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Effect of Ultrasound on the Growth of Probiotics and Bioconversion of Isoflavones in Prebiotic-Supplemented Soymilk

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The objective of the present study was to evaluate the effects of ultrasound on the growth of probiotics and bioconversion of isoflavones in prebiotic-soymilk. Previous studies have shown that ultrasound elevated microbial enzymatic activity and growth by altering cellular membranes. The growth of probiotics was significantly decreased ($P < 0.05$) immediately after ultrasound treatment, attributed to membrane permeabilization, cell lysis, and membrane lipid peroxidation upon ultrasound treatment. The ultrasound treatment also caused alteration at the acyl chain, polar head, and interface region of the probiotic membrane phospholipid bilayers. The cells treated with ultrasound showed recovery from injury with subsequent increase in growth upon fermentation in soymilk ($P < 0.05$). Ultrasound treatment at 100 W for 2 and 3 min also enhanced ($P < 0.05$) the intracellular and extracellular β-glucosidase activity of probiotics, leading to increased ($P < 0.05$) bioconversion of glucosides to aglycones in the prebiotic-soymilk. Our present study illustrated that ultrasound treatment could produce bioactive synbiotic-soymilk with increased concentrations of bioactive aglycones.

KEYWORDS: Ultrasound; probiotics; soymilk; isoflavones; synbiotics

INTRODUCTION

Probiotics are defined as "live microorganisms" that "when administered in adequate amount confer a health benefit on the host" (*1*). Products containing probiotics have gained much attention in recent years attributed to their well documented health-promoting effects such as activation of the immune system, suppression of traveler's diarrhea, regulation of microbial balance in the gut, and antihypertensive effect.

Soybean (*Glycine max*) is well known in a healthy diet as it contains appreciable amounts of essential amino acids and low quantities of saturated fat. The consumption of soy-based foods has increased tremendously due to epidemiological and clinical evidence which suggests the health beneficial effects of soy. Past studies have revealed that soy-based products are effective in the prevention of postmenopausal symptoms, cardiovascular disease, bone health problems, and breast, prostate, and colon cancers (*2*). Clinical data have attributed these health effects of soy to isoflavones that occur naturally in soy.

Isoflavones are a group of phytoestrogens which are structurally similar to the human estrodiol and exist in soybean as aglycones and glucosides, malonyl- and acetyl-glucosides. Setchell et al. (*3*) demonstrated that aglycones are absorbed more efficiently than glucoside conjugates in humans. However, the existing forms of isoflavones in unfermented soybean present predominantly as glucosides which are less bioactive and nonbioavailable. We have previously demonstrated that β-glucosidase producing probiotics could biotransform the isoflavone glucosides to biologically potent aglycones (*4*).

The transformation of isoflavone glucosides to aglycones by probiotics is often hindered by the cellular membrane. This is mainly due to the permeability of the cellular membrane which retards the efficient transfer of isoflavones and β-glucosidase across the membrane. In addition, the semipermeable cellular membrane could also act as a barrier for the secretion of transformed products out of the cellular system for an easy recovery (*5*). Therefore, permeabilization of the cellular membrane is essential to enhance the biotransformation of isoflavones and the recovery of aglycones in soymilk.

Ultrasound technology has attracted much interest in various applications including biotechnological processes involving live cells. It has been reported that ultrasound treatment stimulated acid production by starter culture and reduced the fermentation time in yogurt without inactivating the starter culture (*6*). The viability and β-galactosidase activity of *Lactobacillus* and *Bifidobacterium* in milk was also increased upon ultrasonication, leading to increased lactose hydrolysis in milk (*7*). In another study, ultrasound treatment followed by static incubation was reported to increase the growth of *Brevibacterium* sp. and the production of cholesterol oxidase (*8*). All of these were attributed to the transient alteration of cell membrane permeability upon ultrasound treatment which promoted the mass transfer of substrates and the secretion of metabolites, indicating that ultrasound treatment is a useful tool to elevate the enzymatic activity of whole cells; however, to our knowledge no attempt has been made to utilize such treatments to improve the β-glucosidase activity of probiotics and bioconversion of isoflavones in soymilk.

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Our previous studies have demonstrated that supplementation of prebiotics significantly enhanced the growth and bioactive properties of probiotics in soymilk (*4*, *9*). Among these prebiotics, mannitol illustrated the strongest effect in enhancing the growth characteristics of probiotics and the bioconversion of isoflavones in soymilk. Therefore, mannitol was selected to be supplemented into the soymilk in our current study. Mannitol, a sugar alcohol has been classified as a prebiotic due to its indigestible properties (*10*). They are less likely to be absorbed by humans due to their additional hydroxyl group compared to other sugars and thus escape undigested into the large intestine (*11*). Mannitol could also alter the large intestinal microbiota and produce short chain fatty acids in the large intestine (*12*). The addition of this prebiotic into the probiotic fermented soymilk could be termed synbiotics, a mixture that contains both probiotics and prebiotics which interact to provide a synergistic effect for the maintenance of intestinal microbiota.

The aim of our present study was to evaluate the effect of ultrasound treatment on the membrane properties of probiotics and the bioconversion of isoflavones in soymilk supplemented with a prebiotic (mannitol). In our present study, four strains of probiotics, namely, *Bifidobacterium* FTDC 8943, *B. longum* FTDC 8643, *Lactobacillus* sp. FTDC 2113, and *L. casei* ATCC 393 were incorporated into soymilk supplemented with mannitol. Our previous study showed that these strains demonstrated the highest production of bioactive aglycones in fermented mannitol-soymilk (*4*). Membrane properties of probiotics such as membrane permeability, fluidity, and lipid peroxidation as well as cell characteristics such as viability, β-glucosidase activity, and concentration of isoflavones in fermented prebiotic-soymilk were also determined.

MATERIALS AND METHODS

Bacterial Cultures. Pure cultures of *Bifidobacterium* FTDC 8943, *B. longum* FTDC 8643, *Lactobacillus* sp. FTDC 2113, and *L. casei* ATCC 393 were obtained from the Culture Collection Centre of Universiti Sains Malaysia (USM). Stock cultures were stored at −20 °C in sterile 40% (v/v) glycerol. The microorganisms were grown successively three times in rehydrated de Mann Rogosa Sharpe (MRS) broth (Hi media, Mumbai, India) supplemented with 0.15% (w/v) L-cysteine·HCl (Hi media, Mumbai, India) at 37 °C for 24 h prior to use.

Preparation of Soymilk. The soymilk was prepared as described previously (*9*). Briefly, whole soybeans were washed and soaked overnight in distilled water prior to blending with distilled water at a ratio of 1:3 (w/v). The resultant slurry was filtered to yield soymilk and was pasteurized at 63 °C for 30 min. Upon cooling to 40 °C, soymilk was then supplemented with 0.15% (w/v) filter-sterilized L-cysteine·HCl and 1% (w/v) of mannitol (Mannogem, SPI Polyols Inc., New Castle, USA).

Ultrasound Treatment. Activated cell cultures (5% v/v) were suspended into phosphate buffer (50 mM, pH 6.5) and sonicated at a frequency of 30 kHz (LABSONICM, Sartorius Stedim Biotech, Goettingen, Germany). The intensities of treatment were adjusted to 20 W, 60 W, or 100 W with continuous ultrasound action. In all reactions, the tip of the sonotrode (titanium probe 3 mm × 80 mm) was located at 1 cm depth of the 10 mL liquid medium and sonicated at 25 °C. The sonication period varied at 1, 2, and 3 min. The treated samples were then added into the soymilk and fermented at 37 °C for 24 h. Soymilk with untreated cell cultures was used as the control.

Cell Growth. The growth of probiotic cultures was determined via the pour plate method using MRS agar supplemented with 0.15% (w/v) L-cysteine·HCl. Plates were incubated anaerobically at 37 °C for 24 h in an anaerobic jar with gas generating kits (Merck, Darmstadt, Germany).

Determination of Intracellular β-Glucosidase Activity. Crude enzyme extract for β-glucosidase activity was prepared and determined as previously described (*4*). Briefly, cells were collected upon fermentation and suspended in cold sodium citrate buffer (50 mM, pH 5.5) containing sterile glass beads of 0.25- to 0.30-mm diameter (Sigma, Steinheim, Germany). The cell suspension was sonicated in an ice bath for 15 min to release the cellular extract for the determination of intracellular β-glucosidase activity. The β-glucosidase activity was determined on the basis of the rate of hydrolysis of ρ-nitrophenyl β-D-glucopyranoside (ρNPG) (Sigma Chemical Co., Missouri, USA). The amount of ρ-nitrophenol released was measured spectrometrically at 420 nm. One unit of enzyme activity is defined as the amount of β-glucosidase activity that released 1 mM ρ-nitrophenol from the substrate ρNPG per milliliter per minute. The protein concentration of the crude enzyme extract was quantified using the methods of Bradford (*13*). Specific activity was expressed as unit (U) of β-glucosidase activity per mg of protein.

Determination of Extracellular β-Glucosidase Activity. β-Glucosidase activity of the control and ultrasound-treated cells was determined as previously described (*4*). Briefly, fermented soymilk was centrifuged at 14000*g* for 30 min at 4 °C, and the supernatant was analyzed for β-glucosidase activity. The β-glucosidase activity was determined as mentioned above.

Determination of Isoflavones. Extractions and determination of isoflavones were performed as previously described (*4*). Briefly, 1 mL of samples was added to 10 mL of 80% (v/v) methanol and 1 mL of 100% (v/v) acetonitrile. Subsequently, 100 μL each of Carrez I (150 g/L potassium hexacyanoferrate (II)-3-hydrate) and Carrez II (300 g/L zinc sulfate-7-hydrate) were added to the samples, vortexed, and held in a water bath (50 °C) for 120 min. The samples were filtered (0.2 μm) and analyzed for concentrations of isoflavones using high performance liquid chromatography (HPLC). The HPLC system was equipped with a UV/vis detector (Jasco 875-UV, Tokyo, Japan) set at 259 nm. An Inertsil ODS-3 column (150 × 3 mm, 5 μm, GL Sciences, Tokyo, Japan) was maintained at 40 °C. The degassed mobile phase consisted of solvent A (water/phosphoric acid, 1000:1, v/v) and solvent B (water/acetonitrile/phosphoric acid, 200:800: 1, v/v/v) and was used at a flow rate of 1 mL/min. The gradient was as follows: solvent A 100% (2 min) → 65% (29 min) → 50% (31 min) → 100% (45 min) → 100% (50 min). HPLC grade genistein, glycitin, daidzin, glycitin (LC Laboratories, Massachusetts, USA), glycitein, daidzein (Sigma Chemical Co., Missouri, USA), malonyl daidzin, acetyl daidzin, malonyl glycitin, acetyl glycitin, malonyl genistin, and acetyl genistin (Wako Chemicals, Osaka, Japan) were used as standards.

Membrane Permeabilization. The determination of the permeabilized cell was performed according to the method of Tryfona and Bustard (*14*) with some modifications. Briefly, one milliliter of sample was added with 60 μL of propidium iodide (Sigma Chemical Co., Missouri, USA; 1 mg in 50 mL of phosphate buffered saline, PBS, 100 mM, pH 7) and mixed thoroughly. The mixture was then incubated at 25 °C in the dark for 15 min and centrifuged at 3500*g* and 4 °C for 10 min. Subsequently, the labeled cell pellet was resuspended into 3 mL of sterile PBS and measured using a fluorescence spectrophotometer (Cary eclipse, Varian, California, USA). The fluorescence emission was determined at an excitation wavelength of 538 nm and an emission wavelength of 617 nm. The percentage of permeabilized cells was then calculated as:

$$\%\text{reversible permeabilized cells} = (A - B)/A \times 100\%$$

where A is the fluorescence emission of labeled cells immediately after treatment; and B is the fluorescence emission of labeled cells 30 min following treatment.

Membrane Lipid Peroxidation. The lipid peroxidation of probiotics cells was determined according to the method of Giamarellos-Bourboulis et al. (*15*). The lipid peroxidation reaction was estimated on the basis of the detection of malondialdehyde (MDA) using the thiobarbiturate assay. Briefly, a 0.6 mL aliquot of the sample was mixed with trichloroacetic acid 20% (w/v) at a ratio of 1:1 (v:v). The mixtures were centrifuged (12000*g*, 4 °C, 10 min), and 1 mL of the supernatant was incubated with 1 mL of PBS (100 mM, pH 7.0) and 1 mL of thiobarbituric acid 0.6% (w/v) (Merck, Darmstadt, Germany) at 90 °C for 20 min. Upon incubation, the concentration of malondialdehyde was determined spectrometrically at 535 nm on the basis of a standard curve created with 1,1,3,3-tetramethoxypropane (Merck, Darmstadt, Germany).

Fluorescence Anisotropy. The membrane lipid order of probiotic cells upon treatment was determined via fluorescence anisotropy (FAn) according to the method of Lye et al. (*16*). Fluorescence probes used include 1,6-diphenyl-1,3,5-hexatriene (DPH; Sigma-Aldrich), 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene (TMA-DPH; Sigma-Aldrich), and 8-anilino-1-napthalenesulfonic acid (ANS; Sigma-Aldrich). DPH and

Table 1. Growth of Control and Ultrasound-Treated Probiotic Strains in Mannitol-Soymilk Immediately after Treatment[a]

	viability ($\log_{10}$ CFU/mL)			statistical significances of effect: *P*		
	time (min)					
intensity (W)	1	2	3	*T*	*I*	*T* × *I*
Lactobacillus sp. FTDC 2113	control: 6.76 ± 0.64			0.003	<0.001	<0.001
20	6.61 ± 0.09	6.22 ± 0.01	6.57 ± 0.09			
60	6.72 ± 0.17	6.79 ± 0.28	6.68 ± 0.17			
100	6.26 ± 0.03	6.68 ± 0.52	6.53 ± 0.37			
L.casei ATCC 393	control = 5.41 ± 0.85			<0.001	<0.001	0.007
20	6.51 ± 0.45	6.54 ± 0.35	6.15 ± 0.08			
60	6.26 ± 0.19	6.29 ± 0.34	6.07 ± 0.62			
100	6.62 ± 0.77	6.96 ± 0.44	6.87 ± 0.33			
Bifidobacterium sp. FTDC 8943	control = 6.33 ± 0.11			0.004	0.316	0.050
20	5.66 ± 0.06	5.70 ± 0.33	5.95 ± 0.07			
60	5.72 ± 0.03	6.48 ± 0.39	5.89 ± 0.29			
100	5.76 ± 0.23	5.81 ± 0.19	5.94 ± 0.05			
B.longum FTDC 8643	control = 7.52 ± 0.45			0.014	<0.001	0.067
20	7.21 ± 0.68	7.11 ± 0.07	6.77 ± 0.41			
60	6.43 ± 0.78	6.52 ± 0.45	6.63 ± 0.43			
100	6.36 ± 0.46	6.29 ± 0.37	6.16 ± 0.42			

[a] Results are expressed as the mean ± standard deviation of the mean. Values are the mean of duplicates from three separate runs ($n = 3$). *T*, effect of sonication time; *I*, effect of sonication intensity; *T* × *I*, interaction between sonication time and intensity.

TMA-DPH were dissolved in tetrahydrofuran (Lab-Scan, Dublin, Ireland) to a final concentration of 2 μM and ANS in ethanol to a final concentration of 6 μM. The probe stock solutions were diluted (1:1000) in 0.155 M NaCl with vigorous mixing to produce working solutions. Cell suspensions (5% v/v) in phosphate buffer (50 mM, pH 6.5) with an optical density of 0.3 were added into working solutions at a ratio of 1:3 (v/v) and incubated at 37 °C. The incubation time for DPH and TMA-DPH was 60 min, while that for ANS was 90 min. Fluorescence anisotropy was measured using a fluorescence spectrophotometer. The excitation wavelength for DPH and TMA-DPH was 365 nm, while that for ANS was 390 nm. Emission was determined at 445 nm for DPH and TMA-DPH, and 490 nm for ANS. An unlabeled cellular membrane was used as a blank. Fluorescence anisotropy was calculated according to the equation below:

$$Ar = (I_{vv} - GI_{vh})/(I_{vv} + 2GIvh)$$

where I_{vv} and I_{vh} are the fluoroscence intensities obtained from a vertical polarizer and a vertical and horizontal analyzer, respectively, while G is the instrumental grating factor. $G = I_{hv}/I_{vh}$, where I_{hv} is the intensity measured from a horizontal polarizer and a vertical analyzer.

Scanning Electron Microscopy. The morphology of probiotic cells prior to and after treatment was observed using scanning electron microscopy (SEM). A cell pellet was fixed for 2 h with phosphate buffer (0.1 M, pH 7.2) containing McDowell Trump fixative (4% (v/v) formaldehyde and 1% (v/v) glutaraldehyde). The samples were then centrifuged, and the cell pellet was washed twice with phosphate buffer (0.1 M, pH 7.2) prior to resuspending in phosphate buffer containing 1% (w/v) osmium tetroxide (Sigma−Aldrich, Bellefonte, PA, USA) for 1 h. Subsequently, the cell pellet was washed twice with distilled water followed by a series of ethanol washes (50% ethanol 10 min, 75% ethanol 10 min, 90% ethanol 10 min, and 100% ethanol 10 min). The washed cell pellet was dehydrated with hexamethyldisilazane (HMDS, Supelco, Bellefonte, PA, USA) for 15 min. The cell pellet was air-dried at 25 °C for 48 h. The dried cells were then mounted onto a SEM specimen stub, coated with gold in a Sputter coater (Polaran, Walford, UK), and examined under a scanning electron microscope (Leo Supra, Carl Zeiss, Oberkochen, Germany).

Statistical Analysis. Data were analyzed statistically with SPSS Inc. software (version 15.0) (SPSS Inc., Chicago, Illinois, USA). Univariate was employed to analyze the statistical difference between sample means. The statistical level of significance was preset at $\alpha = 0.05$. The multiple comparisons of means were assessed by Tukey's test. All data presented were mean values of duplicates, obtained from three separate runs, unless stated otherwise.

RESULTS

Viability of Probiotics Immediately after Treatment. The average number of cells initially added into the soymilk was 6.51 $\log_{10}$ CFU/mL (**Table 1**). There was a reduction in the viable counts of probiotics in mannitol-soymilk immediately upon treatment ($P < 0.05$), which was inversely proportional to the intensity and time of the treatment. Our results showed that the treatment at 100 W was more detrimental to the growth of probiotics compared to the control and cells treated with other lower intensities ($P < 0.05$). This was most prevalent in soymilk fermented by *B. longum* FTDC 8643, which showed a lower ($P < 0.05$) viability (1.16−1.36 $\log_{10}$ CFU/mL) than the control immediately after treatment. The viability of cells treated for 2 and 3 min was also extensively decreased ($P < 0.05$) compared to the control.

Viability of Probiotics after Fermentation. The ability of probiotics to regain growth during fermentation is an important indication of the recovery and replication of cells after ultrasound treatment. Our results showed that all probiotic strains treated with ultrasound were able to grow well in mannitol-soymilk fermented at 37 °C for 24 h (**Table 2**) with viability above the therapeutic level (8 $\log_{10}$ CFU/mL). Increasing the treatment intensities to 100 W significantly ($P < 0.05$) enhanced the growth of probiotics in mannitol-soymilk after fermentation at 37 °C for 24 h. This was clearly observed in mannitol-soymilk fermented by *Lactobacillus* sp. FTDC 2113 and *Bifidobacterium* sp. FTDC 8943 with an increased viability of 0.49−0.57 $\log_{10}$ CFU/mL and 0.26−0.57 $\log_{10}$ CFU/mL, respectively, compared to the control ($P < 0.05$). The treatment at 60 W also significantly ($P < 0.05$) promoted the growth of probiotics in mannitol-soymilk upon fermentation, and this was most prevalent in *L. casei* ATCC 393 (0.07−0.57 $\log_{10}$ CFU/mL increased in growth upon treatment). Our results also showed that increasing the treatment duration effectively increased ($P < 0.05$) the growth of probiotics. *Lactobacillus* sp. FTDC 2113 treated for 3 min at all intensities showed the highest ($P < 0.05$) growth compared to the control and all other strains studied.

Intracellular β-Glucosidase. Ultrasound treatment significantly ($P < 0.05$) elevated the intracellular β-glucosidase specific activity of probiotics compared to that of the control (**Table 3**). Treatment at 100 W exerted the highest ($P < 0.05$) effect in promoting the production of intracellular β-glucosidase of

Table 2. Growth of Control and Ultrasound-Treated Probiotic Strains in Mannitol-Soymilk after Fermentation at 37 °C for 24 h[a]

	viability ($\log_{10}$ CFU/mL)			statistical significances of effect: *P*		
	time (min)					
intensity (W)	1	2	3	T	I	T x I
Lactobacillus sp. FTDC 2113	control: 8.40 ± 0.17			0.029	0.010	0.452
20	8.57 ± 0.23	8.32 ± 0.08	8.87 ± 0.15			
60	8.65 ± 0.38	8.77 ± 0.07	8.86 ± 0.22			
100	8.97 ± 0.04	8.90 ± 0.49	8.89 ± 0.60			
L.casei ATCC 393	control = 8.26 ± 0.11			0.001	0.034	0.452
20	8.28 ± 0.23	8.67 ± 0.37	8.48 ± 0.01			
60	8.64 ± 0.17	8.74 ± 0.30	8.33 ± 0.60			
100	8.43 ± 0.03	8.80 ± 0.29	8.33 ± 0.77			
Bifidobacterium sp. FTDC 8943	control = 8.49 ± 0.51			0.021	0.044	0.329
20	8.60 ± 0.60	9.44 ± 0.33	8.43 ± 0.54			
60	8.86 ± 0.45	9.08 ± 0.43	7.74 ± 0.60			
100	9.03 ± 0.67	9.06 ± 0.48	8.70 ± 0.71			
B.longum FTDC 8643	control = 8.44 ± 0.48			0.210	0.141	0.008
20	8.57 ± 0.16	8.50 ± 0.16	8.43 ± 0.33			
60	8.10 ± 0.57	8.93 ± 0.23	9.24 ± 0.13			
100	9.18 ± 0.25	8.38 ± 0.29	8.83 ± 0.45			

[a] Initial inoculum of *Lactobacillus* sp. FTDC 2113: 6.76 ± 0.64 $\log_{10}$ CFU/mL; *L. casei* ATCC 393: 5.41 ± 0.85 $\log_{10}$ CFU/mL. *Bifidobacterium* sp. FTDC 8943: 6.33 ± 0.11 $\log_{10}$ CFU/mL. *B. longum* FTDC 8643: 7.52 ± 0.45 $\log_{10}$ CFU/mL. Results are expressed as the mean ± standard deviation of the mean. Values are the mean of duplicates from three separate runs ($n = 3$). *T*, effect of sonication time; *I*, effect of sonication intensity; $T \times I$, interaction between sonication time and intensity.

Table 3. Intracellular β-Glucosidase Specific Activity of Control and Ultrasound-Treated Probiotic Strains in Mannitol-Soymilk after Fermentation at 37 °C for 24 h[a]

	intracellular β-glucosidase activity (U/mg protein)			statistical significances of effect: *P*		
	time					
intensity (W)	1	2	3	*T*	*I*	$T \times I$
Lactobacillus sp. FTDC 2113	control = 0.080 ± 0.001			0.006	0.015	<0.001
20	0.082 ± 0.002	0.093 ± 0.006	0.075 ± 0.005			
60	0.081 ± 0.006	0.089 ± 0.004	0.080 ± 0.002			
100	0.077 ± 0.003	0.086 ± 0.005	0.093 ± 0.008			
L. casei ATCC 393	control = 0.069 ± 0.003			<0.001	0.001	0.123
20	0.077 ± 0.007	0.088 ± 0.016	0.071 ± 0.002			
60	0.083 ± 0.003	0.089 ± 0.017	0.080 ± 0.005			
100	0.079 ± 0.007	0.077 ± 0.003	0.071 ± 0.001			
Bifidobacterium sp. FTDC 8943	control = 0.055 ± 0.001			<0.001	<0.001	0.001
20	0.063 ± 0.004	0.073 ± 0.001	0.067 ± 0.009			
60	0.067 ± 0.009	0.066 ± 0.003	0.065 ± 0.002			
100	0.080 ± 0.004	0.073 ± 0.009	0.079 ± 0.008			
B.longum FTDC 8643	control = 0.056 ± 0.001			<0.001	<0.001	<0.001
20	0.061 ± 0.001	0.068 ± 0.004	0.061 ± 0.003			
60	0.061 ± 0.003	0.069 ± 0.005	0.065 ± 0.008			
100	0.058 ± 0.000	0.075 ± 0.005	0.068 ± 0.006			

[a] Results are expressed as the mean ± standard deviation of the mean. Values are the mean of duplicates from three separate runs ($n = 3$). *T*, effect of sonication time; *I*, effect of sonication intensity; $T \times I$, interaction between sonication time and intensity.

probiotics. The effect was most prevalent in *Bifidobacterium* sp. FTDC 8943 where treatment at 100 W increased ($P < 0.05$) the intracellular β-glucosidase specific activity of this strain by 32.7−45.5% compared to that of the control. The intracellular β-glucosidase specific activity of probiotics was highest ($P < 0.05$) when treated for 2 min, and the specific activity increased by 7.5−33.9% compared to that of the control upon treatment for 2 min.

Extracellular β-Glucosidase. Extracellular β-glucosidase activity of probiotics is an important attribute for the easy recovery of bioactive aglycones in soymilk. All strains exhibited varying levels of extracellular β-glucosidase specific activity of probiotics ranging from 1.92 to 4.94 U/mg protein (**Table 4**). The specific activity of extracellular β-glucosidase was increased ($P < 0.05$) upon treatment with ultrasound. Increasing the treatment intensities to 100 W increased ($P < 0.05$) the extracellular β-glucosidase specific activity of probiotics in mannitol-soymilk by 6.25−60.9% compared to that of the control. The duration of ultrasound treatment also significantly ($P < 0.05$) affected the level of extracellular β-glucosidase specific activity. Treating the cells for 1 min showed little effect on the extracellular β-glucosidase specific activity of probiotics. However, increasing the treatment duration to 2 min significantly ($P < 0.05$) increased the excretion of β-glucosidase by probiotics in mannitol-soymilk (20.3−85.4% higher than the control). Further increasing the treatment duration to 3 min also significantly ($P < 0.05$) increased the release of β-glucosidase in mannitol-soymilk. This was most prevalent in soymilk fermented by *L. casei* ATCC 393 and *B. longum* FTDC 8643 where the extracellular β-glucosidase specific activity increased ($P < 0.05$) by 21.6−53.7% and 24.0−64.1%, respectively, compared to that of the control.

Concentration Isoflavones Glucosides. Acetyl daidzin and acetyl genistin were not detected in soymilk prior to and after fermentation

Table 4. Extracellular β-Glucosidase Specific Activity of Control and Ultrasound-Treated Probiotic Strains in Mannitol-Soymilk after Fermentation at 37 °C for 24 h[a]

	extracellular β-glucosidase activity (U/mg protein)			statistical significances of effect: P		
	time					
intensity (W)	1	2	3	T	I	$T \times I$
Lactobacillus sp. FTDC 2113	control = 4.94 ± 0.81			<0.001	<0.001	<0.001
20	5.61 ± 0.52	9.16 ± 0.10	7.89 ± 0.06			
60	7.28 ± 0.35	5.98 ± 0.58	6.51 ± 0.35			
100	7.91 ± 0.80	7.95 ± 0.38	7.06 ± 0.59			
L.casei ATCC 393	control = 4.67 ± 0.21			<0.001	<0.001	<0.001
20	4.43 ± 0.13	6.15 ± 0.75	5.68 ± 0.53			
60	5.52 ± 0.16	6.47 ± 0.16	6.65 ± 0.53			
100	6.31 ± 0.26	6.58 ± 0.09	7.18 ± 0.19			
Bifidobacterium sp. FTDC 8943	control = 2.25 ± 0.10			<0.001	<0.001	0.343
20	2.43 ± 0.17	3.03 ± 0.58	2.61 ± 0.35			
60	2.89 ± 0.12	3.27 ± 0.73	2.93 ± 0.09			
100	3.04 ± 0.74	3.19 ± 0.72	3.08 ± 0.79			
B.longum FTDC 8643	control = 1.92 ± 0.42			0.001	0.004	0.082
20	2.01 ± 0.30	2.56 ± 0.05	3.15 ± 0.58			
60	2.04 ± 0.25	2.28 ± 0.41	2.29 ± 0.22			
100	2.04 ± 0.05	2.31 ± 0.05	2.38 ± 0.34			

[a] Results are expressed as the mean ± standard deviation of mean. Values are the mean of duplicates from three separate runs ($n = 3$). T, effect of sonication time; I, effect of sonication intensity; $T \times I$, interaction between sonication time and intensity.

at 37 °C for 24 h (**Table 5**). The concentration of acetyl glycitin in mannitol-soymilk fermented by control probiotics ranged from 0.10 to 0.15 μg/mL. Acetyl glycitin was completely hydrolyzed by probiotics upon treatment with ultrasound where it was no longer detected in mannitol-soymilk fermented by treated probiotics.

Probiotics were able to hydrolyze β-glucosides including daidzin, glycitin, and genistin in mannitol-soymilk and the hydrolysis of β-glucosides especially genistin was increased ($P < 0.05$) upon treatment with ultrasound. The concentration of genistin in soymilk fermented by probiotics treated at 100 W were 10.3−56.4% lower ($P < 0.05$) than that of the control. Increasing the treatment duration to 3 min significantly ($P < 0.05$) promoted the hydrolysis of genistin by probiotics in mannitol-soymilk. This was clearly observed in mannitol-soymilk fermented by *Lactobacillus* sp. FTDC 2113 which showed a decrease ($P < 0.05$) in the concentration of genistin by 23.9−56.4% compared to that of the control upon ultrasound treatment for 3 min.

In addition to β-glucosides, soymilk also contains a considerable amount of malonyl glucosides. All types of malonyl glucosides including malonyl daidzin, malonyl glycitin, and malonyl genistin were hydrolyzed in mannitol-soymilk upon fermentation at 37 °C for 24 h. The hydrolysis of malonyl glucosides by probiotics increased ($P < 0.05$) upon treatment with ultrasound. The hydrolysis of malonyl glucosides by probiotics in mannitol-soymilk was the highest ($P < 0.05$) upon treatment at 100 W, particularly in soymilk fermented by *Lactobacillus* sp. FTDC 2113. Upon treatment at 100 W, the concentration of malonyl daidzin, malonyl glycitin, and malonyl genistin in mannitol-soymilk fermented by *Lactobacillus* sp. FTDC 2113 were 38.4−50.9%, 17.6−26.5%, and 46.7−63.3% lower ($P < 0.05$), respectively, than the control. Increasing the treatment duration to 3 min at any intensity significantly ($P < 0.05$) enhanced the hydrolysis of malonyl glucosides by probiotics in mannitol-soymilk.

Concentrations of Isoflavone Aglycones. The concentration of aglycones including daidzein, glycitein, and genistein in mannitol-soymilk significantly ($P < 0.05$) increased upon treatment with ultrasound (**Table 6**). The treatment at all intensities and durations studied significantly ($P < 0.05$) increased the production of daidzein by probiotics in fermented mannitol-soymilk. Among the intensities studied, treatment at 100 W showed the highest ($P < 0.05$) effect in promoting the production of daidzein in mannitol-soymilk fermented by probiotics. The effect was clearly observed in mannitol-soymilk fermented by *Lactobacillus* sp. FTDC 2113, *Bifidobacterium* sp. FTDC 8943, and *B. longum* FTDC 8643. Upon treatment at 100 W, the concentration of daidzein in probiotics-fermented mannitol-soymilk was 4.3−69.9% higher ($P < 0.05$) than that of the control. The production of daidzein by probiotics in mannitol-soymilk improved with increased treatment time where 2 and 3 min showed the highest ($P < 0.05$) effect.

The effect of ultrasound on the concentrations of glycitein and genistein showed a trend similar to that of daidzein, where the treatment at 100 W and 3 min showed the highest ($P < 0.05$) conversion to aglycones. The increase in concentration of glycitein was most prevalent in soymilk fermented by *Bifidobacterium* sp. FTDC 8943, which was more than 8.1% higher ($P < 0.05$) than that of the control upon treatment at 100 W. Increasing the treatment time to 3 min significantly increased ($P < 0.05$) the concentration of glycitein by up to 44.2% compared to that of the control. A similar treatment (100 W, 3 min) also significantly increased ($P < 0.05$) the production of genistein by as high as 63.0% compared to that of the control, and this was observed in mannitol-soymilk fermented by *Lactobacillus* sp. FTDC 2113.

Membrane Permeability. The membrane of probiotics was permeabilized upon treatment with ultrasound and was clearly observed in all of the probiotic strains studied (**Table 7**). Increasing the intensity and duration of ultrasound treatment substantially increased ($P < 0.05$) the membrane permeability of probiotics. Ultrasound treatment at 100 W showed the highest ($P < 0.05$) effect on increasing the membrane permeability of probiotics. Increasing the duration of treatment to 2 and 3 min significantly ($P < 0.05$) enhanced the membrane permeability of probiotics.

Lipid Peroxidation. Membrane lipid peroxidation was determined on the basis of the estimation of malonyldehyde (MDA) content. The release of MDA by probiotics increased significantly ($P < 0.05$) upon ultrasound treatment, which was directly proportional to the intensity and time of treatment (**Table 8**). The treatment at 100 W drastically enhanced ($P < 0.05$) the released of MDA by all strains compared to that of the control and other lower intensities. This was clearly observed in *B. longum* FTDC 8643 where the concentration of MDA increased ($P < 0.05$) by 113.3−166.7% upon treatment with 100 W. The concentration of MDA increased ($P < 0.05$) stepwise with increased time of

Table 5. Concentration of Acetyl-, Malonyl-, and β-Glucosidase in Mannitol-Soymilk Fermented by Control and Ultrasound-Treated Probiotics at 37 °C for 24 h[a]

		concentration of glucosides (μg/mL)			statistical significances of effect: *P*		
		time (min)					
glucosides	intensity (W)	1	2	3	*T*	*I*	*T* × *I*
daidzin	*Lactobacillus* sp. FTDC 2113	control = 0.13 ± 0.01			<0.001	<0.001	<0.001
	20	0.09 ± 0.01	0.07 ± 0.00	0.09 ± 0.01			
	60	0.13 ± 0.06	0.11 ± 0.01	0.07 ± 0.00			
	100	0.07 ± 0.00	0.08 ± 0.00	0.09 ± 0.01			
	L.casei ATCC 393	control = 0.12 ± 0.07			0.693	0.274	0.980
	20	0.11 ± 0.06	0.13 ± 0.02	0.14 ± 0.02			
	60	0.12 ± 0.02	0.16 ± 0.04	0.10 ± 0.01			
	100	0.15 ± 0.07	0.15 ± 0.05	0.16 ± 0.06			
	Bifidobacterium sp. FTDC 8943	control = 0.02 ± 0.00			0.038	0.035	0.527
	20	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00			
	60	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00			
	100	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00			
	B.longum FTDC 8643	control = 0.03 ± 0.01			0.912	0.600	0.509
	20	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.00			
	60	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.00			
	100	0.03 ± 0.00	0.02 ± 0.01	0.03 ± 0.01			
glycitin	*Lactobacillus* sp. FTDC 2113	control = 0.02 ± 0.00			0.006	0.018	0.388
	20	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00			
	60	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00			
	100	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00			
	L.casei ATCC 393	control = 0.02 ± 0.00			0.013	0.033	0.604
	20	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00			
	60	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00			
	100	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00			
	Bifidobacterium sp. FTDC 8943	control = 0.01 ± 0.00			0.528	0.379	0.951
	20	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00			
	60	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00			
	100	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00			
	B. longum FTDC 8643	control = 0.02 ± 0.00			<0.001	<0.001	<0.001
	20	0.02 ± 0.00	0.00 ± 0.00	0.00 ± 0.00			
	60	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00			
	100	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00			
genistin	*Lactobacillus* sp. FTDC 2113	control = 2.89 ± 0.41			0.029	0.012	0.674
	20	2.30 ± 0.34	2.11 ± 0.48	2.20 ± 0.11			
	60	2.29 ± 0.26	2.17 ± 0.75	1.50 ± 1.03			
	100	1.46 ± 0.84	1.53 ± 0.95	1.26 ± 0.67			
	L. casei ATCC 393	control = 2.61 ± 0.03			0.012	0.007	0.412
	20	2.52 ± 0.19	2.20 ± 0.12	2.26 ± 0.23			
	60	2.03 ± 0.11	2.07 ± 0.24	2.22 ± 0.14			
	100	2.34 ± 0.17	2.24 ± 0.33	2.22 ± 0.45			
	Bifidobacterium sp. FTDC 8943	control = 2.54 ± 0.29			0.027	0.023	0.741
	20	1.77 ± 0.55	2.14 ± 0.31	1.91 ± 0.40			
	60	1.96 ± 0.51	1.96 ± 0.52	1.55 ± 0.29			
	100	1.72 ± 0.64	1.60 ± 0.46	1.60 ± 0.49			
	B. longum FTDC 8643	control = 2.54 ± 0.06			<0.001	<0.001	0.010
	20	2.76 ± 0.42	2.29 ± 0.27	2.14 ± 0.29			
	60	2.202 ± 0.07	2.03 ± 0.02	1.93 ± 0.03			
	100	2.08 ± 0.12	2.13 ± 0.07	1.58 ± 0.08			
malonyl daidzin	*Lactobacillus* sp. FTDC 2113	control = 2.24 ± 0.09			<0.001	<0.001	0.119
	20	1.63 ± 0.21	1.41 ± 0.11	1.52 ± 0.35			
	60	1.51 ± 0.24	1.46 ± 0.50	1.26 ± 0.40			
	100	1.30 ± 0.18	1.38 ± 0.37	1.10 ± 0.14			
	L. casei ATCC 393	control = 2.48 ± 0.06			0.451	0.018	0.002
	20	2.42 ± 0.17	2.50 ± 0.07	2.58 ± 0.02			
	60	2.35 ± 0.05	2.44 ± 0.11	2.51 ± 0.01			
	100	2.55 ± 0.11	2.34 ± 0.12	2.07 ± 0.01			
	Bifidobacterium sp. FTDC 8943	control = 1.51 ± 0.06			<0.001	<0.001	0.004
	20	1.23 ± 0.04	1.32 ± 0.16	1.19 ± 0.11			
	60	1.23 ± 0.03	1.17 ± 0.03	0.94 ± 0.10			
	100	1.06 ± 0.06	0.96 ± 0.03	0.76 ± 0.24			
	B. longum FTDC 8643	control = 1.46 ± 0.10			<0.001	<0.001	0.036
	20	1.38 ± 0.11	1.35 ± 0.12	1.19 ± 0.11			
	60	1.20 ± 0.08	1.17 ± 0.10	1.12 ± 0.07			
	100	1.16 ± 0.08	1.12 ± 0.05	0.84 ± 0.11			
malonyl glycitin	*Lactobacillus* sp. FTDC 2113	control = 0.34 ± 0.01			<0.001	<0.001	0.154

Table 5. Continued

		concentration of glucosides (μg/mL)			statistical significances of effect: *P*		
		time (min)					
glucosides	intensity (W)	1	2	3	*T*	*I*	*T* × *I*
20	0.28 ± 0.00	0.26 ± 0.00	0.28 ± 0.02				
	60	0.27 ± 0.01	0.27 ± 0.05	0.26 ± 0.04			
	100	0.26 ± 0.02	0.28 ± 0.04	0.25 ± 0.02			
	L. casei ATCC 393	control = 0.38 ± 0.03			0.232	0.231	0.980
	20	0.43 ± 0.08	0.447 ± 0.03	0.45 ± 0.02			
	60	0.41 ± 0.03	0.47 ± 0.76	0.41 ± 0.01			
	100	0.40 ± 0.01	0.43 ± 0.02	0.42 ± 0.03			
	Bifidobacterium sp. FTDC 8943	control = 0.38 ± 0.04			0.036	0.021	0.728
	20	0.34 ± 0.04	0.32 ± 0.03	0.33 ± 0.07			
	60	0.33 ± 0.03	0.33 ± 0.04	0.28 ± 0.03			
	100	0.30 ± 0.01	0.29 ± 0.02	0.27 ± 0.09			
	B. longum FTDC 8643	control = 0.36 ± 0.02			0.003	0.002	0.121
	20	0.38 ± 0.02	0.34 ± 0.00	0.31 ± 0.03			
	60	0.32 ± 0.00	0.31 ± 0.01	0.31 ± 0.01			
	100	0.32 ± 0.02	0.31 ± 0.02	0.28 ± 0.03			
malonyl genistin	*Lactobacillus* sp. FTDC 2113	control = 2.59 ± 0.11			<0.001	<0.001	0.001
	20	1.87 ± 0.04	1.60 ± 0.19	1.70 ± 0.23			
	60	1.76 ± 0.00	1.55 ± 0.42	1.34 ± 0.33			
	100	1.38 ± 0.11	1.36 ± 0.18	0.95 ± 0.17			
	L. casei ATCC 393	control = 7.63 ± 0.06			<0.001	<0.001	<0.001
	20	7.11 ± 0.32	7.57 ± 0.30	7.80 ± 0.16			
	60	7.06 ± 0.11	7.21 ± 0.13	7.40 ± 0.12			
	100	7.30 ± 0.16	6.47 ± 0.19	6.20 ± 0.16			
	Bifidobacterium sp. FTDC 8943	control = 3.05 ± 0.23			<0.001	<0.001	0.008
	20	2.91 ± 0.17	2.81 ± 0.13	2.76 ± 0.27			
	60	2.82 ± 0.11	2.72 ± 0.17	2.20 ± 0.28			
	100	2.41 ± 0.03	2.14 ± 0.11	1.82 ± 0.05			
	B. longum FTDC 8643	control = 2.18 ± 0.31			0.011	0.004	0.393
	20	2.12 ± 0.29	2.09 ± 0.06	1.84 ± 0.24			
	60	1.83 ± 0.06	1.76 ± 0.09	1.68 ± 0.07			
	100	1.76 ± 0.04	1.67 ± 0.02	1.20 ± 0.11			

[a] Initial concentration of daidzin, 0.15 ± 0.11 μg/mL; glycitin, 0.75 ±0.08 μg/mL; genistin, 4.15 ± 0.04 μg/mL; malonyl daidzin, 4.15 ± 0.04 μg/mL; malonyl glycitin, 0.91 ± 0.00; malonyl genistin, 8.69 ± 0.01 μg/mL μg/mL; acetyl glycitin, 0.16 ± 0.01 μg/mL. Acetyl daidzin and acetyl genistin were not detected in mannitol-soymilk prior to and after fermentation. The concentration of acetyl glycitin in mannitol-soymilk fermented by untreated *Lactobacillus* sp. FTDC 2113, 0.23± 0.00 μg/mL; *L. casei* ATCC 393, 0.47 ± 0.02 μg/mL; *Bifidobacterium* sp. FTDC 8943, 0.12± 0.01 μg/mL; *B. longum* FTDC 8643, 0.07 ± 0.00 μg/mL. Acetyl glycitin was not detected in mannitol-soymilk fermented by all treated-probiotics. Results are expressed as the mean ± standard deviation of the mean. Values are the mean of duplicates from three separate runs (n = 3). *T*, effect of sonication time; *I*, effect of sonication intensity; *T* × *I*, interaction between sonication time and intensity.

treatment where probiotics treated for 3 min showed the highest ($P < 0.05$) concentration of MDA (7.5−166.7% higher compared to that of the control).

Fluorescence Anisotropy. The ultrasound treatment significantly ($P < 0.05$) decreased the FAn of ANS for all strains except *L. casei* ATCC 393 (**Table 9**). Among the treatment intensities, 20 W had the strongest effect ($P < 0.05$) on lowering FAn of ANS and was clearly observed in *B. longum* FTDC 8643 and *Bifidobacterium* sp. FTDC 8943. The FAn of ANS also significantly ($P < 0.05$) decreased upon treatment for longer duration. This was most prevalent in *B. longum* FTDC 8643 where 2 and 3 min of treatment with ultrasound decreased ($P < 0.05$) the FAn values by 8.0−34.0% compared to that of the control.

The effect of ultrasound on FAn of TMA-DPH was highly strain dependent and was most prevalent in *L. casei* ATCC 393 ($P < 0.05$; **Table 9**). FAn of TMA-DPH for *L. casei* ATCC 393 was substantially enhanced ($P < 0.05$) by ultrasound treatment and was the highest upon treatment at 100 W for 3 min, where FAn of TMA-DPH for *L. casei* ATCC 393 was 80.8% higher compared to that of the control ($P < 0.05$).

The effect of ultrasound on FAn of DPH was also strain dependent where the effect was more prevalent in lactobacilli strains than in bifidobacteria strains (**Table 9**). In both lactobacilli strains, the ultrasound treatment significantly ($P < 0.05$) increased the FAn of DPH. The FAn values for probiotics were the highest ($P < 0.05$) upon treatment with ultrasound at 100 W and were the most prevalent for *Lactobacillus* sp. FTDC 2113 and *L. casei* ATCC 393 where FAn values were increased by more than 48.6% compared to those of the control ($P < 0.05$).

Scanning Electron Micrograph. The morphology of probiotic cells was altered upon ultrasound treatment (**Figure 1**). SEM showed the rupturing of the probiotic cellular membrane upon ultrasound treatment. In addition, the membranes of probiotics also showed permeabilized zones indicating the formation of pores on the cellular surface upon ultrasound treatment.

DISCUSSION

Ultrasound is a form of energy generated by sound waves frequencies and has conventionally been used for the disintegration of biological cell walls to liberate intracellular contents. In addition, ultrasound has also been successfully used to produce positive effects on growth (*17*), enzyme activity of cells (*7*), and delivery of therapeutically beneficial macromolecules into cells (*18*). These positive effects were mainly attributed to the stress induced by ultrasound on the cellular membrane. The application of ultrasound technology in various fields including bioprocess, biotechnology, and food processing has increased tremendously in recent years.

Table 6. Concentration of Aglycone in Mannitol-Soymilk Fermented by Control and Ultrasound-Treated Probiotics at 37 °C for 24 h[a]

		concentration of aglycones (μg/mL)			statistical significances of effect: P		
		time (min)					
aglycone	intensity (W)	1	2	3	T	I	T × I
daidzein	*Lactobacillus* sp. FTDC 2113	control = 3.50 ± 0.21					
	20	3.56 ± 0.32	1.93 ± 0.17	3.91 ± 0.08	0.005	<0.001	0.007
	60	4.02 ± 0.46	4.88 ± 1.21	5.44 ± 0.28			
	100	4.58 ± 0.93	4.64 ± 0.50	4.98 ± 0.13			
	L. casei ATCC 393	control = 4.62 ± 0.24					
	20	4.77 ± 0.30	4.93 ± 0.45	5.12 ± 0.26	0.185	0.165	0.605
	60	5.04 ± 0.49	4.98 ± 0.71	5.47 ± 0.26			
	100	5.84 ± 0.09	4.99 ± 0.91	4.95 ± 0.44			
	Bifidobacterium sp. FTDC 8943	control = 4.47 ± 0.13					
	20	3.69 ± 0.34	4.49 ± 0.03	4.15 ± 0.20	0.019	<0.001	0.006
	60	4.74 ± 0.17	5.22 ± 0.64	4.47 ± 0.11			
	100	4.66 ± 0.77	5.15 ± 0.07	6.07 ± 0.35			
	B. longum FTDC 8643	control = 4.39 ± 0.89					
	20	5.45 ± 0.31	5.07 ± 0.13	5.41 ± 0.59	0.004	0.001	0.160
	60	4.88 ± 0.59	5.40 ± 0.03	6.08 ± 0.52			
	100	5.56 ± 0.34	6.85 ± 0.52	7.46 ± 0.43			
glycitein	*Lactobacillus* sp. FTDC 2113	control = 1.89 ± 0.93					
	20	1.87 ± 0.97	1.64 ± 1.00	1.93 ± 1.01	0.982	0.927	0.999
	60	1.79 ± 0.84	1.95 ± 0.89	1.82 ± 0.81			
	100	1.88 ± 0.88	2.01 ± 0.89	2.15 ± 0.97			
	L. casei ATCC 393	control = 1.94 ± 0.47					
	20	1.90 ± 0.37	2.06 ± 0.66	2.19 ± 0.60	0.941	0.978	0.998
	60	2.32 ± 0.75	1.86 ± 0.46	2.04 ± 0.41			
	100	2.16 ± 0.28	2.01 ± 0.69	2.01 ± 0.57			
	Bifidobacterium sp. FTDC 8943	control = 1.97 ± 0.20					
	20	2.12 ± 0.07	2.29 ± 0.33	2.26 ± 0.19	0.016	0.011	0.084
	60	2.54 ± 0.03	2.64 ± 0.34	2.34 ± 0.09			
	100	2.13 ± 0.18	2.18 ± 0.27	2.84 ± 0.26			
	B. longum FTDC 8643	control = 2.34 ± 0.32					
	20	2.96 ± 0.11	2.55 ± 0.33	2.44 ± 0.36	0.042	0.010	0.214
	60	2.54 ± 0.46	2.72 ± 0.29	3.03 ± 0.09			
	100	2.84 ± 0.17	3.25 ± 0.04	3.19 ± 0.11			
genistein	*Lactobacillus* sp. FTDC 2113	control = 4.05 ± 0.82					
	20	6.06 ± 1.12	6.05 ± 1.16	6.26 ± 1.32	<0.001	<0.001	0.028
	60	6.35 ± 1.64	6.76 ± 0.16	6.58 ± 0.31			
	100	6.59 ± 0.36	6.60 ± 1.14	6.60 ± 0.72			
	L. casei ATCC 393	control = 4.34 ± 0.01					
	20	4.55 ± 0.17	4.84 ± 0.36	4.86 ± 0.22	0.001	<0.001	0.134
	60	4.79 ± 0.07	4.93 ± 0.71	5.14 ± 0.44			
	100	5.45 ± 0.06	5.54 ± 0.06	5.48 ± 0.42			
	Bifidobacterium sp. FTDC 8943	control = 5.45 ± 0.11					
	20	5.50 ± 0.66	5.73 ± 0.07	6.04 ± 0.06	0.015	0.003	0.384
	60	6.13 ± 0.47	6.82 ± 0.32	6.24 ± 0.60			
	100	6.39 ± 1.05	6.96 ± 0.34	7.03 ± 1.09			
	B. longum FTDC 8643	control = 5.89 ± 0.45					
	20	6.31 ± 0.48	5.91 ± 0.41	5.71 ± 0.34	0.123	0.009	0.267
	60	6.03 ± 0.51	6.60 ± 0.91	6.70 ± 0.23			
	100	6.47 ± 0.30	7.09 ± 0.44	7.41 ± 0.15			

[a] Initial concentration of daidzein, 1.48 ± 0.03 μg/mL; glycitein, 1.09 ± 0.05 μg/mL; genistein, 1.96 ± 0.47 μg/mL. Results are expressed as the means ± standard deviation of the means. Values are the means of duplicates from three separate runs ($n = 3$). *T*, effect of sonication time; *I*, effect of sonication intensity; *T* × *I*, interaction between sonication time and intensity.

In our present study, ultrasound treatment exerted a strong impact on the growth of probiotics immediately after treatment. A reduction in the viable counts of probiotics in mannitol-soymilk was observed immediately upon treatment and was more prevalent at higher intensities and durations. This was attributed to the increased stress with increased intensity of ultrasound waves. The mechanism of ultrasound-induced stress is rather complex and was predominantly due to cavitation (*19*). Cavitation refers to the growth, oscillation, and collapse of microbubbles in an acoustic field. The collapse of cavitation bubbles releases a shock wave that cause structural alteration in the surrounding cells and eventually leads to the localized rupture of membrane lipid bilayers (*19*). Such a disruption of the lipid membrane affects several cellular functions including nutrient transport, enzymes activities, and cell proliferation (*20*). Therefore, the alteration of the membrane upon treatment with ultrasound may account for the decrease in cell viability of probiotics immediately after treatment.

It has been reported that stress induced by ultrasound would only transiently or reversibly traumatize the cells. The viable cells treated with ultrasound could recover from cellular membrane injury and regain their metabolic activity upon fermentation.

Table 7. Membrane Permeability of Control and Ultrasound-Treated Probiotic Cells Immediately after Treatment[a]

	% permeabilized			statistical significances of effect: *P*		
	time (min)					
intensity (W)	1	2	3	*T*	*I*	*T* × *I*
Lactobacillus sp. FTDC 2113	control = 1.82 ± 0.29			<0.001	<0.001	<0.001
20	28.15 ± 1.23	34.55 ± 1.10	57.95 ± 4.85			
60	38.45 ± 3.07	54.50 ± 2.43	64.75 ± 2.56			
100	51.90 ± 2.78	38.58 ± 2.35	42.98 ± 1.04			
L. casei ATCC 393	control = 1.53 ± 0.10			<0.001	<0.001	<0.001
20	39.90 ± 7.32	47.50 ± 1.11	53.40 ± 6.17			
60	39.99 ± 0.24	49.14 ± 4.08	52.42 ± 4.85			
100	49.09 ± 6.52	57.36 ± 3.01	40.97 ± 7.61			
Bifidobacterium sp. FTDC 8943	control = 2.41 ± 0.09			<0.001	<0.001	<0.001
20	48.82 ± 3.23	41.31 ± 2.14	60.77 ± 2.32			
60	56.57 ± 1.31	60.66 ± 11.35	32.43 ± 1.68			
100	47.52 ± 9.69	66.13 ± 1.68	38.355 ± 3.19			
B. longum FTDC 8643	control = 2.83 ± 0.92			<0.001	<0.001	<0.001
20	58.24 ± 1.22	45.51 ± 10.22	67.27 ± 2.18			
60	44.68 ± 8.87	64.57 ± 2.82	50.56 ± 4.96			
100	46.79 ± 8.82	55.77 ± 1.29	47.13 ± 3.71			

[a] Results are expressed as the mean ± standard deviation of the mean. Values are the mean of duplicates from three separate runs ($n = 3$). *T*, effect of sonication time; *I*, effect of sonication intensity; *T* × *I*, interaction between sonication time and intensity.

Table 8. Membrane Lipid Peroxidation of Control and Ultrasound-Treated Probiotic Cells Immediately after Treatment[a]

	concentration of malondialdehyde (g/mL)			statistical significances of effect: *P*		
	time (min)					
intensity (W)	1	2	3	*T*	*I*	*T* × *I*
Lactobacillus sp. FTDC 2113	control = 0.40 ± 0.01			0.018	0.042	0.358
20	0.42 ± 0.04	0.43 ± 0.01	0.43 ± 0.03			
60	0.43 ± 0.06	0.41 ± 0.01	0.43 ± 0.04			
100	0.44 ± 0.03	0.41 ± 0.03	0.48 ± 0.09			
L. casei ATCC 393	control = 0.13 ± 0.05			0.001	0.001	0.440
20	0.17 ± 0.04	0.17 ± 0.02	0.18 ± 0.03			
60	0.18 ± 0.01	0.18 ± 0.02	0.21 ± 0.02			
100	0.18 ± 0.00	0.21 ± 0.00	0.24 ± 0.01			
Bifidobacterium sp. FTDC 8943	control = 0.24 ± 0.02			<0.001	<0.001	<0.001
20	0.27 ± 0.00	0.30 ± 0.00	0.28 ± 0.02			
60	0.25 ± 0.01	0.32 ± 0.01	0.38 ± 0.03			
100	0.32 ± 0.00	0.34 ± 0.00	0.38 ± 0.02			
B. longum FTDC 8643	control = 0.15 ± 0.03			0.002	<0.001	0.208
20	0.17 ± 0.06	0.22 ± 0.06	0.23 ± 0.06			
60	0.21 ± 0.09	0.25 ± 0.04	0.27 ± 0.05			
100	0.35 ± 0.09	0.32 ± 0.05	0.40 ± 0.08			

[a] Results are expressed as the mean ± standard deviation of the mean. Values are the mean of duplicates from three separate runs ($n = 3$). *T*, effect of sonication time; *I*, effect of sonication intensity; *T* × *I*, interaction between sonication time and intensity.

Our present study showed that *Lactobacillus* and *Bifidobacterium* treated with ultrasound grew effectively in mannitol-soymilk upon fermentation at 37 °C for 24 h. In agreement with our finding, Yang et al. (*8*) reported that ultrasound exposure on *Brevibacterium* sp. without fermentation significantly reduced the cell viability, but subsequent static fermentation substantially promoted the growth of the bacteria. Likewise, Wang et al. (*7*) also demonstrated that viable cells of *Lactobacillus* decreased during ultrasound treatment but subsequently increased after ultrasound processing ceased. In another study, Nguyen et al. (*17*) also demonstrated that ultrasound was able to promote the growth of *Bifidobacteria* in milk upon fermentation at 37 °C, suggesting that ultrasound had no detrimental effect on the propagation ability of surviving cells.

One of the proposed mechanisms to explain the enhanced growth of bacterial cells after treatment with ultrasound is the pore formation and changes in the permeability of cell membrane upon treatment. It has been reported that ultrasound (20 kHz; 200 W/cm^2) treatment effectively increased the permeability of bacterial cells (*Brevibacterium* sp.) due to pore formation on the cellular membrane (*8*). These pores on the cellular membrane accelerated oxygen and nutrient transport into the cells and increased the transport of waste products from cells, thus enhancing growth (*21*).

Various cellular components containing growth factors are also released into the culturing medium upon cell lysis after treatment with ultrasound. Treatment of cells with ultrasound exhibited varying levels of cellular damage where the effects ranged from apparently unaffected cells to permeabilized cells to lysed cells and cell debris. Lysed cells provided components that could stimulate the growth of remaining bacterial cultures which were not extensively damaged (*17*). Gaudreau et al. (*22*) previously reported that cell lysates contained many growth factors such as amino acids, vitamins, and nucleotides which stimulated

Table 9. Fluorescence Anisotropy (ANS, TMA-DPH, and DPH) of Control and Ultrasound-Treated Probiotic Cells Immediately after Treatment[a]

		FAn			statistical significances of effect: *P*		
		time (min)					
fluorescence probe	intensity (W)	1	2	3	*T*	*I*	*T* × *I*
ANS	*Lactobacillus* sp. FTDC 2113	control = 0.34 ± 0.05			0.018	0.046	0.613
	20	0.29 ± 0.07	0.28 ± 0.09	0.27 ± 0.07			
	60	0.26 ± 0.05	0.28 ± 0.09	0.31 ± 0.06			
	100	0.25 ± 0.06	0.29 ± 0.08	0.32 ± 0.06			
	L. casei ATCC 393	control = 0.61 ± 0.08			0.076	0.187	0.858
	20	0.61 ± 0.08	0.60 ± 0.11	0.52 ± 0.11			
	60	0.56 ± 0.09	0.51 ± 0.13	0.49 ± 0.17			
	100	0.62 ± 0.15	0.53 ± 0.12	0.53 ± 0.08			
	Bifidobacterium sp. FTDC 8943	control = 0.47 ± 0.07			0.005	0.008	0.565
	20	0.40 ± 0.10	0.37 ± 0.09	0.34 ± 0.06			
	60	0.39 ± 0.04	0.34 ± 0.09	0.41 ± 0.10			
	100	0.41 ± 0.09	0.37 ± 0.07	0.41 ± 0.09			
	B. longum FTDC 8643	control = 0.51 ± 0.11			0.018	0.003	0.668
	20	0.40 ± 0.08	0.35 ± 0.10	0.33 ± 0.09			
	60	0.50 ± 0.10	0.41 ± 0.14	0.46 ± 0.12			
	100	0.46 ± 0.10	0.43 ± 0.07	0.43 ± 0.10			
TMA-DPH	*Lactobacillus* sp. FTDC 2113	control = 0.67 ± 0.12			<0.001	0.245	0.009
	20	0.50 ± 0.07	1.34 ± 0.24	0.71 ± 0.28			
	60	0.62 ± 0.06	0.83 ± 0.29	0.50 ± 0.12			
	100	0.44 ± 0.15	0.98 ± 0.44	0.98 ± 0.30			
	L. casei ATCC 393	control = 0.78 ± 0.14			0.020	0.016	0.132
	20	0.83 ± 0.20	0.83 ± 0.15	0.83 ± 0.43			
	60	0.81 ± 0.28	0.61 ± 0.15	0.82 ± 0.43			
	100	0.73 ± 0.22	0.88 ± 0.29	1.41 ± 0.35			
	Bifidobacterium sp. FTDC 8943	control = 0.54 ± 0.35			0.926	0.051	0.073
	20	0.84 ± 0.37	0.29 ± 0.09	0.62 ± 0.20			
	60	0.28 ± 0.15	0.45 ± 0.10	0.54 ± 0.15			
	100	0.74 ± 0.38	1.18 ± 0.62	0.61 ± 0.17			
	B. longum FTDC 8643	control = 0.69 ± 0.34			0.162	0.068	0.507
	20	0.67 ± 0.27	0.85 ± 0.05	0.96 ± 0.21			
	60	1.04 ± 0.25	0.96 ± 0.29	0.84 ± 0.10			
	100	1.30 ± 0.61	0.88 ± 0.08	1.04 ± 0.23			
DPH	*Lactobacillus* sp. FTDC 2113	control = 0.35 ± 0.18			<0.001	<0.001	0.045
	20	0.63 ± 0.13	0.63 ± 0.27	1.25 ± 0.50			
	60	1.02 ± 0.44	0.96 ± 0.34	0.93 ± 0.43			
	100	0.92 ± 0.52	1.08 ± 0.22	1.03 ± 0.41			
	L. casei ATCC 393	control = 0.37 ± 0.06			0.006	<0.001	0.001
	20	0.40 ± 0.17	0.40 ± 0.09	0.35 ± 0.09			
	60	0.70 ± 0.19	0.51 ± 0.18	0.40 ± 0.12			
	100	0.55 ± 0.16	0.60 ± 0.07	0.74 ± 0.10			
	Bifidobacterium sp. FTDC 8943	control = 0.58 ± 0.07			0.097	0.016	0.769
	20	0.75 ± 0.34	0.55 ± 0.26	0.62 ± 0.27			
	60	0.95 ± 0.51	1.00 ± 0.44	0.82 ± 0.13			
	100	1.07 ± 0.54	1.04 ± 0.58	0.81 ± 0.34			
	B. longum FTDC 8643	control = 0.68 ± 0.19			0.596	0.455	0.996
	20	0.57 ± 0.28	0.54 ± 0.21	0.63 ± 0.13			
	60	0.48 ± 0.30	0.52 ± 0.21	0.50 ± 0.12			
	100	0.62 ± 0.34	0.59 ± 0.36	0.53 ± 0.28			

[a] Results are expressed as the mean ± standard deviation of the mean. Values are the mean of duplicates from three separate runs ($n = 3$). *T*, effect of sonication time; *I*, effect of sonication intensity; *T* × *I*, interaction between sonication time and intensity.

the growth of *L. rhamnosus* culture in milk. In our present study, both mechanisms may have contributed to the enhanced growth of treated probiotics in mannitol-soymilk. This was strongly evidenced by our SEM micrograph which showed pore formation on the cellular surface of probiotics and cells lysis upon treatment with ultrasound.

Isoflavones are components that occur naturally in soy and have attracted much attention due to their various beneficial health effects. However, not all isoflavones are equally bioactive and bioavailable, with only aglycones that have been reported to play a critical role in promoting human health such as decreasing the risk of osteoporosis, postmenopause disorder, and breast cancer. In order for the transformation of isoflavones to take place, β-glucosidase must be essentially present for the hydrolysis of β-glycosidic bonds of glucosides to produce bioactive aglycones. Our results showed that treatment with ultrasound especially at 100 W for 2 min significantly enhanced the intracellular β-glucosidase activity of the probiotics studied. This was in tandem with the increased growth of probiotics after fermentation at 37 °C for 24 h, indicating a growth-dependent characteristic.

Extracellular β-glucosidase plays an important role in accelerating the biotransformation of isoflavones in soymilk. This was due to the fact that extracellular biocatalysis often occurs at a faster rate due to the absence of the cellular membrane as a barrier.

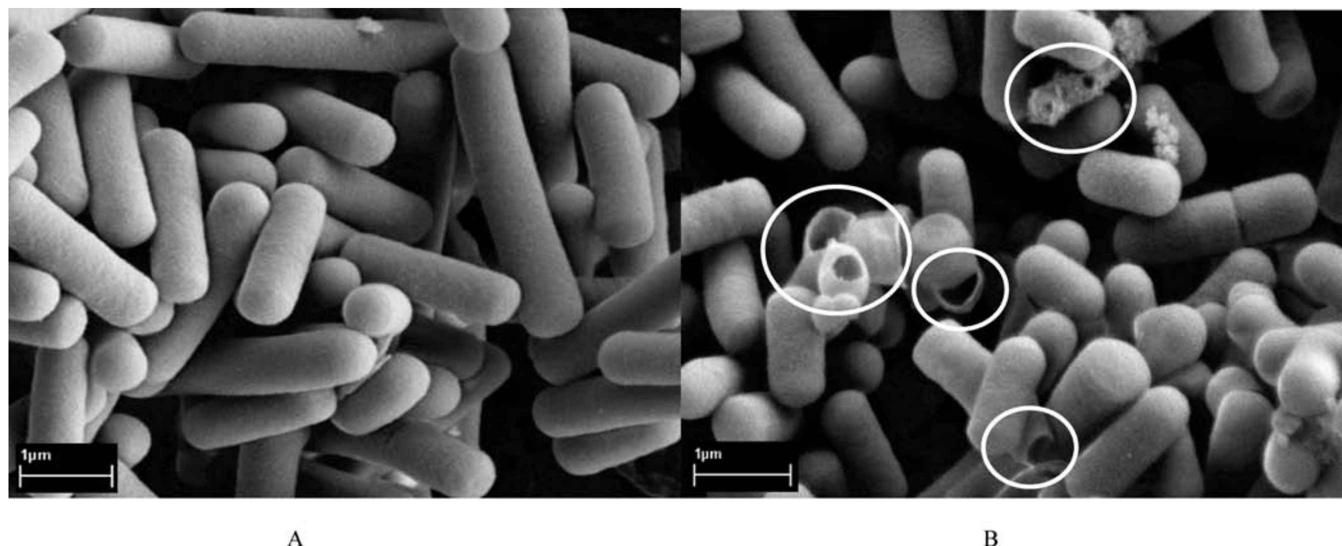

Figure 1. Scanning electron micrographs of probiotics without treatment (**A**) and probiotics treated with ultrasound (**B**). Circles show ruptured cells and cells with pores.

A previous study has suggested that an extracellular enzyme showed a higher catalytic activity than that occurring inside the cells (*7*). In our present study, the extracellular β-glucosidase activity of probiotics in mannitol-soymilk was enhanced upon treatment with ultrasound, which was beneficial for the faster biotransformation of isoflavones. Wang et al. (*7*) also previously reported that ultrasound treatment increased the extracellular β-galactosidase activity of *Lactobacillus* strains in milk. This was due to the release of β-galactosidase from the cells to the culture medium (milk) and subsequently increased extracellular enzyme activity. The release of enzyme into the culture medium was caused by an alteration of membrane permeability upon ultrasound treatment. Ultrasound treatment could generate powerful shockwaves or shooting flows which led to the damage of the cell wall and the reversible change in permeability of the cell membrane (*8*). The alteration of membrane permeability facilitated enzyme excretion and thus enhanced extracellular enzyme activity. Our results showed that the membrane of probiotics was permeabilized upon treatment with ultrasound, leading to increased excretion of β-glucosidase.

With the increased intracellular and extracellular β-glucosidase upon treatment with ultrasound, we further evaluated the bioconversion of isoflavones in mannitol-soymilk by treated probiotic cells. Glucosides such as genistin, acetyl glycitin, malonyl daidzin, malonyl genistin, and malonyl glycitin was substantially hydrolyzed in mannitol-soymilk fermented by ultrasound-treated probiotics. Hydrolysis of these glucosides by probiotics was most prevalent when treated at high intensity (100 W) and long duration (2 and 3 min). The increased hydrolysis of glucosides upon treatment at this intensity and time was also accompanied by an increase in the concentration of their corresponding aglycones. This was well correlated with our β-glucosidase activity results which showed major enhancement upon treatment at high intensity and time. In addition to increased enzymic activity upon ultrasound treatment, the increased bioconversion by whole cells could also be due to other factors. Bar previously demonstrated that ultrasound irradiation at 20 kHz and a power output of 22 kW m^{-2} enhanced the rate of mass transfer across the cells *Rhodococcus erythropolis* (*23*). Therefore, the enhanced bioconversion of isoflavones in mannitol-soymilk could be due to the increased transfer of substrate (glucosides) into the cells and hydrolysis by intracellular β-glucosidase, and also the increased transport of transformed products (aglycones) outside the cell.

The cellular membrane is one of the main targets in ultrasound treatment. Therefore, in order to better understand the mechanism involved, membrane properties of probiotic cells were evaluated in our present study. Our SEM micrograph and membrane permeability results indicated that membrane of probiotics was altered upon treatment with ultrasound. The alteration of membrane structure by ultrasound has mainly been attributed to lipid peroxidation (*24*). The membrane lipid peroxidation of probiotics in our present study increased significantly upon treatment and was higher when treated with stronger intensity and longer time, mainly attributed to the increased production of free radicals. During ultrasound treatment, especially at high intensity, cavitation bubbles whose size is close to the resonant size for the applied frequency would begin to oscillate nonlinearly and eventually collapse. The collapse of the cavitation bubbles resulted in a shock wave that produces extremely high temperature and pressure which in turn fragment water and other molecules into free radicals (*25*). These free radicals react with polyunsaturated fatty acids of the membrane resulting in peroxidation and deterioration of the lipid bilayer, and alteration of the membrane lipid composition (*20*).

Although our present results indicated that ultrasound treatment affected the lipid content of the membrane by lipid peroxidation, little information is available on the location where ultrasound could affect the membrane. Thus, we further evaluated the possible location of alteration in the membrane bilayer by three different fluorescence probes. ANS probe, a type of anionic probe, binds at the interface between the apolar tail and the polar head of the phospholipid membrane and has been associated with the reduced effective negative charge of the membrane surface (*26*). Our experimental data showed that FAn of ANS for probiotic cells treated with ultrasound was significantly lower than that of the control. This suggested that ultrasound treatment caused an alteration in the polar head of the phospholipid membrane and increased the effective negative charge on the membrane surface of probiotics. This led to increased electrostatic repulsion of the headgroups which pushes them apart, making the membrane surface more permeable (*27*). This was in tandem with our observation from the SEM micrograph and data from our membrane permeability test.

TMA-DPH is a hydrophobic probe with a TMA cationic group anchored to the polar heads of the phospholipids and DPH incorporated to the upper region of the acyl group due to the presence of cationic TMA group (*28*). The binding of TMA-DPH provides valuable information about water penetration into the membrane and has been associated with membrane fluidity at the upper region of the phospholipid bilayer (*29*). Ultrasound treatment drastically increased the FAn value of TMA-DPH for the membrane of probiotics in our present study indicating increased rigidity of the acyl region and reduced water penetration into the membrane. A reduced penetration of water into the membrane subsequently increased the hydrophobicity of the membrane and formed an effective hydrophobic barrier for polar molecules (*30*). Such an alteration of the membrane hydrophobicity could affect the ability of viable cells to propagate effectively. This was in tandem with the decreased growth of probiotics immediately upon treatment with ultrasound in our present study.

DPH is a fluorophore incorporated into apolar regions of the membrane. Unlike TMA-DPH probes, DPH (lacking the polar group) is embedded deeper in the hydrophobic part of the bilayer, providing indications of membrane rigidity and fluidity (*28*). The FAn of DPH is a directly proportional to membrane rigidity and inversely proportional to membrane fluidity. In our present study, ultrasound treatment substantially increased the FAn of DPH for probiotics indicating that the apolar phospholipid tail of the membrane of probiotics was more rigid immediately after treatment with ultrasound. Alteration of the membrane fluidity could affect the cellular function of cells where the reduction of membrane fluidity inhibited cell growth and induced cell death (*20*). This reduction of membrane fluidity was well-correlated with the decreased growth of our probiotic strains immediately after treatment with ultrasound. The decrease of membrane fluidity was due to lipid peroxidation which resulted in deterioration of phospholipid composition and an increase in the ratio between cholesterol and phospholipids. Increased cholesterol content has been associated with restricted molecular motion in the hydrophobic portion of the membrane lipid bilayer and consequently reduced membrane fluidity (*20*). In our present study, the increased FAn of DPH was in tandem with membrane lipid peroxidation.

In conclusion, ultrasound treatment caused membrane permeabilization and cell lysis from lipid peroxidation and physical disruption. This treatment altered the acyl chain, polar head, and interface region of the phospholipid bilayer of probiotics. Considering that cellular function is affected by the membrane, such an alteration in the membrane led to decreased growth of probiotics immediately after treatment with ultrasound. However, the cells treated with ultrasound showed recovery from cellular membrane injury and regained the ability to proliferate upon fermentation at 37 °C for 24 h. The intracellular and extracellular β-glucosidase activity of probiotics was also higher upon ultrasound treatment, leading to enhanced hydrolysis of glucosides and the production of aglycones in mannitol-soymilk. Results from our present study provided information on the potential application of ultrasound in the production of bioactive synbiotic-soymilk.

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