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EFFECTS OF ULTRASONICATION ON THE PRODUCTION OF HYALURONIC ACID BY LACTOBACILLI

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The oral administration of hyaluronic acid has been shown to improve skin health. This study aimed at utilizing ultrasound treatment to enhance the production of hyaluronic acid by lactobacilli. Among the 16 strains of lactobacilli screened, a higher production of hyaluronic acid ($P<0.05$) was observed from seven strains namely, *Lactobacillus casei* BT 2113, *Lactobacillus casei* BT 1268, *Lactobacillus casei* BT 8633, *Lactobacillus casei* FTDC 8033, *Lactobacillus acidophilus* FTDC 1231, *Lactobacillus gasseri* FTDC 8131 and *Lactobacillus rhamnosus* FTDC 8313. Upon ultrasonic treatment, hyaluronic acid concentration increased significantly ($P<0.05$) in all the seven strains studied. The use of ultrasonication at 100% amplitude increased the hyaluronic acid content ($P<0.05$) up to 51.6 % with concentrations ranging from 0.262 mg ml^{-1} to 0.385 mg ml^{-1}, while an amplitude of 20% yielded a lower increase ranging from 0.227 mg ml^{-1} to 0.310 mg ml^{-1}. Increasing duration of ultrasonic treatment (from 1 min to 3 min) also significantly ($P<0.05$) improved the concentration of hyaluronic acid in some strains. Our data demonstrated that a sub-lethal physical treatment, such as ultrasonication, can be applied to increase the membrane permeability of lactobacilli cells and subsequently increase the release of bioactive metabolites.

Keywords: Lactobacilli, hyaluronic acid, ultrasonication

Lactobacilli have long been known to exhibit functional characteristics typically associated with probiotic bacteria ranging from gut health to metabolic diseases (Liong et al., 2007), attributed to their abilities to produce bioactive metabolites such as acids and enzymes (Liong & Shah, 2005; Yeo & Liong, 2010). In this case, probiotics can be defined as 'living microorganisms, which present a benign efficacy in terms of physical well-being to the host when administered in sufficient quantity' (Holzapfel & Schillinger, 2002). Recent evidences have shown an apparent rise in researches on lactobacilli detailing a host of other ancillary benefits. In a clinical study carried out by Kalliomaki and co-workers (2001), it was discovered that *Lactobacillus* GG possesses a high efficacy in preventing atopic eczema, a form of dermatitis amongst high risk children. As such, the dermal benefits associated with lactobacilli is an interesting aspect to investigate, considering that Arck and co-workers (2010) postulated that lactobacilli have the potential to regulate dermal health from preventing skin disorders to mitigating skin ageing. This notion was further corroborated by Pinto and co-workers (2011), who found that *Lactobacillus plantarum* produces bioactive compounds that can aid tissue regeneration. In a way, the bioactive compounds produced by lactobacilli can enhance the bodily response to improve skin health, and have been demonstrated to be especially favourable in wound healing (Brachkova et al., 2011).

Hyaluronic acid (HA) is one such dermal bioactive constituent being produced by lactobacilli. There are numerous therapeutic advantages of HA as detailed by Price and co-workers (2007), such as in the field of ophthalmic surgery, aesthetic augmentation, epithelial

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regeneration and drug delivery. Fujikawa (2008) demonstrated that the oral administration of HA at more than 120 mg per day improved skin dryness, itchiness, erosion and exfoliation. In another randomized, double-blind, placebo controlled study involving 40 healthy subjects with joint discomfort, oral supplementation of 80 mg per day of HA for 90 days significantly improved joint mechanics and muscle function, thus attenuating risk factors and alleviating symptoms of knee osteoarthritis (Martinez-Puig et al., 2012). Conventionally, HA is extracted from avian and bovine sources, animal-derived sources pose a high risk of cross-species infection with viruses and pathogens as well as potential contamination, which can affect the quality of the end product (Chong et al., 2005). Microbial fermentation may be a preferred alternative, considering the lower production cost. It has been reported for the first time in 2009 that HA is produced in milk broth through fermentation by a putative probiotic strain *Streptococcus thermophilus* YIT2084 (Izawa et al., 2009). In a more recent study, it is reported that certain strains of lactobacilli, such as *L. rhamnosus* FTDC 8313 and *L. gasseri* FTDC 8131, were also capable of producing HA at concentrations of more than 1 g l^{-1} when cultivated in skimmed milk, suggesting its potential use in dermatological field (Lew et al., 2013). Hyaluronic acid is produced intracellularly and excreted to the extracellular matrix via transportation across the plasma membrane; hence, the permeability of the cell membrane plays a crucial part in facilitating transfers of dermal bioactive metabolites extracellularly. Mild and sub-lethal physical treatments, such as ultrasonication, has been reported to increase membrane permeability (Ewe et al., 2012; Lye et al., 2012) and transport of metabolites across microbial membranes. The effects of ultrasound on cells are, however, dependent on the strength and frequency of waves, cell wall structure and treatment environment (Tabatabaie & Mortazavi, 2008). Although extensive research has been carried out on the many therapeutic benefits displayed by lactobacilli, not much emphasis has been given on dermatological aspects and the production of dermal bioactives, such as HA. We thus hypothesized that the yield of HA obtained from lactobacilli can be increased upon the application of sub-lethal ultrasonic treatments.

1. Materials and methods

1.1. Bacterial cultures

Lactobacillus casei BT 2113, *L. casei* BT 1311, *L. casei* BT 1268, *L. casei* BT 8633, *L. casei* FTDC 8033, *L. casei* BT 314, *L. acidophilus* FTDC 0291, *L. acidophilus* FTDC 1733, *L. acidophilus* FTDC 1231, *L. bulgaricus* FTDC 8011, *L. bulgaricus* FTDC 1211, *L. gasseri* FTDC 8131, *L. gasseri* CHO 220, *L. rhamnosus* FTDC 8313, *L. fermentum* FTDC 8219 and *L. fermentum* FTDC 8312 were obtained from the Culture Collection Centre of Bioprocess Technology Division, Universiti Sains Malaysia (Penang, Malaysia). The stock cultures were stored at –20 °C in 40% (v/v) sterile glycerol. The strains were activated in sterile de Man, Rogosa and Sharpe (MRS) broth (Hi-Media, Mumbai, India) for three consecutive times using 10% (v/v) inoculum and incubated at 37 °C for 24 h ahead of use.

1.2. Determination of hyaluronic acid

Ten millilitres of sterile MRS broth was inoculated with 10% (v/v) inoculum and incubated at 37 °C for 20 h. Standardization was carried out on the fermentation broth by way of the absorbance to 0.3 at 600 nm using a UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan). It

was then centrifuged at 10,000 *g* for 15 min at 25 °C. The supernatant was collected and stored at –20 °C prior to analyses. Hyaluronic acid concentrations were determined using the cetyltrimethylammonium bromide (CTAB) turbidimetric method (Chen & Wang, 2009). The sample was first mixed with 2.5 volumes of absolute ethanol and rested at 4 °C for an hour. The sample was then centrifuged at 10 000 *g* for 15 min at 4 °C and the sediment was dissolved in five volumes of distilled deionised water. CTAB reagent was prepared by dissolving 2.5 g CTAB (Sigma-Aldrich) in 100 ml of 0.2 M NaCl solution. One millilitre of HA standard and sample were mixed gently with 2.0 ml of CTAB reagent and allowed to rest for 10 min before measuring the absorbance at 400 nm using a UV-Vis spectrophotometer (Shimadzu).

1.3. Physical treatment via ultrasonication

Activated cultures were subjected to ultrasonication using an Ultrasonic Homogenizer (Sartorius Stedim Biotech, Goettingen, Germany) with a frequency of 30 kHz and nominal power of 115 W cm^{-2}. The treatment intensities were adjusted to three different levels, namely 20%, 60% and 100%. In all treatments, the tip of the sonotrode (at 80 mm in length and 3 mm in diameter) was positioned at the same position, that is immersed at a 10 mm depth of the 10 ml culture medium. The samples were then ultrasonicated at three different durations namely, 1, 2 and 3 min at 25 °C and followed by cooling at 4 °C for 15 min. Untreated cells were used as control.

1.4. Scanning electron microscopy

The cell pellet was fixed with the McDowell Trump fixative agent (4%, v/v formaldehyde, Sigma-Aldrich; 1%, v/v glutaraldehyde, Sigma-Aldrich) and 1% (w/v) osmium tetroxide (Sigma-Aldrich). Following that, the pellet was dehydrated alternately with a series of ethanol and hexamethyldisilazane (Supelco, Bellefonte, PA, USA). The dried cells were mounted onto a scanning electron microscopy specimen stub, coated with gold in a Sputter coater (Polaran, Walford, UK) and examined under a scanning electron microscope (Leo Supra, Oberkochen, Germany).

1.5. Determination of viability

Viability (log CFU ml^{-1}) of the treated and control cells were evaluated via the pour plate method in MRS agar. The plated MRS agar was incubated at 37 °C for 48 hours.

1.6. Statistical analysis

Data analysis was performed using SPSS Inc. software (Version 20.0) (Chicago, IL, USA). One-way and two-way analysis of variance (ANOVA) were used to evaluate the significant differences between sample means, with significance level at α=0.05. Mean comparisons were assessed by Tukey's test and all data presented were mean values obtained from three separate runs. P-values were stipulated for both amplitude and time to indicate the general inclination of the factors studied on the response variables with the respective statistical significance.

2. Results and discussion

2.1. Screening of Lactobacillus *sp. for production of hyaluronic acid*

All the 16 strains of lactobacilli studied were able to produce varying concentrations of HA. A higher concentration of HA was detected from seven out of the 16 strains of lactobacilli studied, namely *L. casei* BT 2113, *L. casei* BT 1268, *L. casei* BT 8633, *L. casei* FTDC 8033, *L. acidophilus* FTDC 1231, *L. gasseri* FTDC 8131 and *L. rhamnosus* FTDC 8313 ($P<0.05$), with concentrations ranging from 0.236 mg ml^{-1} to 0.303 mg ml^{-1}. These strains were selected for the subsequent evaluation using ultrasonication. Considering that HA is produced intracellularly before being transported into the extracellular matrix (Izawa et al., 2009), the permeability of the cellular membrane would be a significant barrier in the transportation of HA across the membrane.

Hyaluronic acid is postulated to be released extracellularly because of its gel-like characteristics, which aid microorganisms in adherence to existing environment as well as providing protection against reactive oxides (Yamada & Kawasaki, 2005). Extracellular HA lends its gel-like properties to the microbial colonies formed with the slimy translucent outer layer (Chong et al., 2005). Not all strains of lactobacilli screened exhibit similar HA producing capabilities. The capability of lactobacilli to excrete HA is uniquely strain dependent, some species do not produce HA, while others have been found to be excellent producers of HA (Lew & Liong, 2013).

2.2. Effect of ultrasonication on the concentration of hyaluronic acid

In this present study, all treated strains showed a significant increase ($P<0.05$) in the concentration of HA upon ultrasonic treatment (Table 1). Hyaluronic acid in the extracellular matrix is an important aspect for an improved and uncomplicated recovery of the bioactive metabolite from the fermentation media. Correspondingly, the use of ultrasonication at 100% amplitude increased ($P<0.05$) the production of HA up to 51.6% with concentrations ranging from 0.262 mg ml^{-1} to 0.385 mg ml^{-1}. On the other hand, an amplitude of 20% contributed to a lower increase in HA content, with concentrations ranging from 0.227 mg ml^{-1} to 0.310 mg ml^{-1}. Increasing the intensity of the ultrasonic treatment also increased the production of HA in all strains studied ($P<0.05$). The duration of ultrasonic treatment also significantly ($P<0.05$) affected the concentration of HA. Cells treated at 20% amplitude for 1 min showed little changes in the concentration of HA. However, in strains, such as *L. casei* BT 1268, *L. casei* BT 2113, *L. gasseri* FTDC 8131, *L. rhamnosus* FTDC 8313 and *L. casei* BT 8633, treatment for 3 min produced a significant increase in HA concentration compared to shorter durations studied ($P<0.05$).

Ultrasonication has long been used as a sub-lethal physical treatment to enhance membrane permeabilisation allowing for improved mass transfer across the membrane (Yeo & Liong, 2011; Lye et al., 2012). Results obtained from the current study justified the significant increase of HA concentration after ultrasonic treatment. This may be attributed to the increase in cross membrane transfer of HA from inside the cell to the extracellular matrix. We postulate that the cross-membrane movement that was previously hindered by the plasma membrane has been enhanced upon ultrasonication. Evidently from the results obtained, by increasing the intensity of the ultrasonic treatment, the concentration of HA increased proportionally as well.

Table 1. Concentration of hyaluronic acid in extracellular extracts of control and ultrasound-treated lactobacilli cultured in MRS broth for 20 h at 37 °C

Amplitude (%)	Extracellular hyaluronic acid concentration (mg ml^{-1} of MRS)			Statistical significance of effect: P		
	Time (min)					
	1	2	3	A	T	A x T
L. acidophilus FTDC 1231	Control[a]=0.280±0.003			<0.001	0.116	0.003
20	0.290±0.001	0.310±0.002	0.283±0.015			
60	0.284±0.001	0.299±0.002	0.291±0.002			
100	0.357±0.010	0.385±0.012	0.357±0.017			
L. casei BT 1268	Control[a]=0.258±0.005			<0.001	< 0.001	<0.001
20	0.265±0.003	0.266±0.002	0.273±0.002			
60	0.272±0.002	0.277±0.004	0.285±0.003			
100	0.300±0.001	0.335±0.009	0.330±0.002			
L. casei BT 2113	Control[a]=0.225±0.003			<0.001	< 0.001	<0.001
20	0.227±0.001	0.227±0.001	0.231±0.001			
60	0.236±0.001	0.245±0.003	0.252±0.001			
100	0.262±0.002	0.300±0.001	0.341±0.001			
L. casei FTDC 8033	Control[a]=0.243±0.001			<0.001	<0.001	<0.001
20	0.249±0.001	0.255±0.001	0.260±0.001			
60	0.268±0.001	0.277±0.002	0.287±0.001			
100	0.295±0.002	0.305±0.003	0.294±0.001			
L. gasseri FTDC 8131	Control[a]=0.242±0.002			<0.001	<0.001	0.722
20	0.248±0.001	0.252±0.001	0.257±0.001			
60	0.267±0.001	0.271±0.001	0.276±0.001			
100	0.284±0.002	0.293±0.001	0.294±0.001			
L. rhamnosus FTDC 8313	Control[a]=0.257±0.001			<0.001	<0.001	<0.001
20	0.258±0.002	0.266±0.002	0.268±0.002			
60	0.300±0.001	0.297±0.001	0.319±0.002			
100	0.297±0.003	0.334±0.002	0.358±0.003			
L. casei BT 8633	Control[a]=0.269±0.002			<0.001	<0.001	0.008
20	0.269±0.001	0.267±0.002	0.276±0.002			
60	0.282±0.002	0.290±0.001	0.299±0.002			
100	0.339±0.001	0.385±0.001	0.360±0.002			

Results are expressed as mean±standard deviation. The values are means of repeated measurements from three separate runs (n=3).

[a] Control: untreated cells

2.3. Scanning electron microscopy

Scanning electron microscopy was performed to inspect the effect of ultrasonic treatment on the morphology of the lactobacilli cells (Fig. 1). Untreated cells (control; Fig. 1A) showed a

smoother and intact plasma membrane surface without any pronounced physical effect, while ultrasonic treated cells at 100% amplitude for 3 min showed the formation of pores in the cell membrane (Fig. 1B). It can be suggested that the formation of pores on the membrane of the lactobacilli cells allowed the uncomplicated channelling of HA into the extracellular matrix. This view was supported by the concept of ultrasonication, where mechanical waves produced from the tip of the sonotrode could stimulate acoustic cavitation, hence the appearance of temporary, non-specific pores on the cellular membrane (Ewe et al., 2012). It has been reported that cells were able to return to their impermeable state immediately after ultrasound exposure with pore opening that last within milliseconds to seconds (Mehier-Humbert et al., 2005). However, the temporarily formed pores did not alter the growth of cells during fermentation, and cells from the subsequent passage showed intact membrane morphology (data not shown), supporting the fact that the treatment was sub-lethal and cells resumed their normal growth.

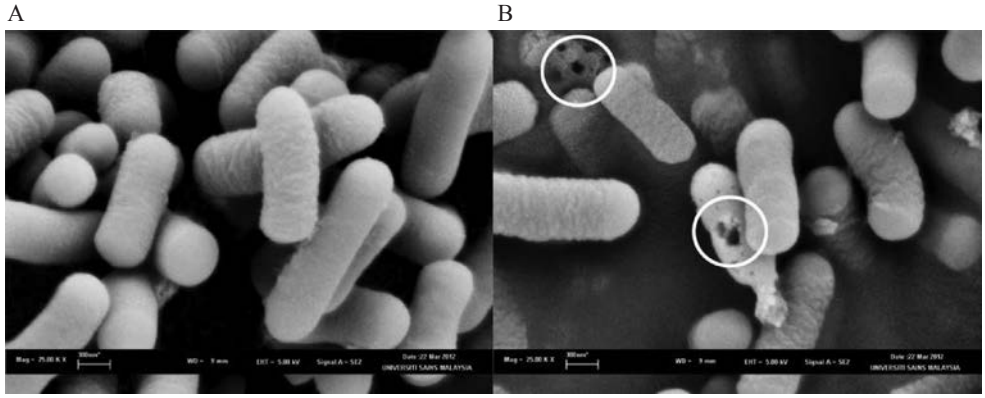

Fig. 1. Scanning electron micrographs of *Lactobacillus acidophilus* FTDC 1231 without treatment (A) and lactobacilli treated with ultrasound at 100% amplitude for 3 min (B).
Circles indicate ruptured cells and cells with pores

2.4. Viability of Lactobacillus *sp. immediately upon treatment*

The effect of ultrasonic treatment on the viability of lactobacilli was strain dependent (Table 2). Ultrasonic treatment at 100% amplitude showed detrimental effects on the growth of cells especially *L. casei* BT 8633, compared to the control and cells treated at other lower intensities. Some strains showed a significant increase ($P<0.05$) in viability at 100% amplitude, such as *L. gasseri* FTDC 8131 and *L. rhamnosus* FTDC 8313. At 20% amplitude, the viability of *L. gasseri* FTDC 8131 was significantly increased ($P<0.05$) in contrast to the control. In addition, increasing the duration of the ultrasonic treatment also resulted in an increase ($P<0.05$) in viability of cells and this was most prevalent for *L. rhamnosus* FTDC 8313. In contrast, *L. casei* BT 8633 showed a lower ($P<0.05$) viability immediately after treatment at 100% amplitude for 3 min compared to the control. When lactobacilli were treated at lower amplitude of 20% for 3 min, viability was higher ($P<0.05$) than that of control, and this was most prevalent for *L. casei* BT 1268, *L. casei* FTDC 8033, *L. rhamnosus*

FTDC 8313 and *L. gasseri* FTDC 8131. All treated cells were inoculated into a fresh batch of medium and showed an increase in growth after fermentation (growth was comparable within strains; data not shown).

Table 2. Viability of control and ultrasound-treated lactobacilli after fermentation in sterile MRS broth for 20 h at 37 °C

Amplitude (%)	Viability (log CFU ml^{-1})			Statistical significance of effect		
	Time (min)					
	1	2	3	A	T	A x T
L. acidophilus FTDC 1231		Control[a]=7.21±0.14		<0.001	<0.001	0.008
20	7.45±0.17	7.35±0.21	7.15±0.14			
60	7.22±0.27	7.20±0.08	7.11±0.10			
100	7.23±0.12	7.01±0.14	7.23±0.09			
L. casei BT 1268		Control[a]=7.12±0.18		0.035	0.008	<0.001
20	6.88±0.06	6.96±0.15	7.20±0.08			
60	6.93±0.11	7.08±0.03	7.05±0.11			
100	7.19±0.18	7.01±0.09	6.98±0.03			
L. casei BT 2113		Control[a]=7.16±0.05		<0.001	<0.001	0.424
20	6.67±0.21	7.06±0.06	6.98±0.25			
60	6.90±0.03	7.35±0.12	7.05±0.15			
100	6.87±0.13	7.19±0.15	7.01±0.08			
L. casei FTDC 8033		Control[a]=6.97±0.11		0.027	0.138	0.399
20	6.88±0.05	7.21±0.10	7.18±0.09			
60	7.01±0.14	7.22±0.17	7.27±0.21			
100	6.93±0.09	7.17±0.25	7.04±0.15			
L. gasseri FTDC 8131		Control[a]=6.82±0.16		<0.001	<0.001	0.722
20	6.91±0.11	6.93±0.08	6.89±0.11			
60	6.95±0.18	7.04±0.12	7.17±0.22			
100	7.10±0.21	7.11±0.26	7.09±0.09			
L. rhamnosus FTDC 8313		Control[a]=7.17±0.25		<0.001	<0.001	<0.001
20	7.32±0.27	6.98±0.12	7.26±0.15			
60	7.35±0.22	7.10±0.05	7.18±0.10			
100	7.32±0.28	7.19±0.18	7.29±0.19			
L. casei BT 8633		Control[a]=7.28±0.19		<0.001	<0.001	0.047
20	7.25±0.21	7.31±0.05	7.22±0.13			
60	7.10±0.16	7.08±0.06	7.11±0.09			
100	7.12±0.25	7.15±0.12	7.12±0.16			

Results are expressed as mean±standard deviation. The values are means of repeated measurements from three separate runs (n=3).

[a] Control: untreated cells

In this study, the ultrasonication parameters used were sublethal, thus would not have terminated or killed all cells. Even though there are many benefits in utilising ultrasonication to obtain higher yields of HA, inactivation of the released bioactive compounds and cell lysis may nevertheless occur (Lateef et al., 2007). This explains the detrimental effects of ultrasonic treatment at 100% amplitude on the growth of certain lactobacilli strains studied. Ultrasonication has been reported to cause increased structural changes to the cells leading to the interference of regular cellular functions, such as nutrient transport and cell division as well as the disruption of the phospholipid membrane (Yeo & Liong, 2011). However, for certain strains, such as *L. gasseri* FTDC 8131 and *L. rhamnosus* FTDC 8313, cell viability increased after treatment at 100% amplitude. Our results also showed that a higher viability was observed upon treatment at higher amplitudes and longer durations. Ewe and co-workers (2012) mentioned that since lactobacilli exhibit aggregating properties in nature, ultrasonication often de-clump bacterial clusters thereby increasing the number of viable cell counts upon fermentation.

3. Conclusions

In conclusion, the permeability and fluidity of the membrane play significant role in influencing both extracellular HA concentration and cellular viability. Upon ultrasonic treatment, HA concentration increased significantly ($P<0.05$) in all strains of lactobacilli studied.

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