorbance of the OPA final solution (mixed of OPA reagent with TCA filtrate) with the non-inoculated sterile skim milk (reference sample) was subtracted from each sample reading. OPA final solution was used as a blank to calibrate the spectrophotometer.

2.6. Statistical Analysis

Differences of least square means were used to determine significant differences at $P < 0.05$ for main effects (mild sonication intensity, time and temperature), two way interaction effects (mild sonication intensity ∗ temperature and mild sonication intensity ∗ time) and three way interaction effects (mild sonication intensity ∗ time ∗ temperature). Data are presented as mean ± standard deviation of the means. Significant differences were determined at $\alpha = 0.05$. Significant differences ($P < 0.05$) among the main effects were analyzed using Tukey's adjustment. Data were analyzed using Proc Mixed model of Statistical Analysis System (SAS®).

3. Results and Discussion

3.1. Growth

The growth characteristics of *Lactobacillus delbrueckii* ssp. *bulgaricus* as influenced by various mild sonication intensities at different temperatures (4˚C, 22˚C and 40˚C) for 12 h are shown in **Figure 1**. Therewassignificant ($P < 0.001$) interaction between mild sonication intensities ∗ time ∗ temperature and between mild sonication intensity ∗ time (**Table 1**). Viable counts increased over time from 0 to 12 h (**Figure 1**). The mild sonication intensity ∗ temperature interaction was also significant ($P < 0.001$) (**Table 1**). All mild sonication intensities and control showed better viable counts at 4˚C than at 22˚C and 40˚C (**Table 2**). The temperature and mild sonication intensity had significant ($P < 0.001$) effects (**Table 1**). Mild sonication conditions adversely influenced growth of *Lactobacillus delbrueckii* ssp. *bulgariucs* LB-12.

In the present study, the application of mild sonication intensities to *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 at different temperatures enhanced the exponential growth phase after 2 h of incubation. Liong and Shah [16] found growth of *Lactobacillus bulgaricus* and

Table 1. The Probability > F value of mild sonication intensity, time, temperature and their interactions for growth, bile tolerance and protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12.

Growth	Bile tolerance	Protease activity	
EFFECT	Lb[3]	Lb	Lb
	Pr > F	Pr > F	Pr > F
INT[2]	<0.0001	<0.0001	<0.0001
TIME[1]	<0.0001	<0.0001	<0.0001
TEMP	<0.0001	<0.0001	<0.0001
INT ∗ TIME	<0.0001	0.2318	<0.0001
INT ∗ TEMP	<0.0002	<0.0002	0.0368
INT ∗ TIME ∗ TEMP	<0.0001	0.6765	<0.0001

[1]Time = Incubation period of 24 h for protease activity; [2]INT = Mild sonication intensity; [3]L. b = *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12.

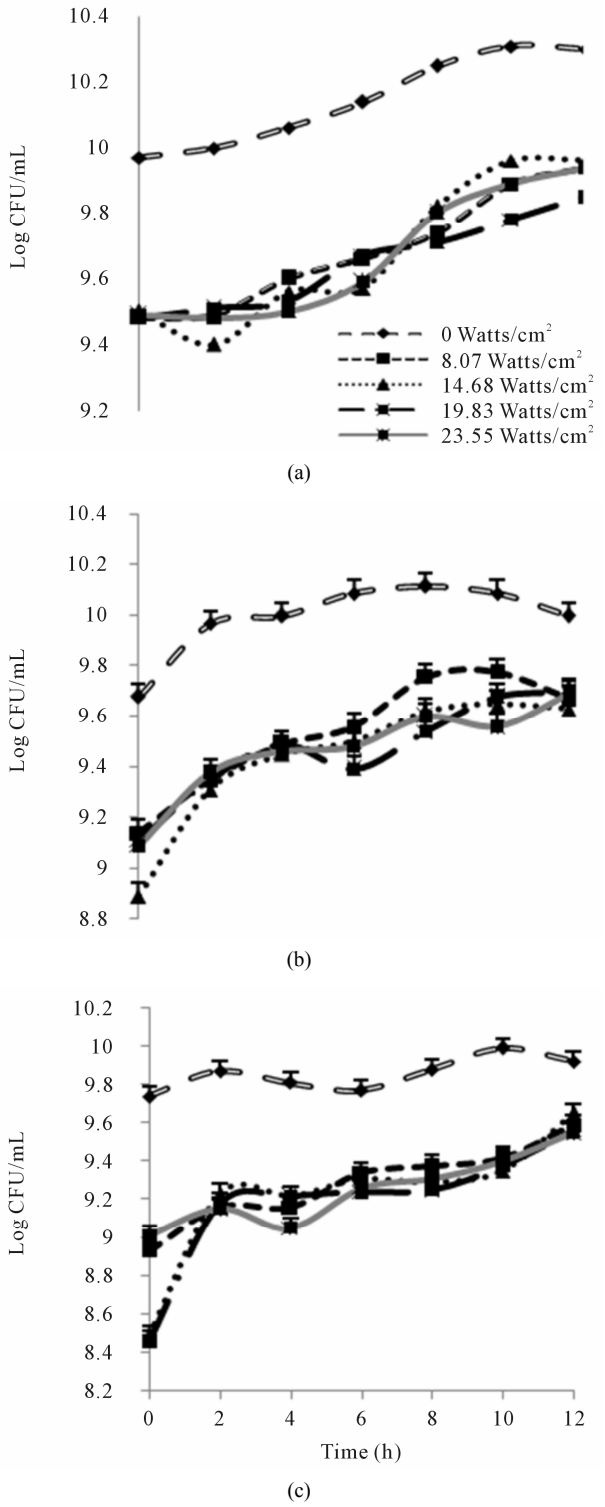

Figure 1. Growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 at 4˚C (a), 22˚C (b) and 40˚C (c).

Lactobacillus acidophilus to be predominant in the first 9 - 15 hours. Simova *et al.* [17] reported the growth of *Streptococcus thermophilus* T15 and *Lactobacillus bulgaricus* HP1 by pre-incubating both cultures for 5.5 h before inoculation and found that growth reached exponential phase in the first 5 h and stationary phase in 8 - 12 h. Additionally, Kobayashi *et al.* [18] indicated that low intensity of pulsed ultrasound treatments may stimulate cell propagation and production of proteoglycan in human nucleus pulposus cell line, possibly by enhancement of growth factor-related genes.

Table 2. The least square means (log_{10}) for growth, bile tolerance and protease activity of bacteria as influenced by mild sonication intensities.

Parameter	Intensity (W/cm^2)	*Lactobacillus bulgaricus*		
		4˚C	22˚C	40˚C
		LS mean	LS mean	LS mean
Growth	0	$10.15^{A,a}$	$9.99^{A,b}$	$9.86^{A,c}$
Growth	8.07	$9.69^{B,a}$	$9.54^{B,b}$	$9.28^{B,c}$
Growth	14.68	$9.68^{B,a}$	$9.43^{D,b}$	$9.22^{CD,c}$
Growth	19.83	$9.65^{B,a}$	$9.47^{C,b}$	$9.18^{D,c}$
Growth	23.55	$9.67^{B,a}$	$9.47^{C,b}$	$9.25^{BC,c}$
Bile tolerance	0	$7.73^{A,c}$	$8.43^{A,a}$	$8.15^{A,b}$
Bile tolerance	8.07	$7.68^{B,b}$	$7.96^{C,a}$	$7.60^{D,c}$
Bile tolerance	14.68	$7.71^{A,b}$	$7.95^{C,a}$	$7.59^{D,c}$
Bile tolerance	19.83	$7.51^{C,c}$	$8.13^{B,a}$	$7.74^{B,b}$
Bile tolerance	23.55	$7.52^{C,c}$	$8.10^{B,a}$	$7.66^{C,b}$
Protease activity	0	$0.27^{C,b}$	$0.34^{C,a}$	$0.37^{B,a}$
Protease activity	8.07	$0.32^{BC,c}$	$0.40^{BC,b}$	$0.52^{A,a}$
Protease activity	14.68	$0.40^{A,b}$	$0.38^{BC,b}$	$0.52^{A,a}$
Protease activity	19.83	$0.34^{AB,b}$	$0.44^{AB,a}$	$0.47^{A,a}$
Protease activity	23.55	$0.35^{AB,b}$	$0.46^{A,a}$	$0.52^{A,a}$

^{ABCD}LS means containing a common letter within the same column for the same parameter are not significantly different. ^{abc}LS means containing a common letter within the same row are not significantly different.

3.2. Bile Tolerance

The bile tolerance of *Lactobacillus delbrueckii* ssp. *Bulgaricus* LB-12 asinfluencedbyvariousmild sonication intensities at three different temperatures (4˚C, 22˚C and 40˚C) is shown in **Figure 2**. There was no significant ($P = 0.677$) interaction formild sonication intensity ∗ time ∗ temperature and the interaction for mild sonication intensity ∗ time was also not significant ($P = 0.232$) (**Table 1**). Viable counts decreased from 0 to 12 h (**Figure 2**). At 4˚C, bile tolerance of the control culture and the culture subjected to 14.68 W/cm^2 was significantly ($P < 0.05$) higher than the rest of the mild sonication intensities (**Table 2**).

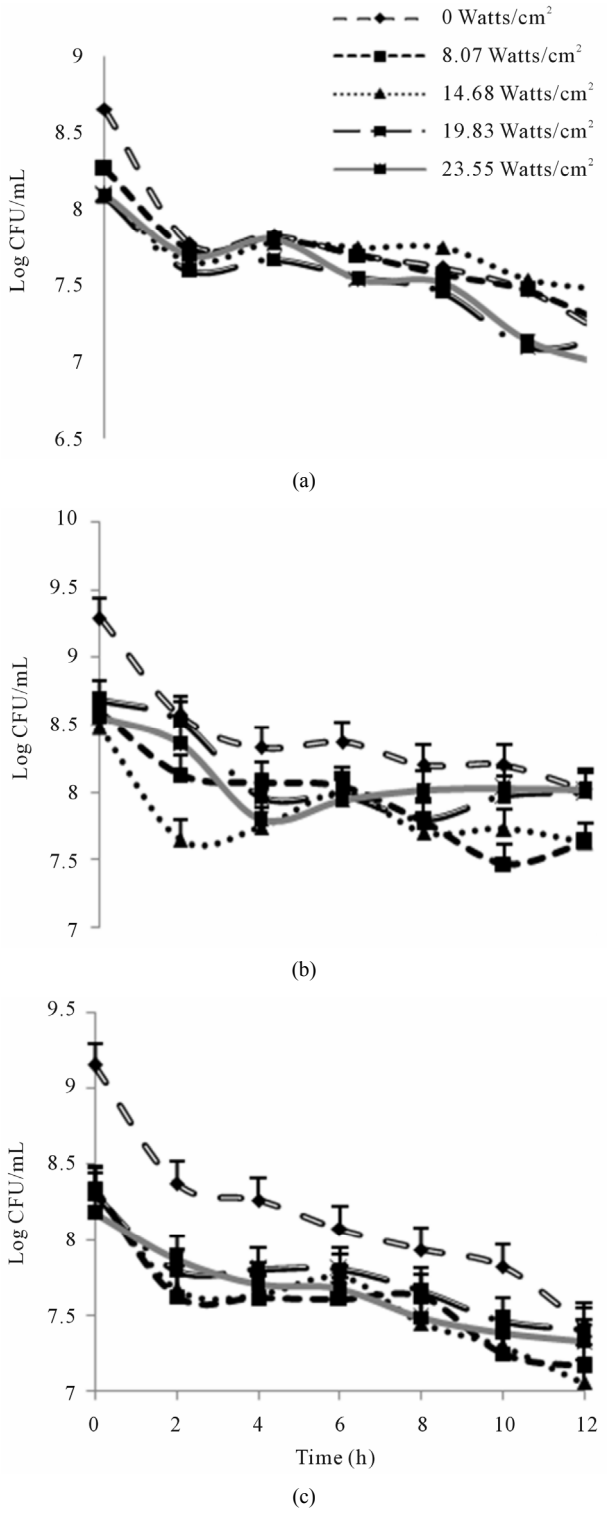

Figure 2. Bile tolerance of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 at 4˚C (a), 22˚C (b) and 40˚C (c).

The mild sonication intensity ∗ temperature interaction was significant ($P < 0.001$) (**Table 1**). The control and all mild sonication intensities showed better ($P < 0.05$) bile tolerance at 22˚C than at 4˚C and 40˚C during 12 h of incubation (**Table 2**). The temperature had a significant ($P < 0.001$) effect (**Table 1**).

Mean log reduction of the viable counts of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 subjected to various intensities at three different temperatures (4˚C, 22˚C and 40˚C) was obtained by subtracting counts at 12 h from 0 h as shown in **Table 3**. In **Table 3**, a high number indicates high bacterial death and a lower number indicates lower bacterial death. The log reduction at 4˚C, 22˚C and 40˚C showed that control had the highest bacterial death for *Lactobacillus bulgaricus* compared to the mild sonication intensities.

Mild sonication intensities had a significant ($P < 0.001$) effect (**Table 1**). Taking log reduction values over 12 h into consideration, the treated samples performed better than control (**Table 3**).

Shah and Jelen [7] have attributed increased bile tolerance of Lactobacilli strains to their rigid cell wall. There could be other factors responsible for increased bile tolerance of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* when subjected to mild sonication conditions. In addition, Clark and Martin [19] reported that *B. longum* and *L. bulgaricus* in the presence or absence of bile acid (Oxgall), resisted bile concentrations as high as 4.0%. Lick *et al.* [20] found that *Streptococcus thermophilus* and *Lactobacillus bulgaricus* strains are able to survive gastrointestinal passage *in vivo* and detected viable *Streptococcus thermophilus* in human duodenal samples after fresh yogurt ingestion.

3.3. Protease Activity

The protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12as influenced by various mild sonication intensities at different temperatures (4˚C, 22˚C and 40˚C) is shown in **Figure 3**. There was a significant ($P <$ 0.001) interaction between mild sonication intensity ∗ time ∗ temperature and between mild sonication intensity

Table 3. The mean log reduction of the viable counts of the control and mildly sonicated cultures obtained by subtracting viable log cfu/ml between 0 h and 12 h of incubation in the presence of bile acid (Oxgall) for bile tolerance.

Parameter	Intensity (W/cm^2)	*Lactobacillus bulgaricus* Log cfu/ml		
		4˚C	22˚C	40˚C
Bile tolerance	0	1.51	1.27	1.72
Bile tolerance	8.07	1.03	0.96	1.18
Bile tolerance	14.68	0.62	0.85	1.28
Bile tolerance	19.83	0.93	0.68	0.90
Bile tolerance	23.55	1.12	0.55	0.86

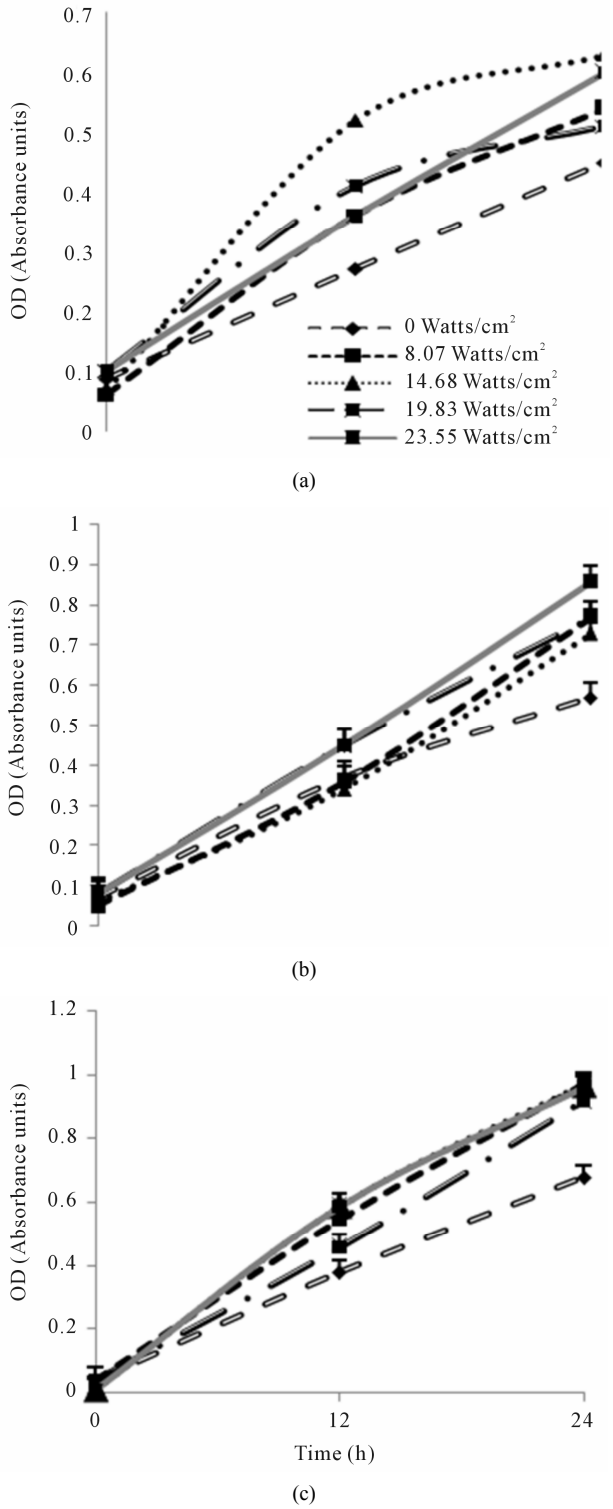

Figure 3. Protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 at 4˚C (a), 22˚C (b) and 40˚C (c).

∗ time (**Table 1**). Absorbance units increased over time from 0 to 24 h (**Figure 3**). At 4˚C, OD values of cultures subjected to 14.68 W/cm^2 were significantly ($P < 0.005$) higher than OD values for the control at 12 and 24 h (**Figure 3(a)** and **Table 4**). At 22˚C, cultures subjected to 23.55 W/cm^2 had significantly ($P \leq 0.001$) higherprotease activity than the control at 12 and 24 h (**Figure 3(b)** and **Table 4**). At 12 and 24 h, the OD values were 0.45 and 0.86 absorbance units and 0.37 and 0.57 absorbance units for the 23.55 W/cm^{2}treatments and the control, respectively. At 40˚C, cultures subjected to 8.07, 14.68 and 23.55 W/cm^2 showed a significant ($P < 0.005$) increase in protease activity compared to the control at 12 and 24 h (**Figure 3(c)**, **Table 4**).

The mild sonication intensity ∗ temperature interaction was significant ($P < 0.05$) (**Table 1**). Cultures subjected to 8.07 and 14.68 W/cm^2 showed better protease activity at 40˚C than at 4˚C and 22˚C (**Table 3**). The temperature also had a significant ($P < 0.001$) effect (**Table 1**). The cultures sonicated at 40˚C showed higher protease activity compared to the 40˚C control culture (**Table 2**). The mild sonication intensities had a significant ($P < 0.001$) effect (**Table 1**). Some mild sonication conditions increased protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12.

This study of mild sonication intensities at three different temperatures (4˚C, 22˚C and 40˚C) showed that *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 exhibited higher OD values, hence higher protease activity. *Lactobacillus bulgaricus* exhibited higher *β*-galactosidase activity than *Streptococcus thermophilus* and *Lactobacillus acidophilus* [7]. Additionally, Wang *et al.* [8] sonicated samples of *Lactobacillus bulgaricus* B-5b and reported that the highest amount of *β*-galactosidase released by sonication-fermentation was after 4 h of the culture incubation in milk fermentation. This could indicate that the intracellular enzyme was not released into the medium during conventional fermentation, but was released during sonicated fermentation.

4. Conclusion

Mild sonication conditions included 1) mild sonication intensities; 2) temperatures and 3) times, all three of which played a role in influencing the desirable attributes of *Lactobacillus bulgaricus*. Of all the mild sonication intensities studied, 14.68 W/cm^2 had the best overall influence at certain time points for improving the bile tolerance and growth at 4˚C and protease activity at 40˚C. Mild sonication intensity of 23.55 W/cm^2 had the best overall influence at certain time points for protease activity of at 22˚C. Some mild sonication conditions could be recommended for improvement of some characteristics of *Lactobacillus delbrueckii* ssp. *bulgaricus*.

5. Acknowledgements

Approved for publication by the Director of the Louisiana Agricultural Experiment Station as manuscript

Table 4. The probability > F value of protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 at various mild sonication intensities compared to the control (0 W/cm^2).

		4˚C				22˚C				40˚C			
Culture	Time	Intensity (W/cm^2)				Intensity (W/cm^2)				Intensity (W/cm^2)			
	(h)	8.07	14.68	19.83	23.55	8.07	14.68	19.83	23.55	8.07	14.68	19.83	23.55
Lb[1]	0	0.639	0.742	0.783	0.751	0.778	0.971	0.751	0.846	0.953	0.664	0.920	0.586
Lb	12	0.104	0.001	0.012	0.127	0.851	0.566	0.158	0.001	0.003	0.001	0.163	0.001
Lb	24	0.117	0.002	0.257	0.011	0.001	0.005	0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1]*Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12.

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