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The effect of electroporation on the growth characteristics and antimicrobial activity of lactic acid bacteria

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ABSTRACT

Aims: This study aimed to evaluate the effect of electroporation on the growth characteristics and antimicrobial activity of lactic acid bacteria (LAB) including *Bifidobacterium longum* ATCC 15707, *Lactobacillus acidophilus* ATCC 314, *Lactobacillus casei* ATCC 393 and *Lactobacillus fermentum* ATCC 14931.
Methodology and results: Electroporation with the strength of electric field at 1.0–3.0 kV/cm for 2-4 millisecond were applied on the bacterial cultures. All bacterial cultures showed significant (P<0.05) increased in cell viability (40%-325%) upon electroporation. Such treatment also increased the acidity of the cell where the pH of cells decreased upon treatment. In tandem with the increased viability, electroporated bacterial cultures also showed higher proteolytic activity compared to the control (P<0.05). The electroporation treatment also increased (P<0.05) the bacteriocin activity of treated cells compared to the control. However, the molecular weight of bacteriocins produced were not affected by electroporation. Treated cells also possessed better antimicrobial activity. According to the results collected, all treated LAB strains showed 11.5%-113.8% higher (P<0.05) inhibitory activity compared to untreated control against tested pathogenic bacteria, *Escherichia coli* and *Listeria monocytogenes* that commonly associated with food contamination. Microarray data analysis showed that electroporation regulated the entities encoding for surface protein and transporter.
Conclusion, significance and impact of study: The results from this study suggested that electroporation could enhance the growth characteristics and antimicrobial activity of LAB by modifying the surface regions of the cells. This result may serve as the reference for food manufacturers to opt for effective biopreservation method and produce food with extended shelf life.

Keywords: Lactic acid bacteria, bacteriocin, electroporation, growth, antimicrobial

INTRODUCTION

Lactic acid bacteria (LAB) are common occupant in our gastrointestinal tract and the most common LAB available are from the genera of bifidobacteria and lactobacilli (Smith and Jones, 2012). This type of bacteria has been included in various products and shown to exert probiotic properties. Probiotics are living organism which has the ability to confer health benefits to the host through modulating of intestinal microbiota when taken in sufficient dosage (Gutiérrez *et al*., 2016). In addition to its capability to regulate gastrointestinal microbiota composition by inhibiting the growth of undesirable pathogens, probiotics can also cause stimulation to the intestinal barrier capacity against foreign antigens and exert immunomodulatory effect including phagocytosis, activation of humoral immunity and the formation of anti-inflammatory substances (Masood *et al*., 2011). In order to be included in products, probiotics must be non-toxic, non-pathogenic, remain stable and can maintain a sufficient viable counts for extended period of storage even under harsh conditions (Yang *et al*., 2014).

Most of the beneficial effect of LAB are due to its capability in producing bacteriocins. Bacteriocins are ribosomally synthesized peptides that commonly exhibit active antibacterial activity especially against pathogens that is closely related to the species of the producer microorganisms. While the bacteriocins can exert strong antibacterial properties, the producer microorganism will not be harmed by the bacteriocins due to the protection

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by specific immunity proteins (Taheri *et al*., 2012). These antibacterial peptides are generally active against Gram-positive bacteria and are able to prevent the growth of several foodborne microorganism and slowdown spoilage (Barbosa *et al*., 2017). The bacteriocins produced by bifidobacteria and lactobacilli are more widely acceptable due to their safe use and efficiency in the food manufacturing industry. Many recent studies focus on the application of bacteriocins in food processing and/ or preservation, which aims to increase food storage time/ shelf life, treat pathogenic illness and even for cancer therapy and at the same time maintain human health. Hence, along with its food preservative effect, bacteriocins was suggested to have the potential to replace antibiotics in the future for treatment against pathogens with multiple drugs resistance. This has inevitably attracted a considerable attention and demand for higher yield of bacteriocins. However, past literature has reported that the production and yield of bacteriocin is usually low (<20%) (Zacharof and Lovitt, 2012). While the antimicrobial mechanism of action for bacteriocin has been extensively studied and documented previously (Yang *et al*., 2010), the mechanism of bacteriocin biosynthesis and secretion by LAB has not been completely elucidated and explored.

Some studies have demonstrated that the production of bacteriocins by LAB is associated with bacterial growth (Trinetta *et al*., 2008) and yield could be increased by altering the membrane properties during separation (Zacharof and Lovitt, 2012). In most cases, environmental factors that can stimulate the growth of LAB including the incubation temperature, pH of the media as well as availability and types of nutrients can influence the production and secretion of bacteriocins. de Arauz *et al*. (2009) demonstrated that nisin biosynthesis can varies throughout the growth phase and were associated with the change in the culture environment. To date, such modification of growth and separation techniques is still unable to achieve an ideal production of bacteriocins. Therefore, investigating the bacteriocin biosynthesis and targeting the most effective aspects on bacteriocin synthesis may seems necessary to improve the production rate.

Physical treatments have been extensively applied in the biotechnological processes involving biological cells. Electroporation has been documented to provide an efficient approach to affect the membrane structure, leading to increased growth of microbial cells (Yeo and Liong, 2012). Many of the metabolic activity and health beneficial effect of microorganism is growth related. Therefore, it is hypothesized that an enhanced in growth by electroporation may subsequently increase the production of various bioactive metabolites/ substance by the microorganism. However, extensive and irreversible membrane injury by electroporation could be harmful to the cell viability resulting in lower enzymatic activity. Hence, an appropriate application of sub-lethal dosage, duration and intensity of electroporation is an important requirement to preserve the viable counts of cells as well as maintaining their bioprocess applications. The most appropriate sub-lethal dosage and intensity may vary with the intended application (Hickisch *et al*., 2016).

Considering that the physical treatment may affect the membrane structure, there is possibility that the bacteriocin production which is membrane associated will also be affected by electroporation. However, there has been limited studies on the application of electroporation to enhance the bacteriocin production and antimicrobial activity. Therefore, the aim of the study is to evaluate the effect of electroporation on the bacteriocin production, growth characteristics and its antimicrobial activity.

MATERIALS AND METHODS

Bacterial cultures

Pure cultures of LAB including *Bifidobacterium longum* ATCC 15707, *Lactobacillus acidophilus* ATCC 314, *Lactobacillus casei* ATCC 393, *Lactobacillus fermentum* ATCC 14931 and pathogens including *Escherichia coli* and *Listeria monocytogenes* were obtained from the culture collection centre of Taylor's University (Selangor, Malaysia). All stock cultures were kept in sterile 40% (v/v) glycerol and stored at –20 °C prior to use. Lactobacilli and *Bifidobacterium* were activated by incubating for 24 h at 37 °C aerobically and anaerobically, respectively in sterile de Mann Rogosa Sharpe (MRS) broth (Merck, Billerica, USA) supplemented with 0.15% (w/v) L-cysteine-HCL (Merck, Billerica, USA) for three times consecutively prior to use. *Escherichia coli* and *Listeria monocytogenes* were activated in tryptone soya broth (TSB) (Merck, Billerica, USA) at 37 °C for 24 h prior to experimental use. Activated LAB were centrifuged at 4000× *g* for 10 min at 4 °C and the resultant cell pellets were collected. The cell pellets were washed with buffered peptone water (Merck, Billerica, USA) twice. The cell pellets were then resuspended with sterile MRS broth to achieve an OD_{600}=2.0.

Electroporation

The LAB cultures (in exponential phase) were electroporated by applying pulsed electric field at a field strength set at 1.0 kV/cm, 2.0 kV/cm or 3.0 kV/cm for a duration of 2.0 ms or 4.0 ms with a Micropulser (Bio-Rad Laboratories, Hercules, USA). The micropulser was equipped with a 2 mm electrode gap cuvette (Bio-rad Laboratories, Hercules, CA, USA) (Yeo *et al*., 2014). Upon treatment, 10% (v/v) treated cell cultures were immediately added into fresh sterile MRS broth and incubated at 37 °C for 24 h. Untreated cell cultures was used as the control.

Growth of lactic acid bacteria

Viable cell monitoring

Determination of viable cell count was done via pour plate method on MRS agar supplemented with L-cysteine (Boczek *et al*., 2014) and incubated for 24 h at 37 °C.

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Lactobacilli cells were plated aerobically, and *Bifidobacterium* cells were plated anaerobically. After incubation, the number of colonies formed were enumerated and reported as colony forming unit per mL (CFU/mL).

Determination of pH

The pH of the media containing treated and control culture were determined using Oakton pH 700 benchtop pH meter equipped with glass electrode (Oakton Instruments, USA). Prior to analysis, the pH meter was first calibrated with standard solution of pH 4.01, 7.00 and 10.01.

Proteolytic activity and bacteriocin production

Measurement of antimicrobial activity

The antimicrobial activity of the samples against selected pathogens were evaluated by disc diffusion assay (Tham *et al*., 2011). The activated pathogens were adjusted to OD_{600}=0.3 in sterile buffered peptone water. The activated pathogens (0.1 mL) were spread evenly on tryptone soya agar (TSA). Supernatant (crude extract of bacteriocins) were collected after centrifugation of cell cultures at 4000× *g* for 10 min. Supernatant were slowly (20 µL) dispensed and absorbed into the sterile filter paper disc (6 mm in diameter). Thereafter, sterile filter paper disc containing 20 µL of supernatant were gently placed on the surface of TSA. All TSA plates were incubated at 37 °C for 24 h. After the incubation period, the antimicrobial activity of the crude extract of bacteriocins (released by LAB) was measured and expressed as the diameter of zone of inhibition (mm).

Measurement of bacteriocins protein concentration

Treated and untreated LAB (OD_{600}=2.0) samples (5 mL) were grown in 50 mL of MRS broth and were incubated at 37 °C for 24 h. The sample was centrifuged at 4000× *g* for 10 min at 4 °C and supernatant was collected as crude extract. The crude extract was then precipitated with 50% (v/v) methanol to obtain concentrated bacteriocin. The addition of 50% methanol was done gradually using a magnetic stirrer until precipitates were formed. The mixture was then centrifuged at 4500× *g* for 30 min at 4 °C and the precipitate was collected. The precipitate (concentrated bacteriocin) was then dissolved in 2 mL of 0.1 M phosphate buffer at pH 7 (Arfani *et al*., 2017).

Bacteriocin activity of the concentrated bacteriocin were measured using Bradford assay method where it measures the concentration of the protein in the precipitated bacteriocins (Arfani *et al*., 2017). Precipitated bacteriocin was reacted with Bradford reagent at 1:3 ratio (v/v), vortexed and allowed to stand for 15 min at room temperature (25 °C). Protein content was measured using a spectrophotometer at a wavelength of 595 nm (Arfani *et al*., 2017). The protein concentration was calculated based on a linear regression from Bovine Serum Albumin (BSA) as protein standard.

Bradford reagent was prepared by dissolving 0.0033 g of Coomassie Brilliant Blue G-250 (Merck, Darmstadt, Germany) in 10 mL of 95% (v/v) ethanol. A 34 mL of 85% (v/v) phosphoric acid was then added to the solution prior to homogenization by vigorous shaking. The resultant solution was then filtered with Whatman™ filter paper (Grade 1, Whatman, Dassel, Germany) and kept in dark bottles at room temperature. The prepared Bradford reagent was diluted with distilled water (1:5 v/v) before use for analysis to reduce oxidation (Bradford, 1976).

Measurement of bacteriocin molecular weight

The bacteriocin molecular weight were determined with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Proteolytic activity

The proteolytic activity of LAB were determined with spectroscopic o-phthaldialdehyde (OPA) method (Church *et al*., 1985). The ammonia groups that were being released from amino acids and peptide is an indication of proteolysis. This reaction is specific for primary amines in amino acids, peptides and proteins.

Briefly, treated and control samples were centrifuged at 4000× *g* for 10 min at 4 °C and 150 µL of the supernatant were collected and added to 3 mL of OPA reagent. The mixtures were subsequently incubated at room temperature (25 °C) for 2 min. The absorbance of the solution was measured spectrophotometrically at 340 nm. The proteolytic activity of LAB were expressed as absorbance at 340 nm. Sterile media without any cell culture were used as blank (Donkor *et al*., 2007).

The OPA reagent was prepared by mixing 50 mL of 100 mM sodium tetraborate (Merck, Darmstadt, Germany), 200 µL of β-mercaptoethanol (Sigma- Aldrich, USA), 5 mL of 20% (w/v) sodium dodecyl sulphate (SDS) (Merck, Darmstadt, Germany) and 80 mg of OPA (Merck, Darmstadt, Germany) (dissolved in 1 mL of methanol). This reagent was prepared fresh prior to analysis (Zhu *et al*., 2009). All the analyses were carried out in triplicates.

Microarray data analysis

The most prevalent strain (*L. acidophilus*) along with the best electroporation intensity (3 kV/2 ms) were selected and it were subjected to microarray data analysis using GeneSpring Analysis (Agilent, USA).

Isolation of RNA

Isolation of RNA from cell cultures were performed through RNA extraction where it removes proteins, lipids and DNA from the RNA sample. Briefly, total RNA isolation reagent (Agilent, USA) was used to breakdown the cells so that nucleic acids were released from the cells. RNA is then recovered by alcohol precipitation and

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washed with 75% (v/v) ethanol to remove any salt residual. RNA purity was assessed by spectrophotometer at 280 nm (Bowtell and Sambrook, 2003).

GeneSpring analysis

Following the manufacturer's instruction, the concentration of RNA samples was normalized to 50 ng before preparation of Cyanine-3 (Cy3) labelled cRNA using the One-Color Low Input Quick Amp Labeling Kit (Agilent, USA). Cy3 labeled cRNA was then purified by RNeasy Mini Kit (Qiagen, Valencia, CA) to purify the amplified cRNA samples. Dye incorporation and cRNA yield were checked with NanoVue™ Plus Spectrophotometer (GE Healthcare, UK) at a wavelength of 280 nm. A 0.825 µg of Cy3-labeled cRNA with specific activity ≥6 pmol Cy3/ug cRNA was fragmented at 60 °C for 30 min in a reaction volume of 25 µL containing 25× Agilent fragmentation buffer and 10× Agilent blocking agent following the manufacturer's instructions (Agilent, USA). Upon completion of fragmentation step, 25 µL of the mixture were then combined with 25 µL of 2× Hi-RPM Hybridization Buffer. This was then immediately hybridized to Agilent SurePrint G3 Custom Gene Expression v3 8x60K at 65 °C for 17 h in a rotating Agilent microarray hybridization oven (Agilent, USA). Microarrays were washed after hybridization at room temperature for 1 min with Agilent GE Wash Buffer at 37 °C for 1 min with Agilent GE Wash buffer 2. Slides with a total of 1250 entities were immediately scanned with Agilent SureScan Microarray Scanner of 3 µm resolution. The setting was set at 532 nm (Cy3) wavelength and extended dynamic range (10-100 %). Agilent Feature Extraction Software were used to extract normalized intensities and all the data (in .txt format) files were recorded. The .txt files were then uploaded in Agilent GeneSpring Software for analysis (Agilent, USA).

Statistical analysis

Data in this study were analyzed with version 23.0 SPSS Inc. Software (SPSS Inc., Chicago, Illinois, USA). Statistical difference between sample means were analyzed using one way ANOVA. The level of significance was pre-set at 95% confidence level (α=0.05).

RESULTS AND DISCUSSION

Bacterial cultures

Many LAB have been shown to produce bacteriocins, peptides with antimicrobial activity (Taheri *et al*., 2012). Studies found that bacteriocins have great potential for the use in food industry, to prevent the spoilage of food and also clinical potential due to its activity against human and animal pathogens. Many researches have reported that the production of bacteriocin is growth related and viability of bacterial cultures plays an important role.

Cell viability

Electroporation has been widely applied to increase cell membrane permeability. This is mainly due to electric field induced tension and electrochemical compression that cause localized instabilities in the cellular membrane of microorganisms, forming pores in the cellular membrane (Ewe *et al*., 2012). These transient permeabilization caused by electroporation allows the delivery of nutrients into the cell and at the same time enhance the removal of intracellular protein by microorganism (Kanduser *et al.*, 2006). This lead to the widely use of electroporation in the field of biotechnology which mainly focusses on the introduction of foreign compounds molecules into cells and the removal of molecules including proteins within the cells. This approach involves creation of permeable and diffusible membrane by application of high intensity but short duration pulse electric field on the cells which will cause electric potential difference across the cellular membrane (Rols, 2006). Upon removal of the pulsed electric field, the cell permeabilization state is reversible, thus, most of the cells will recover from its injury at the membrane and resume back to its normal physiological activity (Rols, 2006). However, when electric pulse exceeds its critical threshold value, cell membrane will not recover and results in membrane disruption and cell death (Russell *et al*., 2000). Therefore, selection of intensity and duration of electroporation is very important in maintaining the viability of the cell.

In our study, the cell viability of LAB was promoted by electroporation treatment (Table 1). LAB treated with 1-3 kV for 2 ms and 4 ms showed increased viability compared to the control (P<0.05) after 24 h fermentation. Other studies have also reported a strain-dependent effect with a higher intensity of electroporation (5-7.5 kV). Yeo *et al*. (2014) demonstrated that electroporation at 7.5 kV for 3.5 ms significantly promoted the growth of lactobacilli and bifidobacteria due to enhanced membrane permeability. Additionally, Lye *et al*. (2011) also reported that electric field strength at 5.0-7.5 kV increased the growth of *L. acidophilus.*

Based on the collected data (Table 1), all electroporated LAB showed increased viability by 40%-325 % (P<0.05) when compared to its control after fermentation at 37 °C for 24 h. In this study, it was demonstrated that the cell viability of LAB increased proportionally with the intensity of pulsed electric field (1–3 kV) especially when treated for 2 ms. All treated cells showed best viability when it is treated at 3 kV for 2 ms (3 kV/2 ms) as compared to treatment at other parameters. This agrees with the study conducted by Lye *et al*. (2011) who stated that a greater membrane permeabilization effect (P<0.05) was observed in cells when they were electroporated at a field strength of 7.5 kV/cm. This could be due to the electrical charges that causes current accumulation and voltage transmission across the membrane. Changes in membrane voltage will then lead to formation of pores and changes to the structure and membrane permeability (Deng *et al*., 2003). Following the alteration of membrane permeability, the uptake of

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nutrients from media also increases thus showing increased cell viability.

Najim and Aryana (2013) reported that mild pulsed electric field (1 kV/cm, 0.5 sec) significantly improved the growth of *L. acidophilus* compared with the control. Since *L. acidophilus* has been widely used as an adjunct culture in approximately 80% of the yogurts in the United States, it is important to identify the best pulsed electric field which can be used for pre-treating cultures to enhance these desirable attributes (Najim and Aryana, 2013). In our results, *L. acidophilus* showed the highest reaction against electroporation with *L. acidophilus* showing an increase of 325% (P<0.05) in viability upon treatment at 3 kV/2 ms. This was then followed by *L. fermentum*, *L. casei* and *B. longum*. Thus, suggesting that this treatment can be used to increase the viability of the common adjunct culture used in yogurt and other products.

The duration of treatment carried out in this study is 2 ms and 4 ms, respectively. Based on the results collected, when comparing cells treated at the same intensity but different duration, it showed that the cells treated at 4 ms always have generally lower viability compared to the cells treated at 2 ms upon fermentation for 24 h. This is in accordance with the result reported by Lye *et al*. (2011) that *L. acidophilus* showed a decrease (P<0.05) in membrane permeability upon treatment at a higher field strength and longer treatment time (7.5 kV/cm for 4 ms). Generally, our result showed that electroporated cells were able to proliferate better when treated at all studied intensity for a shorter duration (2 ms) compared to longer duration (4 ms). This effect is obviously observed in *L. acidophilus* ATCC 314 where cells treated for 2 ms showed higher (P<0.05) cell viability (~2 fold) compared to cells treated for 4 ms. This could be due to the fact that the intensity and duration of treatment applied will affect the degree of membrane organizational changes (Rols *et al*., 1990). The alteration of membrane organization upon electroporation affected cell enzyme activities and pH homeostasis that subsequently led to slower cell growth (Russell *et al*., 2000). Therefore, this could be the reason for lower cell viability of LAB electroporated for 4 ms compared to 2 ms in this study.

Determination of pH

Based on the results collected (Table 2), it showed that the media containing electroporated LAB exhibited a slightly lower pH (P<0.05) when compared to that of untreated control. It was shown that the pH of the media after fermentation is inversely proportional to the intensity of the treatment. From this study, electroporation at 3kV gave a more promising effect in decreasing the pH of the cells. As the intensity of treatment increases, pH reading decreased likely due to organic acids production.

Generally, lower pH was observed when the cells were treated at 3 kV/2 ms compared to treatment at other parameter. This agrees with the result obtained from cell viability. Cell viability is directly correlated with the metabolic activity which may increase production of organic acid, leading to lower pH. This was in alignment with the study carried out by Seratlić *et al*. (2013), when *L. plantarum* cells were exposed to pulsed electric field (below 12 J/cm^3), acidification activity increased and led to decrease in pH of cells. Reversible pore formation leads to the assimilation of certain nutrients from the medium to the cell, which probably affects the growth and cell metabolism upon electroporation (Seratlić *et al*., 2013). The decreased in pH upon electroporation in this study is indeed a favorable phenomenon where such pH may exert antimicrobial effect due to the accumulation of organic acids by LAB (Ajao *et al*., 2018).

Antimicrobial activity

According to Table 3, all strains studied exhibited inhibitory activity against *E. coli* and *L. monocytogenes* and the inhibitory effect was enhanced (P<0.05) upon treatment with electroporation. As the intensity of electroporation increased, the antimicrobial activity of LAB increased. Majority strains showed best antimicrobial activity when the cells were treated at an intensity of 3 kV for a duration of 2 ms (3 kV/2 ms). The antimicrobial activity of LAB against *L. monocytogenes* and *E. coli* was 26.7%-92.9% and 11.5%-113.8% higher compared to the control, respectively (P<0.05). These results indicated that our LAB were able to synthesize inhibitory substances with antimicrobial activity. In a study carried out to investigate the metabolic activity of *Lactobacillus plantarum* upon electroporation, the authors reported that the heat production rate of electroporated cells were higher, indicating the enhanced metabolic activity of treated cells (Seratlić *et al*., 2013). The increased metabolic activity lead to stimulation of antimicrobial metabolites production and thus increases the antimicrobial activity. Yeo *et al.* (2014) claimed that the production of antimicrobial compounds was associated to the growth of cells. This trend was also observed in our study, where the treated cells with higher viability compared to control also exerted a higher antimicrobial effect.

Bacteriocin activity

Electroporation significantly increased (P<0.05) the bacteriocin activity of LAB especially lactobacilli strains (Figure 1). The bacteriocin activity of LAB was promoted by 8.64%-166.42% (P<0.05) compared to the control upon treatment with electroporation at different parameters. The most significant increase in bacteriocin activity was shown by *L. acidophilus* (131.85%-166.42% higher than control), while *B. longum* showed the least increase in bacteriocin activity (8.64%-70.37% higher than control). Studies reported that electroporation at the early stage of the fermentation may increase the concentration of bacteriocins in the environment to the level that required for auto phosphorylation of histidine protein kinase (Loghavi *et al.*, 2008).

Based on the presented chart (Figure 1), it showed that all LAB cells treated with electroporation at 3 kV/2 ms produced the highest bacteriocin activity when compared

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Table 1: Viability of control- and electroporated- lactic acid bacteria after fermentation at 37 °C at 24 h.

Strains	Log_{10} CFU/mL						
	Intensity of electroporation (kV/ms)						
	Control	1 kV/2 ms	1 kV/4 ms	2 kV/2 ms	2 kV/4 ms	3 kV/2 ms	3 kV/4 ms
L. acidophilus ATCC 314	9.86 ± 0.48^{dA}	10.37 ± 0.36^{abA}	10.04 ± 0.69^{cdB}	10.48 ± 0.99^{aA}	10.31 ± 0.84^{bA}	10.49 ± 1.27^{aA}	10.24 ± 0.49^{cA}
B. longum ATCC 15707	9.40 ± 1.25^{dD}	9.57 ± 0.64^{cdD}	9.55 ± 0.86^{cdC}	9.76 ± 1.13^{abC}	9.64 ± 1.49^{cD}	9.92 ± 1.11^{aD}	9.74 ± 1.41^{bC}
L. casei ATCC 393	9.58 ± 1.43^{dC}	10.00 ± 1.85^{cC}	10.04 ± 1.59^{cB}	10.10 ± 1.95^{bB}	10.08 ± 1.62^{bcC}	10.17 ± 2.32^{aC}	10.13 ± 0.96^{abB}
L. fermentum ATCC 14931	9.72 ± 1.10^{eB}	10.12 ± 2.26^{dB}	10.14 ± 1.14^{cdA}	10.21 ± 1.06^{bAB}	10.17 ± 2.32^{bcB}	10.26 ± 2.11^{aB}	10.16 ± 1.53^{cAB}

Results are presented in terms of means ± standard deviation of means in log_{10} CFU/mL; Values stated are means of triplicates from three separate runs (n=3).
^{abcde}Means in a specific row with different lowercase superscripts are significantly different (P<0.05).
^{ABCD}Means in a specific column with different uppercase superscripts are significantly different (P<0.05).

Table 2: pH change of control- and electroporated- lactic acid bacteria after fermentation at 37 °C at 24 h.

Strains	pH						
	Intensity of electroporation (kV/ms)						
	Control	1 kV/2 ms	1 kV/4 ms	2 kV/2 ms	2 kV/4 ms	3 kV/2 ms	3 kV/4 ms
L. acidophilus ATCC 314	3.76 ± 0.07^{aB}	3.23 ± 0.09^{Cc}	3.40 ± 0.04^{bD}	3.19 ± 0.15^{cC}	3.37 ± 0.16^{bC}	3.22 ± 0.18^{cD}	3.58 ± 0.23^{abC}
B. longum ATCC 15707	5.49 ± 0.08^{aA}	5.21 ± 0.19^{bA}	5.30 ± 0.06^{abA}	5.20 ± 0.06^{bA}	5.35 ± 0.02^{abA}	5.17 ± 0.05^{bA}	5.33 ± 0.26^{abA}
L. casei ATCC 393	4.54 ± 0.03^{aA}	4.20 ± 0.02^{bB}	4.25 ± 0.17^{bC}	4.28 ± 0.02^{bB}	4.22 ± 0.05^{bB}	4.12 ± 0.05^{bC}	4.17 ± 0.05^{bB}
L. fermentum ATCC 14931	5.46 ± 0.41^{aA}	5.21 ± 0.16^{abA}	4.75 ± 0.16^{cB}	5.14 ± 0.16^{bA}	5.20 ± 0.16^{abA}	4.94 ± 0.25^{cB}	5.17 ± 0.31^{bA}

Results are presented in terms of means ± standard deviation of means; Values stated are means of duplicates from three separate runs (n=3).
^{abc}Means in a specific row with different lowercase superscripts are significantly different (P<0.05).
^{ABCD}Means in a specific column with different uppercase superscripts are significantly different (P<0.05).

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Table 3: Zone of inhibition (mm) of control- and electroporated-lactic acid bacteria after treatment.

Strains	Pathogens	Zone of inhibition (mm)						
		Intensity of electroporation (kV/ms)						
		Control	1 kV/2 ms	1 kV/4 ms	2 kV/2 ms	2 kV/4 ms	3 kV/2 ms	3 kV/4 ms
L. acidophilus ATCC 314	*E. coli*	7.00 ± 0.00^{cB}	8.50 ± 0.71^{bC}	9.00 ± 1.41^{bE}	11.00 ± 1.41^{abC}	10.50 ± 0.71^{abD}	12.00 ± 0.00^{aCD}	10.50 ± 0.71^{abAB}
	L. monocytogenes	7.50 ± 0.71^{cA}	11.00 ± 1.41^{abA}	9.50 ± 0.71^{bE}	10.50 ± 0.71^{abD}	10.50 ± 0.71^{abD}	11.50 ± 0.71^{aD}	10.50 ± 0.71^{abAB}
B. longum ATCC 15707	*E. coli*	6.75 ± 0.50^{dBC}	9.50 ± 1.73^{cB}	10.25 ± 3.30^{bD}	13.25 ± 4.27^{aA}	13.00 ± 4.76^{aB}	13.00 ± 3.83^{aBC}	10.25 ± 1.71^{bB}
	L. monocytogenes	6.50 ± 1.50^{dC}	8.50 ± 0.71^{cC}	11.00 ± 0.00^{abC}	11.00 ± 0.00^{abC}	12.00 ± 0.00^{aC}	11.50 ± 0.71^{aD}	10.00 ± 0.00^{bB}
L. casei ATCC 393	*E. coli*	6.50 ± 1.50^{dC}	7.25 ± 1.50^{cD}	11.75 ± 0.96^{abB}	12.00 ± 2.71^{aB}	12.25 ± 1.50^{aC}	12.50 ± 1.29^{aC}	10.75 ± 5.50^{bA}
	L. monocytogenes	7.00 ± 0.00^{dB}	9.50 ± 0.71^{cB}	11.00 ± 0.00^{bC}	10.50 ± 0.71^{abD}	10.50 ± 0.71^{ab}	13.50 ± 0.71^{aB}	11.00 ± 1.41^{abA}
L. fermentum ATCC 14931	*E. coli*	7.25 ± 1.26^{eAB}	8.75 ± 2.06^{dC}	10.50 ± 4.04^{cCD}	12.50 ± 2.38^{bB}	15.25 ± 2.75^{aA}	15.50 ± 4.36^{aA}	11.00 ± 2.94^{cA}
	L. monocytogenes	7.00 ± 0.00^{dB}	10.50 ± 0.71^{cA}	13.00 ± 1.41^{abA}	12.50 ± 0.71^{bB}	13.00 ± 1.41^{abB}	13.50 ± 0.71^{aB}	10.50 ± 0.71^{cAB}

Results are presented in terms means ± standard deviation of means; Values stated are means of duplicates from three separate runs (n=3).
Initial diameter of disc: 6 mm.
^{abcde}Means in a specific row with different lowercase superscripts are significantly different (P<0.05).
^{ABCDE}Means in in a specific column with different uppercase superscripts are significantly different (P<0.05).

to cells treated at other intensities and control (P<0.05). This agrees with the results obtained from cell viability as well as antimicrobial activity where cells treated at an intensity of 3 kV/2 ms showed higher (P<0.05) cell viability and better antimicrobial properties. This suggested that electroporation induced the bacteriocin activity of LAB by stimulating their growth, which subsequently lead to an increased in antimicrobial activity.

Bacteriocin molecular weight

Based on the results obtained from SDS-PAGE (Figure 2), it showed that the molecular weight of bacteriocin produced were not affected by electroporation. All bacteriocins produced by LAB upon treatment with electroporation in this study were of the same class when compared to the control. Single protein band was observed at 14.2-20 kDa. This molecular weight can be classified into Class III bacteriocins. Class III bacteriocins are large, heat-labile (>10 kDa) protein bacteriocins (Mokoena, 2017).

Proteolytic activity

Lactobacilli and bifidobacteria possess a complex proteolytic system that allows them to hydrolyse protein for growth. This is one of the basic metabolic activities of LAB. This proteolytic system includes extracellular proteinases which plays a vital role in the breakdown of proteins into small peptides that were subsequently degraded into amino acids (Cao *et al*.,

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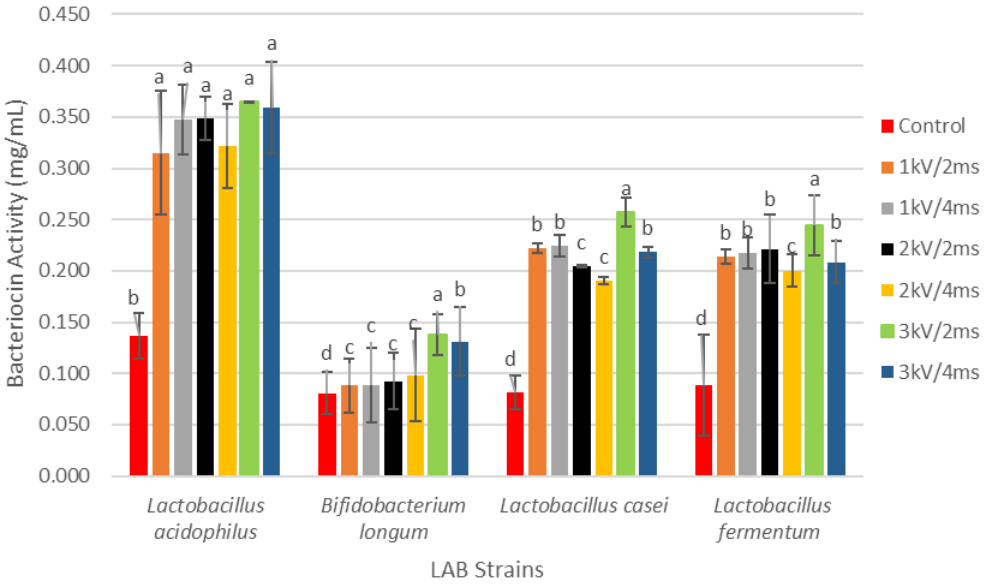

Figure 1: Bacteriocin activity (mg/mL) of lactic acid bacteria treated with electroporation (1-3 kV, 2-4 ms). Data are presented in terms of means ± standard deviation of means (mg/mL). [abcd] Means with different lowercase superscripts are significantly different (P<0.05).

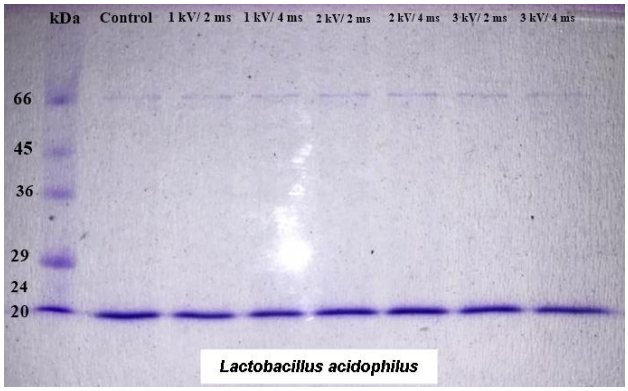

Figure 2: Molecular weight of bacteriocin produced by control and electroporated *L. acidophilus*.

2019). Amino acids are important elements for growth, reproduction and maintenance of organisms. Numerous studies have been carried out on the extracellular proteolytic activities of LAB as a promising tool to hydrolyse extracellular protein molecules into free amino acids, which poses great potential for functional amino acid production (Toe *et al*., 2019). Based on the Table 4, all LAB strains exhibited proteolytic activity. All LAB strains showed different degree of proteolysis based on the liberation of amino acids and peptides. All electroporated LAB showed higher proteolytic activity (P<0.05) when compared to its control. This is in line with the studies carried out by Najim and Aryan (2013) where the study reported that mild pulsed electric field significantly enhanced the protease activity of *L. acidophilus*. Overall, all LAB samples treated at an intensity of 3 kV/2 ms showed highest proteolytic activity (P<0.05). This was most obvious in *L. acidophilus*, where the proteolytic activity was 259.1% higher (P<0.05) than that of the control. Previous study reported by Ng *et al.* (2008) stated that proteolytic activity of lactobacilli and bifidobacteria was directly growth-associated. This is in accordance with the trend of results obtained from viable cell monitoring. As a result, the increased proteolytic activity upon electroporation was most likely attributed to the enhanced growth of LAB strains. It is also likely that the electroporation has caused structural changes that lead to leakage of protease enzyme to the extracellular environment, thus increased its proteolytic activity. Pillar and Antonio (2006) also suggested that mild pulse electric field could originate small conformational changes on LAB cells leading to enhanced proteolytic activity. Based on the data collected (Table 4), it showed that shorter treatment duration resulted in better proteolytic activity when compared to longer duration. The results obtained is consistent with the trend of results obtained from viable cell monitoring.

Microarray data analysis

Microarray data analysis was carried out using GeneSpring Analysis. GeneSpring analysis is a powerful and accessible statistical tool specifically for intuitive data analysis and visualization. The analysis were not carried out on all strains of LAB. However, only the most prevalent strain together with the most prevalent intensity were chosen to run the analysis. In this case, *L. acidophilus* treated with electroporation at 3 kV/2 ms was selected for analysis.

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Table 4: Proteolytic activity of control- and electroporated-lactic acid bacteria.

Strains	Proteolytic activity (U)						
	Intensity of electroporation (kV/ms)						
	Control	1 kV/2 ms	1 kV/4 ms	2 kV/2 ms	2 kV/4 ms	3 kV/2 ms	3 kV/4 ms
L. acidophilus ATCC 314	0.22 ± 0.02^{dB}	0.57 ± 0.06^{bA}	0.27 ± 0.04^{dC}	0.71 ± 0.12^{abA}	0.49 ± 0.04^{cA}	0.79 ± 0.08^{aA}	0.46 ± 0.10^{cB}
B. longum ATCC 15707	0.17 ± 0.05^{cC}	0.25 ± 0.03^{bcC}	0.22 ± 0.01^{bcD}	0.32 ± 0.01^{bC}	0.31 ± 0.02^{bB}	0.40 ± 0.02^{aC}	0.30 ± 0.06^{bD}
L. casei ATCC 393	0.26 ± 0.07^{cAB}	0.56 ± 0.05^{aA}	0.47 ± 0.09^{bA}	0.48 ± 0.04^{bB}	0.47 ± 0.07^{bA}	0.50 ± 0.09^{abB}	0.53 ± 0.03^{aA}
L. fermentum ATCC 14931	0.30 ± 0.03^{cA}	0.37 ± 0.03^{abB}	0.35 ± 0.01^{bB}	0.40 ± 0.08^{abBC}	0.34 ± 0.03^{bB}	0.47 ± 0.02^{aB}	0.37 ± 0.05^{abC}

Results are presented in terms of means ± standard deviation of means; Values stated are means of duplicates from three separate runs (n=3).
^{abcd}Means in a specific row with different lowercase superscripts are significantly different (P<0.05).
^{ABCD}Means in a specific column with different uppercase superscripts are significantly different (P<0.05).

Based on the report obtained from GeneSpring analysis, it showed that *L. acidophilus* ATCC 314 treated with electroporation at 3 kV/2 ms up or down-regulated some of the entities by at least one-fold when compared to the control. Figure 3 shows some of the selected genes that were significantly (P<0.05) regulated by electroporation compared to the control. Whether it is up-regulated or down-regulated, it showed that physical treatment significantly (P<0.05) affected the expression of some of the genes of *L. acidophilus*. Based on the analysis, genes that were up- or down-regulated by at least one-fold when compared to the control includes, hypothetical protein, hydrogenase, acetyl-transferase, alpha-galactosidase, lactate oxidase, transcriptional regulator, esterase and transportase. Most of the genes that were significantly regulated are surface protein and transporter. Cell surface protein act as cellular marker which are embedded on the layer of cell membranes whereas transportase act as a transport protein which allows solutes to flow into and out of the cells (Bausch-Fluck *et al*., 2015). Previously, Yeo and Liong (2012) postulated that physical treatment on *Lactobacillus* bacteria increased the growth of lactobacilli by affecting its membrane permeability which induce more efficient nutrient transfer. This postulation can be confirmed with this DNA microarray data showing effect of physical treatment on surface properties that the effect of treatment is mainly targeted on the membrane of LAB.

It can also be seen that the genes regulating proteolytic activity specifically peptidase were upregulated. This was in alignment with the increased proteolytic activity of LAB upon treatment as shown in the above section. Hence, this could be the reason for increased production of bacteriocin in this study.

Other genes that were up or down-regulated by less than one-fold were characterized as insignificant (P>0.05). Based on the report obtained from GeneSpring analysis, it showed that genes that were regulated by less than one-fold were mainly related to carbohydrate metabolism. There is no significant change to the carbohydrate metabolism of cells although a slight up/down regulation was observed after physical treatment. From this analysis, we can conclude that the effect of treatments is targeted primarily on the surface or membrane of LAB.

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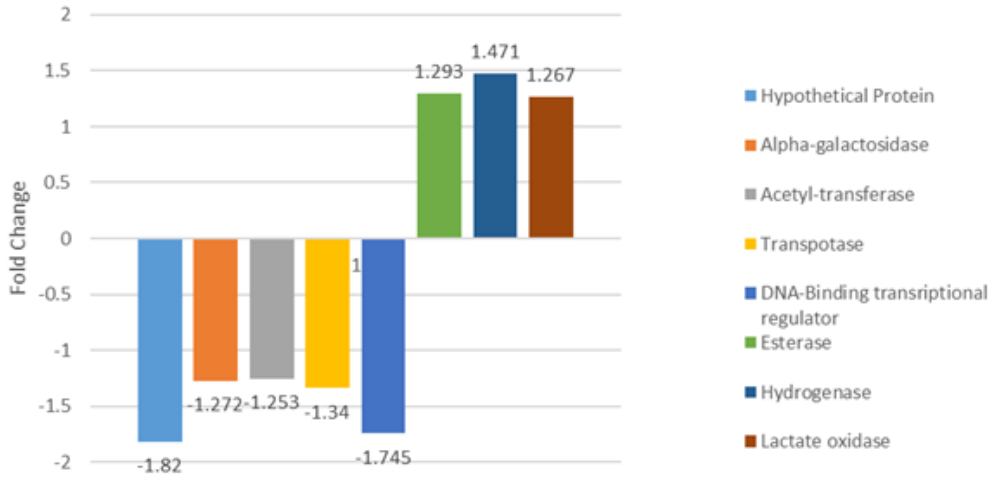

Figure 3: Selected genes of *L. acidophilus* that were significantly (*P*<0.05) regulated by electroporation at 3 kV/2 ms (fold changes compared to control).

CONCLUSION

In summary, this study showed that electroporation can promote the growth of LAB which lead to increased proteolytic activity and antimicrobial activity. This study also demonstrated alteration of the membrane properties by electroporation could increase the production of bacteriocins. This may promote the use of bacteriocin as biopreservation in food industry. Besides, the electroporated cells may also serve as a starter culture for more effective fermentation processes in food production. This will definitely benefits millions of people around the world through improved preservation method to produce safe food for consumption.

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