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Ultrasound treatment (low frequency) effects on probiotic bacteria growth in fermented milk

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Probiotic bacteria, Ultrasound, Fermented milk, β-galactosidase

Abstract

The effect of ultrasonic treatment at 40 kHz for 0, 5, 10, 15 and 20 minutes on the growth of five different strains of probiotic bacteria (*Lactobacillus acidophilus* LA-5*, Lactobacillus casei* LC, *Lactobacillus reuteri* LR-MM53*, Bifidobacterium bifidum* Bb-12 *and Bifidobacterium loungm* BB-536) in fermented milk was investigated. The study findings indicate that ultrasound treatment (10 minutes) increased the viable cells and total acidity for LA-5, LC and LR-MM53 samples but decreased viable cells and total acidity in the Bb-12 and BB-536 samples. All probiotic bacteria strains were ruptured by ultrasound treatment causing an increase in the extracellular release of β-galactosidase enzyme. Increased exposure time led to higher enzymatic activity. 2.9 unit/ml of β-galactosidase was measured in LR-MM53 after ultrasonic treatment for 20 minutes. The fermentation time of LA-5, LC and LR-MM53 samples were reduced after 10 minutes of ultrasound treatment compared with the control sample. Added 5 percent (10^8 CFU/ml) of probiotic bacteria led to reduce at the fermentation time during ultrasonic treatment compared with control sample. The optimal time span of ultrasound treatment (40 kHz, 116 W) was 10 minutes for all fermented milk samples, which can be applied to increase the number of viable cells of probiotic bacteria and β-galactosidase enzyme.

Introduction

Sound waves or acoustic energy with frequencies above 20.000Hz is called ultrasound. Ultrasound causes cavitation in growth media that brings about many chemical and physical changes. Bacterial cells can grow in low intensity of ultrasound treatment because of the following characteristics of ultrasound (Pitt and Ross, 2003). Ultrasound treatment increases the transfer of small particles in the growth media, and incompetence to fully remove microorganism's cells or even non-living molecules from surfaces media. Stable cavitation production results when the low ultrasound intensity is sufficient that the bubbles do not collapse totally during their contraction cycle (Elder, 1959; Chau et al., 2017).

Historically, the effectiveness of low acoustic intensity in inhibition bacterial cells has been limited through the protection provided to the organisms by the food environment. Recently, the systems such as Langevin transducers with high output of ultrasound power at low frequency have greatly increased the effect on bacterial growth, bacterial biological activity and their enzyme production. In general, inhibition of the bacterial cells is more pronounced at high ultrasound treatment by the increasing processing time of this treatment (Pohlman

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et al., 1997; Dong et al., 2017; Wu and Narsimhan, 2017). According to the effects of ultrasound in the in food systems, the ultrasonic treatment frequencies can be divided into low frequency (20-100 kHz), medium frequency (100 kHz-1MHz) and high frequency (1-10 MHz) (Abbas et al., 2013; Al-Hilphy et al., 2016).

Climatic changes affect dairy products in all world zones. Climatic changes are expected to hold back and increasingly adversely affect the dairy industry in the upcoming years. The importance of modern techniques such as Ultrasound to dairy production, therefore, will rise in the coming years in proportion to the changes in ecological temperatures (IPCC, 2007).

Ultrasound power is important in food processing. It is commonly used for controlling the viscosity of food systems, emulsification, sugar crystallization, meat tenderization, drying, brining, cutting processes and improving cleaning and flux during ultrafiltration and microfiltration (Ünver, 2016; Carrillo-Lopez et al., 2017; de Lima Alves et al., 2018; Ojha et al., 2018). Al-Hilphy et al. (2012), using 338 and 430W of ultrasound power (high efficiency) for milk homogenization and to decrease the number of bacteria, found that a higher power (430W) decreased the homogenization index and D-values of bacterial numbers. Low-frequency ultrasound treatment increases the viability of bacteria and the breakdown of some complex compounds such as sugars and protein (Al-Hilphy et al., 2016). Probiotic bacteria are defined as active microorganisms, which, when taken in adequate quantities, grant a health benefit on the host (humans and animals) (Ranadheera et al., 2010). The dose effect of probiotic bacteria is dialectical and the majority of past studies were in-vitro tests. The usual active dose in humans is 10^7-10^9 CFU/mg/day. However, the level of probiotic bacteria in dairy products should include a lower number, 10^6-10^7 CFU/mL, of viable cells at the time of consumption (Gustaw et al., 2011). Probiotics weak growth in milk and dairy products. They need more time to produce their enzymes (such as ß-galactosidase) compared with lactic acid bacteria.. β-galactosidase enzyme hydrolyses lactose (milk sugar) into glucose and galactose (Nguyen et al., 2009). Previous studies show the use of some substances to stimulate probiotic bacteria, such as added gum Arabic and mannan extraction to produce probiotic yogurt (Niamah et al., 2016; Al-Manhel and Niamah, 2017).

The goal of this study was to study the effect of low frequency ultrasound over different time periods on the viability of probiotic bacteria strains, β-galactosidase activity and fermentation time in fermented milk samples.

Materials and Methods

Sources of strains

Freeze-dried probiotic strains of *Lactobacillus acidophilus* (LA-5), *Lactobacillus casei* (LC), *Lactobacillus reuteri* (LR-MM53), *Bifidobacterium bifidum* (Bb-12) and *Bifidobacterium loungm* (BB-536) were purchased from CHr. Hansen company (Denmark). The strains were cultured in deMan Rogosa Sharpe media (Hi-media, India) with 0.05% L-cysteine-HCl (Sigma–Aldrich, Italy) at 37°C for 24-48 hour under anaerobic conditions.

Fermented milk by probiotic bacteria

Restarted skimmed milk (Param dairy, India) of 12% (W: V) was made by dissolving skimmed milk powder (120gm) in 1 litre of water at 50°C. It was pasteurized at 90°C for 5 minutes then cooled at 40°C. 5 percent (10^8CFU/mL) (W: V) of old probiotic strains were added to the pasteurized milk and mixed by vortex with a mixer (VM-300, Germany). The samples were exposed to ultrasonic power (power sonic 405, Korea) at amplitude of 30 percent (conforming to approximately 116 W) and a frequency of 40 kHz for 5, 10, 15 and 20 minutes, respectively. The samples were then incubated at 37°C for 12 hours and cooled at 4°C for 12 hours. Thereafter the following tests were conducted.

Enumeration of probiotic bacteria

Enumeration of viable probiotic cells was conducted by pouring plate method, which used deMan Rogosa Sharpe agar with 0.05% L-cysteine-HCl incubated at 37°C for 24-48 hours under anaerobic conditions. The survival percentage of probiotic bacteria was calculated (Niamah et al., 2017).

Total acidity and pH

Total acidity and pH (pH-meter, SD-300, Germany) of fermented milk samples were measured after fermentation by probiotic strains (Niamah, 2017).

β-galactosidase activity assay

β-galactosidase was measured using O-nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma–Aldrich, Italy) as described by Nguyen et al. (2009). 1 ml of fermented milk by probiotic strains was mixed with 5 ml of sodium phosphate buffer (at pH 7) and incubated at 37°C for 15 minutes. 3 ml of ONPG, prepared by dissolving 0.4gm in 100 ml of sodium phosphate buffer, was added into tubes. The tubes were incubated at 37°C for 15 minutes. To stop the reaction, 2 ml of 1 M Na_2CO_3 (BDH, UK) were added. After cooling 10 ml of acetonitrile (BDH, UK) was added, and the mixture was centrifuged at 5000 rpm for 20 minutes to remove the milk protein and bacterial cells of samples. The samples were measured in absorbance

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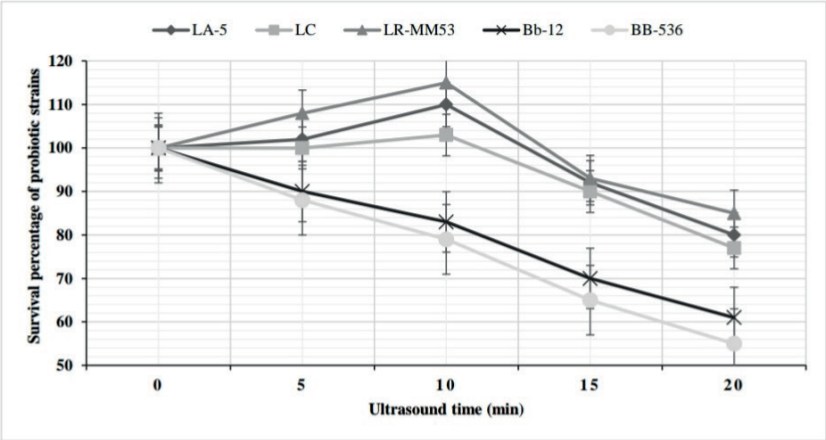

Figure 1: Survival percentage of bacterial strains after ultrasound treatment for 0, 5, 10, 15 and 20 minutes. The results are expressed as mean values with standard deviation (SD), n = 3 for any treatment

at 420 nm by spectrophotometer UV-2900 (Biotech Engineering, UK). A standard calibration curve was created using pure ONP (Sigma-Aldrich, Italy). The activity unit of enzyme was determined by defining the amount of ONP liberated from ONPG.

Water holding capacity (WHC)

In order to determine the WHC percentage of the fermented milk samples after ultrasound treatment, 10 g of fermented milk was weighed and centrifuged at 5000 rpm for 10 minutes at 25°C. The top layer (whey) was removed and weighed. WHC percentage was reported as the ratio of the top layer of the initial fermented milk weight (Niamah et al., 2016).

Fermentation time

After ultrasound treatment, pasteurized milk samples with 1,3 and 5% (10^8CFU/mL) of probiotic bacteria were incubated at 37°C until reaching the pH value of 4.5 ± 0.2, which indicates the end of fermentation time. The results were compared with a non-treated sample (without ultrasound treatment) of each probiotic strain.

Statistical analysis

In order to evaluate the effect of ultrasound treatment on the probiotic bacteria under study, experiments involving fermented milk of each probiotic strain were triplicated. The average of the three values (n=3) for each sample was calculated. An ANOVA table was used to analyse the data in SPSS (version 16.0). The Significance level was set at $p<0.05$ to make comparisons between the means using Least Significant Difference (LSD).Graphs of the findings were created using Microsoft Excel (version 2016). Results were presented as means ± standard deviations.

Results and Discussion

Survival percentage of probiotic bacteria

The changes in survival percentage of probiotic bacteria strains in fermented milk samples after different times of ultrasound treatment are shown in figure 1. The survival percentage of LR-MM53, LA-5 and LC strains increased after 10 minutes of ultrasound treatment as compared with Bb-12 and BB-536 strains (115, 110, 103, 83 and 79 percent survival, respectively). Clearly, the effect of ultrasound treatment on five-probiotic bacteria strain is dependent on the strain type. Their resistance to ultrasound power can be explained by their property's: differences in cell sizes, cell walls, and chemical components of cell walls such as the structure of the phospholipid and protein. Resistance in different strains of bacteria to ultrasonic power with 20 kHz of frequency and 80% amplitude at different time periods, in fermented milk by using *Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris*, *Lactobacillus acidophilus* and *Lactobacillus casei* is detailed in Tabatabaie and Mortazavi (2008). An increase in exposure times to ultrasound treatment leads to inhibition of bacterial cells, and therefore all survival percentages of bacterial strains decrease with increasing the time period of ultrasound treatment. *Bifidobacterium longum* BB536 strain was highly effective with increased exposure time of ultrasound treatment: the survival percentage of this strain was 55% after 20 minutes. This result agrees with the findings of Nguyen et al. (2009) who found the viable cells of *Bifidobacterium* spp. strains decreased when increasing the time of ultrasound treatment. Wang and Sakakibara (1997) reported the decreasing viability of *Lactobacillus* spp. through ultrasound treatment, but that after the ultrasound process stopped, the viability of bacteria was increased in fermented milk. This indicates that the ultrasound treat-

Table 1: Total acidity percentage and pH values of fermented milk samples after ultrasound treatment. The results are expressed as mean values with standard deviations (SD), n = 3 for all

Bacterial strains	Ultrasound treatment time (min)									
	0	5	10	15	20	0	5	10	15	20
	Total acidity (%)					pH values				
LA-5	0.80^{a} ±0.1	0.80^{a} ±0.3	0.82^{a} ±0.2	0.70^{b} ±0.3	0.61^{c} ±0.2	5.0^{a} ±0.1	5.0^{a} ±0.4	4.8^{a} ±0.1	5.7^{c} ±0.1	6.0^{c} ±0.1
LC	0.80^{a} ±0.1	0.81^{a} ±0.1	0.83^{b} ±0.1	0.68^{c} ±0.5	0.62^{d} ±0.5	5.0^{a} ±0.3	5.0^{a} ±0.3	4.9^{a} ±0.1	5.6^{b} ±0.5	6.0^{c} ±0.1
LR-MM53	0.79^{a} ±0.3	0.80^{a} ±0.1	0.81^{a} ±0.2	0.65^{b} ±0.5	0.60^{c} ±0.2	5.1^{a} ±0.2	5.1^{a} ±0.3	5.0^{a} ±0.3	5.7^{b} ±0.3	6.1^{c} ±0.1
Bb-12	0.75^{a} ±0.0	0.65^{b} ±0.2	0.64^{b} ±0.5	0.60^{c} ±0.4	0.55^{d} ±0.1	5.3^{a} ±0.5	5.6^{a} ±0.3	5.5^{b} ±0.3	6.1^{c} ±0.3	6.3^{c} ±0.2
BB-536	0.76^{a} ±0.2	0.64^{c} ±0.1	0.70^{b} ±0.3	0.55^{d} ±0.2	0.50^{f} ±0.0	5.2^{a} ±0.5	5.7^{b} ±0.2	5.9^{b} ±0.2	6.0^{b} ±0.1	6.1^{c} ±0.3

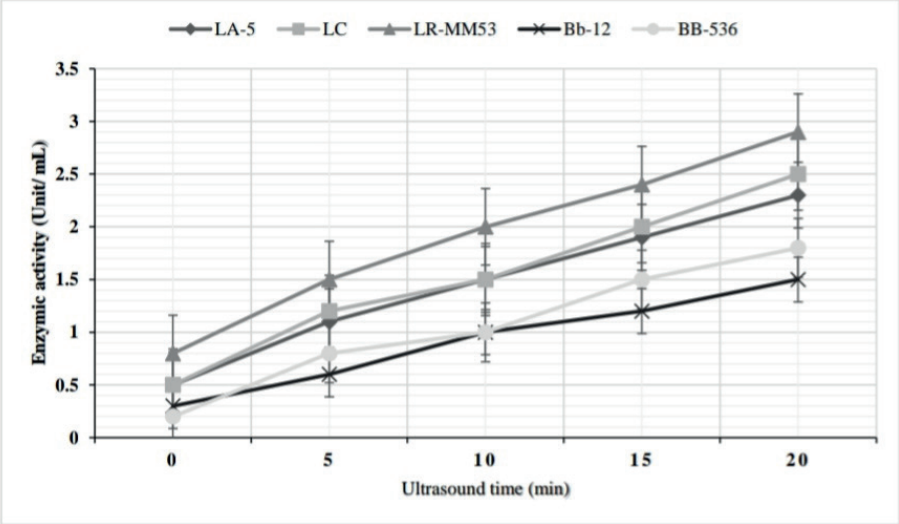

Figure 2: The β-galactosidase enzyme of probiotic bacteria strains after ultrasound treatment for different time periods. The results are expressed as a mean values with standard deviations (SD), n = 3 for all treatments

ment has no effect on the diffusion ability of bacterial cells.

Changes in total acidity and pH

Table 1 shows the changes in total acidity percentage and pH values of fermented milk samples after ultrasound treatment over different time periods. After ultrasound treatment at 10 minutes, the total acidity percentage of milk samples increased while pH values decreased. Increasing acidity of samples can be attributed to the activity of probiotic strains' residual after ultrasound treatment. Higher acidity percentage of fermented milk samples is the result of the encrusting of viable cells of bacteria after 10 minutes of ultrasound treatment. Ultrasound treatment causes cavitation bubbles which have a mechanical effect on the bacterial cells. One possible result is the devastation of the cell wall of the bacterial cells, such that the cells are unable to carry out metabolic activities. Our results were in good agreement with past studies. For example, Shershenkov and Suchkova (2015) reported an increase in the total acidity of yoghurt samples when using 5W of ultrasound treatment (30kHz) for 3 minutes compared with 6W of ultrasound treatment (30kHz) for 1 minute.

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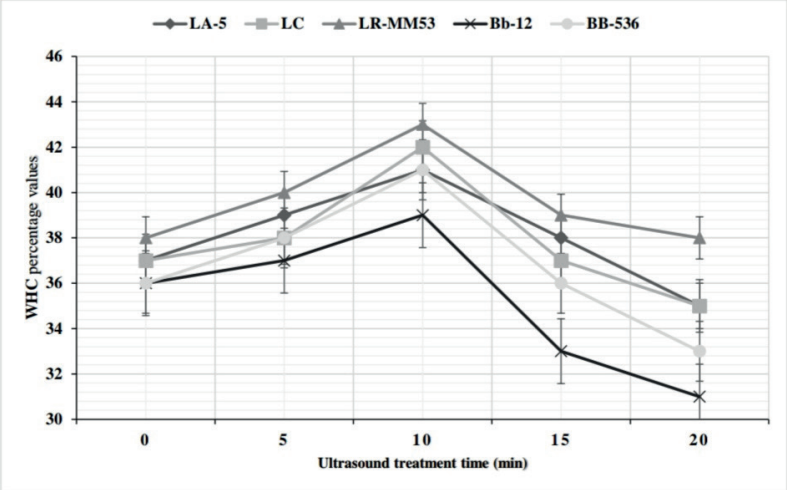

Figure 3: WHC percentage of fermented milk sample by probiotic bacteria after ultrasound treatment for different time periods. The results are expressed as mean values with standard deviations (SD), n = 3 for all treatments

β-galactosidase activity in fermented milk samples

The β-galactosidase activity in sonicated milk fermentation with five probiotic strains increased with increasing time of ultrasonic treatment (figure 2). Increased ultrasound treatment time provided higher β-galactosidase activity in all samples. LA-5, LC and LR-MM 53 strains excelled in enzyme production compared with the Bb-12 and BB-536 strain. The higher β-galactosidase activity of the fermented milk samples after ultrasound treatment identifies the treatment as a preferred agent for dairy fermentation products by probiotic bacteria. Ultrasound treatment effects in milk lead to increase in transfer reactions and lactose hydrolysis. It provides more monosaccharides, such as galactose and glucose.It was reported that lactose utilization by a starter yoghurt consisting of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* were speedier when carbohydrate of milk (lactose) was partially pre-hydrolyzed (O'Leary and Woychik, 1976). Ultrasound treatment method helped in the fast lactose hydrolysis in fermented milk by Bifidobacterium spp (Nguyen et al., 2009). These changes in β-galactosidase enzyme levels and lactose hydrolysis might have enhanced the growth rate of probiotic bacteria and increasing total acidity during milk fermentation. The different activating agents in fermented milk samples by different strains may be imputed to two agents: (1) the viability of bacteria cells under ultrasonic treatment, and (2) the amount of β-galactosidase enzyme produced from these bacteria after ultrasonic treatment.

WHC percentage values of fermented milk

The changes in the water holding capacity (WHC) percentage of fermented milk are presented in figure 3. The WHC percentage values gradually increased after 10 minutes of ultrasound treatment for all probiotic strains, but WHC percentage values of fermented milk decreased after 15 and 20 minutes of ultrasound treatment. The whey separation or lower water holding capacity is partly because of the uneasy gel network of fermented milk. The colloidal linkage of protein molecules is weak and it can not bind water inside a three-dimensional network (Yang and Li, 2010; Niamah et al., 2016). The ultrasound treatment was effected on a three-dimensional network of fermented milk. Vercet et al. (2002) reported milk treatment by manothermosonication process used for the production of yoghurt. This process improved yoghurt texture by breaking the fatty granules and increasing the water holding capacity.

Effect of ultrasonic treatment on fermentation time

The results show a close correlation between the percentage of viability of probiotic bacteria after ultrasound treatment and the decrease of fermentation time, as shown in Table 2. Significant differences were observed at $P \leq 0.05$ between the fermentation time of the control sample (without ultrasound treatment) and the different time periods of ultrasound treatment for all probiotic strains. The data show that a longer period of ultrasound treatment leads to a lower viability percentage of probiotic bacteria. This may be expounded by the reality that when ultrasonic treatment time is increased, more cavitation bubbles were produced and the breakdown of these bubbles imparted chemical and physical effects which break down bacterial cell walls. Therefore, the fermented milk, which was exposed to a longer ultrasonic treatment time, has a lower viability percentage of bacteria (Racioppo et al., 2017; Kassem et al., 2018).

An increase in the initial probiotic bacteria starter (vol-

Table 2: The fermentation time of fermented milk samples by different starter volumes of probiotic strains after ultrasound treatment. The results are expressed as mean values with standard deviations (SD), n = 3 for all treatments

Starter volume (10^8CFU/mL)	Bacterial strains	Ultrasound treatment time (min)				
		0	5	10	15	20
1%	LA-5	$15.0^{c}\pm0.3$	$14.0^{c}\pm0.5$	$13.5^{ab}\pm0.8$	$18.0^{b}\pm0.5$	$21.5^{a}\pm0.1$
	LC	$16.0^{c}\pm0.2$	$15.5^{c}\pm0.6$	$15.0^{c}\pm0.3$	$19.0^{b}\pm0.4$	$22.0^{a}\pm0.5$
	LR-MM53	$14.0^{b}\pm0.1$	$13.5^{b}\pm0.1$	$13.0^{b}\pm0.5$	$16.0^{a}\pm0.5$	$18.0^{a}\pm0.3$
	Bb-12	$20.0^{b}\pm0.1$	$20.5^{b}\pm0.3$	$21.0^{a}\pm0.7$	$22.0^{a}\pm0.4$	$23.0^{a}\pm0.6$
	BB-536	$21.0^{b}\pm0.6$	$22.0^{a}\pm0.8$	$23.0^{a}\pm0.2$	$23.5^{a}\pm0.3$	$24.0^{a}\pm0.1$
3%	LA-5	$14.0^{b}\pm0.5$	$13.5^{c}\pm0.3$	$13.0^{c}\pm0.2$	$16.0^{b}\pm0.2$	$19.0^{a}\pm0.5$
	LC	$15.5^{b}\pm0.3$	$15.0^{b}\pm0.6$	$14.0^{c}\pm0.9$	$17.0^{b}\pm0.3$	$20.0^{a}\pm0.0$
	LR-MM53	$13.0^{b}\pm0.2$	$12.5^{c}\pm0.4$	$12.0^{c}\pm0.5$	$15.0^{b}\pm0.3$	$19.0^{a}\pm0.6$
	Bb-12	$18.0^{b}\pm0.1$	$18.5^{b}\pm0.6$	$19.0^{b}\pm0.4$	$20.0^{a}\pm0.8$	$22.0^{a}\pm0.1$
	BB-536	$19.0^{b}\pm0.1$	$20.0^{b}\pm0.5$	$21.0^{a}\pm0.2$	$23.0^{a}\pm0.1$	$23.5^{a}\pm0.5$
5%	LA-5	$12.5^{c}\pm0.6$	$12.0^{c}\pm0.7$	$11.0^{cb}\pm0.5$	$15.0^{b}\pm0.4$	$18.5^{a}\pm0.2$
	LC	$14.0^{c}\pm0.5$	$13.0^{c}\pm0.2$	$12.5^{c}\pm0.3$	$16.0^{a}\pm0.0$	$18.0^{a}\pm0.5$
	LR-MM53	$12.0^{c}\pm0.9$	$12.5^{c}\pm0.2$	$11.0^{c}\pm0.1$	$13.0^{b}\pm0.4$	$16.0^{a}\pm0.1$
	Bb-12	$16.0^{c}\pm0.1$	$16.5^{c}\pm0.1$	$17.0^{b}\pm0.1$	$19.0^{b}\pm0.6$	$21.5^{a}\pm0.8$
	BB-536	$17.0^{b}\pm0.7$	$18.0^{b}\pm0.6$	$20.0^{a}\pm0.3$	$21.5^{a}\pm0.3$	$22.0^{a}\pm0.2$

ume 5 percent compared with volume 1 percent) led to a greater decrease in fermentation time of fermented milk production because of increasing numbers of bacteria (Table 2). This result agrees with that found by Ojha et al. (2017). This may be expounded by the certainty that in milk fermentation, production of lactic acid is mostly executed by viable cells of lactic acid bacteria. This occurs because the increasing cell number of this bacteria results in a speedy acidification (lactic acid production) when the fermentation process ends. Low frequency ultrasound power was able to catalyse the fermentative activities of probiotic bacteria such as *Lactobacillus* spp. and *Bifidobacterim* spp. in milk. Ultrasound treatment over different time periods caused different effects on lactose metabolism by probiotic bacteria. The fermentation time changes and β-galactosidase enzyme production depend on the survival percentage of probiotic strains in fermented milk samples after ultrasound treatment.

Conclusions

Ultrasound treatment is one of the most promising modern technologies that can be applied in food industries such as dairy production. It is inexpensive compared with other techniques. Low-frequency ultrasound treatment increases the viability of bacteria starters such as lactic acid bacteria and probiotic bacteria. The results of the current study show that increased exposure time of ultrasound treatment leads to decreased viability of probiotic bacteria strains and increased β-galactosidase activity. The best exposure time of ultrasound treatment was 10 minutes. It improved some chemical properties of fermented milk by probiotic bacteria.

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Conflict of Interests

The author hereby declares that there are no conflicts of interest.

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