Effect of salt stress on morphology and membrane composition of *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium bifidum*, and their adhesion to human intestinal epithelial-like Caco-2 cells

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ABSTRACT

The effects of NaCl reduction (10.0, 7.5, 5.0, 2.5, and 0% NaCl) and its substitution with KCl (50% substitution at each given concentration) on morphology of *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium longum* was investigated using transmission electron microscopy. Changes in membrane composition, including fatty acids and phospholipids, were investigated using gas chromatography and thin layer chromatography. Adhesion ability of these bacteria to human intestinal epithelial-like Caco-2 cells, as affected by NaCl and its substitution with KCl, was also evaluated. Bacteria appeared elongated and the intracellular content appeared contracted when subjected to salt stress, as observed by transmission electron microscopy. Fatty acid content was altered with an increase in the ratio of unsaturated to saturated fatty acid content on increasing the NaCl-induced stress. Among the phospholipids, phosphatidylglycerol was reduced, whereas phosphatidylinositol and cardiolipin were increased when the bacteria were subjected to salt stress. There was a significant reduction in adhesion ability of the bacteria to Caco-2 cells when cultured in media supplemented with NaCl; however, the adhesion ability was improved on substitution with KCl at a given total salt concentration. The findings provide insights into bacterial membrane damage caused by NaCl.

Key words: potassium chloride substitution, phospholipids, transmission electron microscopy, CaCO-2

INTRODUCTION

Sodium chloride (table salt) is one of the most important food additives contributing to flavor, texture, and functional properties of food. With increasing awareness about diet and health, numerous attempts

to reduce salt intake have been undertaken due to the high risk of health diseases associated with high sodium intake (Buemi et al., 2002; Kotchen, 2005; Massey, 2005; Heaney, 2006; Albarracin et al., 2011). Substitution of NaCl with other salts, such as KCl, MgCl₂, and CaCl₂, could potentially reduce sodium chloride intake. Several studies have examined the effect of salt reduction on texture and sensory properties of dairy products (Ayyash and Shah, 2011); however, limited literature exists on the changes in structure and membrane composition of dairy bacteria and their functionality as affected by NaCl reduction and its substitution with KCl.

In various food processes, bacterial cells are constantly exposed to different kinds of environmental stresses. Depending on the degree of stress encountered, the bacterial cells develop adaptive responses that allow them to survive in unfavorable conditions. The adaptive mechanisms adopted by the bacteria involve gene regulation, which in turn leads to alterations in phenotypic and physiological characteristics. Morphological changes in bacteria that alter the cell shape are associated with the adaptive mechanisms of bacterial survival (Pianetti et al., 2009). The stability and permeability of cellular membranes play a fundamental role in the adaptation of bacteria to environmental stress. These membrane characteristics are closely associated with the membrane lipids and fatty acid composition. As a response to acidic, osmotic, oxidative, and thermal stress, several changes occur in the cell membrane, particularly in the lipids and fatty acids (FA) of the membrane (Murga et al., 2000; Guerzoni et al., 2001).

Furthermore, in addition to their survival and adaptation to the environmental stress, it is important for the bacteria to maintain their functional properties. Adhesion to intestinal cells is considered one of the important selection criteria for probiotics and is a prerequisite for bacterial colonization. Adhesion is also very important for bacterial-host interactions and for the bacteria to be able to confer their health benefits (Bermudez-Brito et al., 2012). The adhesion ability of bacteria largely depends on their membrane proteins (adhesins), which mediate the attachment of bacteria

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to intestinal mucus layer. Furthermore, exposure to environmental stress or any change on the bacterial membrane surface may alter the adhesion of bacteria to the intestinal cells (Buck et al., 2005). However, before investigation of their functional properties in a complex food matrix with environmental stress, it is important to evaluate their stress responses in a less complex media with limited interfering factors. To the best of our knowledge, no study has evaluated the effect of salt stress on adhesion ability of these potential probiotic bacteria. The objectives of our study were to investigate the effects of NaCl and its substitution with KCl on the viability, morphology, membrane composition, and adhesion ability to Caco-2 cell line of 3 potential probiotic bacteria: *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium longum*.

MATERIALS AND METHODS

Bacterial Cultivation and Experimental Design

Lactobacillus acidophilus (CSCC 2400), *Lb. casei* (ASCC 290), and *B. longum* (CSCC 5089) were obtained from the Australian Starter Culture Collection (Dairy Innovation Australia, Werribee, Australia) and were stored at -80°C . The organisms were activated in sterile de Man, Rogosa, and Sharpe (MRS) broth (Becton Dickinson and Company, Franklin Lakes, NJ) by 1% (vol/vol) inoculation and then incubation at 37°C for 24 h. The activated organisms were used after 3 successive transfers in sterile MRS. The MRS broth was supplemented with various salt concentrations (10.0, 7.5, 5.0, and 2.5% NaCl), and at each salt concentration 50% of NaCl was substituted with KCl. Bacteria were grown individually in MRS broth with and without NaCl and KCl for 24 h at 37°C before each analysis, unless stated otherwise.

Bacterial Cell Viability

Cell viability of all bacteria subjected to varying NaCl concentrations was determined as described before (Gandhi et al., 2014). Briefly, serial dilutions of each sample were made in sterile peptone water (0.15% wt/vol) and were spread on MRS agar plates for *Lb. acidophilus* and *Lb. casei*, and MRS-cysteine agar for *B. longum*. The plates were incubated for 48 h at 37°C (in anaerobic jar for *B. longum*) and the colony-forming units were enumerated.

Transmission Electron Microscopy

The morphological changes in all 3 bacteria were evaluated in the highest NaCl concentration (10.0%

wt/vol) and 50% substitution with KCl at this salt concentration (NaCl:KCl = 1:1). The sections were prepared as described by Pianetti et al. (2009) with some modifications. The 3 bacteria were individually grown in MRS broth containing 0% NaCl/KCl (control), 10.0% NaCl, and 10.0% total salt (5.0% of each NaCl and KCl). After 24 h, the cells were centrifuged ($4,000 \times g$, 10 min, 4°C) and the cell pellet was resuspended in cacodylate buffer (0.1 M sodium cacodylate-HCl buffer, pH 7.4). The cell suspension was fixed in equal volume of 2.5% glutaraldehyde in cacodylate buffer for 8 h at 4 to 8°C . Second fixation was performed using 1% osmium tetroxide (OsO_4) in cacodylate buffer for 30 min at room temperature ($\sim 22^{\circ}\text{C}$). The cell pellet was immersed in 0.5 mL of prewarmed agar solution. The cell pellets were then subjected to successive dehydration on a rotary shaker as follows: 50% ethanol for 10 min, 70% ethanol for 10 min, 90% ethanol for 10 min, 100% ethanol 3 times, 20 min each, and propylene oxide 2 times, 10 min each. The samples were infiltrated with epoxy resin and propylene oxide mixture (1:1) for 1 h 30 min at 37°C , followed by infiltration in epoxy resin and propylene oxide mixture (2:1) overnight at room temperature, and infiltration with fresh epoxy resin for 1 h 30 min at 37°C with the help of vacuum oven. Samples were embedded in fresh epoxy resin and polymerized at 60°C overnight. Thin sections were cut using a diamond knife and stained with uranyl acetate and lead citrate. The sections, mounted on copper grid, were observed using Phillips CM100 electron microscope (Phillips, Eindhoven, the Netherlands).

Extraction and Analysis of Membrane FA by GC

Bacterial membrane proteins were removed with the help of proteinase K for efficient extraction of membrane lipids. The bacteria were grown as described earlier and the cell pellet was collected. The cells were washed twice with PBS buffer (pH 7.4) and resuspended in PBS buffer (0.5 g in 0.5 mL). Proteinase K (25 $\mu\text{g}/\text{mL}$, Sigma-Aldrich, St. Louis, MO) was added to the cell suspension in presence of 5 mM dithiothreitol and incubated for 30 min at 37°C . An aliquot (100 μL) of protease-inhibitor solution was added to stop the protease activity. The protease-inhibitor solution was prepared by dissolving a tablet of EDTA-free protease inhibitor (cOmplete, Roche Applied Science, Penzberg, Germany) in 10 mL of PBS (pH 7.4). The mixture was then centrifuged ($4,000 \times g$, 10 min, 4°C) to collect the cell pellet (0.5 g wet weight) for extraction of FA. Membrane FA were extracted and converted to FAME, as described by Sasser (1990). Decanoic acid (C10:0) was used as the internal standard and was added to the cell pellet before extraction and methylation. The ex-

tracted FA were concentrated by drying under nitrogen and redissolved in GC-grade hexane (Fisher Scientific, Pittsburgh, PA) before analysis.

Separation and Identification of FA by GC-MS

The separation and identification of FA was performed on a gas chromatograph (Agilent 6590N- 5973N GC-MS system; Agilent, Atlanta, GA) equipped with an Agilent 7694E auto-sampler and a capillary DB-wax column (30 mÅ, 0.25 mm id, 0.25 µm film thickness; J&W Scientific, Folsom, CA). The injection volume was 1 µL (splitless mode) and helium as carrier gas was used at a flow rate of 1 mL/min. The injector and detector were held at 250°C. The temperature was increased from 100°C (held for 1 min) to 190°C at a rate of 4°C/min, further increased to 235°C at a rate of 10°C/min, and finally increased to 250°C at a rate of 4°C/min (held for 4 min). The results were expressed as relative molar percentage (mol %) for each FA, and the ratio of unsaturated FA to SFA was also calculated (Zhao and Shah, 2014).

Extraction and Profiling by Thin Layer Chromatography

Enzymatic Digestion of Cell Wall and Cell Surface Proteins. The bacteria were grown as described earlier and the cell pellet was collected after 24 h and washed twice with sterile distilled water (Zhao and Shah, 2014). Approximately 0.5 g of cell pellet was suspended in 0.5 mL PBS (pH 6.2, ~0.18 M optimal for lysozyme; Dickman and Proctor, 1952) and incubated with lysozyme (1 mg/mL; Sigma-Aldrich) for 1 h at 37°C. The cell pellet was washed with and suspended in 0.5 mL of PBS (pH 7.4), followed by digestion with proteinase K (25 µg/mL) in the presence of 5 mM dithiothreitol (DTT) for 30 min at 37°C as described above. The cell pellet was collected and extraction process was performed under nitrogen to minimize oxidation.

Neutral and Acidic Extraction of Membrane Phospholipids. Two step extraction method was used for efficient extraction of major phospholipids (Özbalci et al., 2013). Neutral extraction was carried out by dissolving the cell pellet (collected after digestion of cell wall proteins) in 1,800 µL of chloroform/methanol (1:2, vol/vol) and gently mixing on a rotor for 60 min at room temperature (vortex for 1 min after every 10 min). The solution was centrifuged at $10,000 \times g$ for 5 min at 4°C and the supernatants were collected in a separate tube. Chloroform (600 µL) and 0.8% NaCl solution (1 mL) were added to the supernatant and mixed for 1 min using a vortex. The solution was centrifuged at $5,500$

$\times g$ for 5 min at 4°C and the lower organic phase was collected in a glass tube.

For acidic extraction, the cell pellet was resuspended in 1 mL chloroform/methanol/37% HCl (40:80:1, vol/vol) and gently mixed on a rotor for 30 min at room temperature (vortex for 30 s every 5 min). The tubes were transferred to ice and 250 µL of cold chloroform and 450 µL of cold 0.1 N HCl were added to each tube. The solution was mixed for 1 min using a vortex, and centrifuged at $5,500 \times g$ for 5 min at 4°C. The chloroform-rich phase from neutral and acidic extraction was pooled in a glass tube and dried under an N₂ stream. The weight of dried lipids was determined and stored in chloroform/methanol (90:10, vol/vol; 50 mg/mL) at -20°C until analysis.

Separation of Phospholipids by High-Performance Thin Layer Chromatography

Silica gel 60 thin-layer chromatographic plates (Merck, Darmstadt, Germany) were oven activated for overnight at 100°C before use. The plates were developed with developing solvent before spotting to remove impurities from the adsorbent layer. A filter paper was placed in the developing chamber which was then saturated with the developing solvent for 15 min. Phospholipids were separated on thin layer chromatography plates using chloroform/methanol/acetic acid/water (71:20:6.25:2.5, vol/vol) as the mobile phase. The bands were visualized by spraying with the following reagents: (1) 0.5% (wt/vol) vanillin in ethanol/concentrated H₂SO₄ (97:3, vol/vol) solution for all polar lipids (Rakhuba et al., 2009) and (2) ammonium molybdate/perchloric acid reagent for phospholipids (Nzai and Proctor, 1998). The bands detected with vanillin were much sharper in color and were scanned using Molecular Imager XR+ System (Bio-Rad, Hercules, CA), and analyzed in Image Lab (version 4.0, Bio-Rad). The relative quantity of each phospholipid was determined based on the density of the bands. The major phospholipids were identified by comparing the relative mobility of phospholipid standards and the lipid bands detected in samples. The following standard phospholipids (Sigma-Aldrich) were prepared by dissolving in chloroform/methanol (90:10, vol/vol) and used for identification: cardiolipin (**CL**), phosphatidylcholine (**PC**), lyso-phosphatidylcholine (**LPC**), phosphatidylethanolamine (**PE**), phosphatidylglycerol (**PG**), and phosphatidylinositol (**PI**).

Cell Line

The human intestinal epithelial-like Caco-2 cell line was obtained from the American Type Culture Collec-

tion (ATCC, Manassas, VA). The cells were maintained and cultured in Difco's minimum essential medium (DMEM; Gibco BRL, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and 1% mixture of penicillin-streptomycin solution (Gibco BRL). The cells were incubated at 37°C in a humidified incubator (US Air Flow-NuAire, Plymouth, MN) in an atmosphere of 5% CO₂ and 95% air. All cells used in this study were between 32 and 37 passages. Cells were subcultured at 80% confluence by trypsinization (0.25% trypsin-EDTA, Gibco BRL).

Determination of Adhesion Ability

The adhesion ability of bacteria to Caco-2 cells was measured by the cell adhesion assay as per the method of Parkar et al. (2008) with some modifications. Briefly, Caco-2 cells were seeded at approximately 1×10^4 cells per well in 12-well plates and incubated to obtain confluence before the assay (10–12 d). Bacteria were grown in MRS broth supplemented with varying salt concentrations as described earlier. The bacterial cell pellet was washed with PBS buffer (pH 7.2) and resuspended in antibiotic-free DMEM at a cell density of approximately 10^9 cfu/mL. The cell line monolayer was washed twice with PBS buffer to remove interference of antibiotics. The bacterial cell suspension was added to each well (100:1 bacteria:Caco-2 cells) and incubated at 37°C for 2 h. After the incubation period, the supernatant was removed and wells were gently washed with PBS to remove any nonspecifically bound bacteria. The Caco-2 monolayers were then trypsinized by addition of 0.25% trypsin-EDTA solution and the viable bacteria in each well were counted using appropriate agar plates as described earlier. Adhesion ability was expressed as percentage of bacteria adhered compared with the control using the following equation:

$$\text{Relative adhesion ability (\%)} = \left[\frac{\text{CFU}_S}{\text{CFU}_C} \times 100 \right],$$

where, CFU_S is the number of stressed bacterial cells adhered to Caco-2 cell lines and CFU_C is the number of normal bacteria adhered to Caco-2 cell lines.

Statistical Analysis

All experiments were replicated thrice and all analyses were carried out in duplicate. The data obtained were analyzed using one-way ANOVA at 95% level of significance using SPSS statistics software v 20.0 (IBM Inc., Armonk, NY). Post hoc analysis was performed to further investigate the difference between the means

at different NaCl and KCl concentrations (Oliveira et al., 2012).

RESULTS AND DISCUSSION

Bacterial Cell Viability

The effect of NaCl reduction and substitution on the viable cell count (log₁₀ cfu/mL) of bacteria is shown in Figure 1. An increase in NaCl concentration was inversely associated with the cell viability for all the 3 bacteria. Among the 3 bacteria, *B. longum* was the most affected by salt stress. The lowest viable cell count of *B. longum* was observed to be about 3 log₁₀ cfu/mL when exposed to 10.0% NaCl, whereas *Lb. acidophilus* and *Lb. casei* showed a significant ($P < 0.05$) reduction only after exposure to higher NaCl concentrations (7.5 and 10.0%). At a given salt concentration, substitution with KCl (50%) increased the viable cell count as compared with that of the bacteria grown in medium supplemented with only NaCl. The effect of substitution with KCl was more distinctly observed at higher total salt concentrations (7.5 and 10.0%). Viability of bacteria in stress environment is crucial for functionality of the bacteria in dairy products, particularly those containing high levels of salt or low moisture. Reduced viable count of bacteria was observed on increased exposure to salt stress, possibly owing to the injury caused by salt to the integrity of the bacterial membrane (Gandhi and Shah, 2015). However, KCl substitution relatively improved the cell growth indicative of the protective effect of potassium chloride.

Transmission Electron Microscopy

The morphological changes in the bacterial cells, owing to their growth in media supplemented with salt, were observed by transmission electron microscope (Figure 2). Elongation of bacterial cells grown at 10.0% NaCl (Figure 2b) was observed for all bacteria and was most distinctly observed in *Lb. acidophilus* and *B. longum*. For bacteria grown in media supplemented with 10.0% NaCl, the cell structure was irregular and the membrane showed several deformities. On observing through transmission electron microscopy, the cytoplasmic content appeared coagulated due to salt stress. On the other hand, for the bacteria grown in media supplemented with 10.0% total salt (5.0% of each NaCl and KCl; Figure 2c), these morphological changes were less distinct as compared with the control (0% NaCl/KCl; Figure 2a), possibly owing to the protection offered by KCl.

Morphological changes in microorganisms are visible indicators of their adaptation to environmental stress

Figure 1. Effect of NaCl reduction and substitution with KCl on viable cell count ($\log_{10}$ cfu/mL). Values are means $\pm$ SE of at least 3 replicates ($n \geq 3$). An asterisk (*) means values are significantly different ($P < 0.05$) from control (0%). LA = *Lactobacillus acidophilus*; LC = *Lactobacillus casei*; BL = *Bifidobacterium longum*. Color version available online.

and transmission electron microscopy is a powerful technique to observe such changes in bacterial cells. At high NaCl concentrations (10.0%), elongation of bacterial cells was observed, which is considered as a means of adaptation of the bacteria to unfavorable environmental conditions (McMahon et al., 2007). Elongation of the bacterial cell caused by adaptation to salt stress may be due to several reasons. Low water activity, possibly due to addition of salt in this case, is reported to affect DNA supercoiling, thereby altering the regulation of genes involved in cell division leading to filamentation (Graeme-Cook et al., 1989). The other possible mechanism may be due to the role of cellular turgor pressure during cell division (Csonka and Hanson, 1991), which explains that osmotic stress may alter the degree of cell hydration causing a lack of signals for cells to divide. Substitution of NaCl with KCl proved to be less stressful for the bacteria, as observed by the higher density of the cytoplasmic content and reduced elongation and irregularities in the cell membrane. This may be due to the weaker bonding of potassium ions to the membrane as compared with the sodium ions (Gurtovenko and Vattulainen, 2008), leading to reduced alterations in the cellular membrane.

FA Composition by GC

The relative percentages of major FA and the ratios of unsaturated to saturated FA (U/S) are presented in Figures 3 and 4, respectively. The FA content of the bacteria was altered due to exposure to salt stress. In all the 3 bacteria, a decrease in C14:0 was observed with increasing NaCl concentration; however, substitution with KCl increased the relative percentage of C14:0. Conversely, C16:0, the most abundant FA, was higher in bacteria subjected to high salt concentration. In general, the U/S ratio (Figure 4) increased at higher salt levels (7.5%); however, a slight decline in U/S was observed at 10.0% salt concentrations. The U/S ratio increased on substitution with KCl at a specific salt concentration. *Lactobacillus casei* showed the least difference (almost 1-fold increase) in U/S upon substitution at higher concentration (10.0%), whereas *B. longum* increased about 1.5 times on substitution. The increase in the U/S ratio when the bacteria were subjected to salt stress could be attributed to the increase in the degree of FA unsaturation as an adaptive measure. The trend of U/S ratio for *Lb. casei* was slightly different as compared with the other 2 bacteria, which may be indicative of its resistance to lower salt concentrations (2.5%).

Fatty acids are the major constituents of membrane glycerolipids, and the distribution of unsaturated FA and SFA and the fatty acyl chain conformation af-

Figure 2. Transmission electron micrographs (TEM) of thin sections of bacteria grown in (a) de Man, Rogosa, Sharpe broth (MRS), (b) MRS + 10.0% NaCl, and (c) MRS + 10.0% total salt (5.0% each of NaCl and KCl). Magnification of micrographs is 21,000 fold (black bars below are 200 nm; representative micrographs of bacteria in different conditions). LA = *Lactobacillus acidophilus*; LC = *Lactobacillus casei*; BL = *Bifidobacterium longum*. Arrows indicate membrane irregularities and shrinkage of cytoplasmic contents. Color version available online.

Figure 3. Relative membrane fatty acid composition as affected by NaCl reduction and substitution with KCl. Values are means $\pm$ SE of 3 replicates ($n = 3$). An asterisk (*) means values are significantly different ($P < 0.05$) from control (0.0%). LA = *Lactobacillus acidophilus*; BL = *Bifidobacterium longum*, c = *cis*, t = *trans*. Color version available online.

fects the membrane fluidity. The physical properties of bacteria, for instance membrane fluidity, are directly correlated with the level of unsaturated FA in the membrane (Szalontai et al., 2000). Bacterial cells regulate the membrane FA to adapt to the environmental stress; change in the U/S ratio is the most commonly observed mechanism in cells to modulate membrane fluidity. It has been shown that successful adaptation of bacteria to environmental stress conditions, such as acid stress (Wu et al., 2012), bile stress (Kimoto-Nira et al., 2009), and cold stress (Wang et al., 2005), increases the U/S ratio.

Studies have also correlated an increase in presence of unsaturated FA with a decrease in responsiveness of the stress response promoter element-driven gene to heat and salt stress (Chatterjee et al., 2000). Furthermore, the changes in FA unsaturation may be linked with the stress response proteins in the bacteria (Török et al., 1997). The findings from our study present further support the hypothesis of Chatterjee et al. (2000) and Guerzoni et al. (2001) that FA unsaturation is possibly involved in stress signal transduction, thereby affecting the bacterial stress response mechanisms.

Figure 4. Profiles of unsaturated fatty acid-to-SFA ratio (US/S) as affected by NaCl reduction and substitution with KCl. Values are means $\pm$ SE of 3 replicates ($n = 3$). An asterisk (*) means values are significantly different ($P < 0.05$) from control (0%). LA = *Lactobacillus acidophilus*; LC = *Lactobacillus casei*; BL = *Bifidobacterium longum*. Color version available online.

Phospholipid Composition by Thin Layer Chromatography

The changes in the phospholipids were evaluated using thin layer chromatography and quantified based on the intensity of the bands (Figure 5). Phosphatidylglycerol was the major phospholipid present in the bacterial cells. Cardiolipin and PI contents increased in *Lb. acidophilus* and *Lb. casei* when subjected to 10.0% NaCl concentration. These findings are similar to those observed by López et al. (2000), where PG content decreased and CL increased when *Bacillus subtilis* was grown in hypertonic medium, suggesting that increase in CL acts as a barrier against high ionic level. An increase in PI was observed in *B. longum* when subjected to higher salt concentrations (10.0%); however, CL content was not affected by substitution in *Bifidobacterium*. The most significant ($P < 0.05$) change in PI was observed in *Lb. casei* with increasing salt concentration. In general, no significant changes were observed in less abundant phospholipids (PE, LPC, and PC).

Owing to the closely related cross-regulation between the different membrane components, dramatic alteration in individual constituents may affect the cell membrane, thereby causing cell death. The membrane phospholipids are the most adaptable molecules in response to environmental stress. Stability of membrane depends on the stability of the lipid bilayer conformation that depends on the geometrical shape of the lipids. When exposed to salt stress, bacteria respond by alterations in the phospholipid composition. In all the 3 bacteria the most abundant phospholipid was PG, and thus the lipid bilayer was more negative due to higher concentration of PG (anionic) as compared with PC (zwitterionic). However, when subjected to salt stress the content of PG was reduced in all bacteria, whereas PC remained unaffected and PI and CL were increased in all bacteria. This shift in the membrane lipid metabolism toward the reduced synthesis of anionic phospholipids may reduce the electrostatic repulsion between the lipid bilayer and outer media environment

Figure 5. Phospholipid profile of bacteria as affected by NaCl reduction and substitution with KCl. Values are means $\pm$ SE of 3 replicates ($n = 3$). An asterisk (*) means values are significantly different ($P < 0.05$) from control (0%). LA = *Lactobacillus acidophilus*; LC = *Lactobacillus casei*; BL = *Bifidobacterium longum*; CL = cardiolipin; PE = phosphatidylethanolamine; PC = phosphatidylcholine; PG = phosphatidylglycerol; PI = phosphatidylinositol; LPC = lyso-phosphatidylcholine. Color version available online.

(Lewis and McElhaney, 2000), which could be attributed to the adaptive response of the bacteria to salt stress. An important component of osmotic tolerance is restoration and stabilization of the bacterial membrane lipid bilayer phase, which is attained by an increase in anionic lipids and a decrease in zwitterionic lipids (Beales, 2004). The negative charge on the surface of the membrane increases and thus, an increase of positively charged molecules occurs near the surface (Lee, 2004). This change is important for osmotic adaptation of bacterial cells, as the first stress response in bacteria is the uptake of K^+ into the cytoplasm (McLaggan et al., 1994).

Adhesion Ability to Caco-2 Cell Line

The bacteria were examined for their adhesion ability to human intestinal epithelial-like Caco-2 cells, as affected by varying NaCl and KCl concentrations (Figure 6). The adhesion ability of stressed bacteria was expressed as a percentage of the control (bacteria grown in 0% NaCl). In general, the adhesion ability of all 3 bacteria decreased when subjected to salt stress. The least adhesion was observed at 10.0% NaCl, which was observed to be 52% for *Lb. casei* and approximately 40% for *Lb. acidophilus* and *B. longum*. However, at a particular total salt concentration, substitution with KCl increased the adhesion ability of all bacteria. The increased effect of substitution was observed at higher total salt concentration (10.0%); highest adhesion ability was at 7.5% total salt (3.75% of each NaCl and KCl) for *Lb. casei* and *Lb. acidophilus*, which was almost double of the percentage adhesion of bacteria grown in 7.5% NaCl only. Significant ($P < 0.05$) reductions in adhesion ability of *Lb. acidophilus* and *B. longum* were noted at all salt concentrations, whereas in *Lb. casei* the adhesion ability was significantly ($P < 0.05$) reduced when subjected to salt concentrations higher than 5.0%. Among the 3 bacteria, the adhesion ability of *Lb. casei* was the highest at all NaCl and KCl concentrations.

Human intestinal epithelial-like Caco-2 cell line is a well-characterized colon carcinoma cell line that has been extensively used to study the organization and function of human intestinal cells in vitro. With proper cultivation, spontaneous differentiation, and formation of polarized epithelial cell monolayer, Caco-2 cells mimic the mature enterocyte lining of the small intestine functionally and morphologically (Sambuy et al., 2005). Bacterial adhesion to epithelial intestinal cells is important to prevent immediate washout of bacteria by peristalsis, and thus for colonization by microorganisms (Falkow et al., 1992). Bacterial adhesion is also likely to

be involved in competitive exclusion of enteropathogens and immunomodulation of the host (Plant and Conway, 2002; Lee et al., 2003).

The adhesion ability of stressed bacteria to Caco-2 cells varied with the organism. In general, the adhesion ability of the bacteria was reduced when grown in media supplemented with NaCl. On the other hand, substitution with KCl increased the adhesion ability at a given total salt concentration as compared with only with NaCl. This may be due to weaker bonding of KCl ions to the cell membrane as compared with the stronger bonding of NaCl ions (Gurtovenko and Vattulainen,

Figure 6. Effect of NaCl reduction and substitution with KCl on adhesion ability of bacteria to Caco-2 cells. Values expressed as percentage of control (0%). Values are means $\pm$ SE of 3 replicates ($n = 3$). An asterisk (*) means values are significantly different ($P < 0.05$) from control (0%). LA = *Lactobacillus acidophilus*; LC = *Lactobacillus casei*; BL = *Bifidobacterium longum*. Color version available online.

2008). *Lactobacillus casei* 290 was found to be the most adherent bacteria at all NaCl and KCl concentrations. This may be attributed to the resistance of this strain to salt, as revealed by our previous study on membrane characteristics using flow cytometry (Gandhi and Shah, 2015). *Lactobacillus casei* showed the highest adhesion ability, with 91% relative adherence at 7.5% NaCl + KCl (1:1), which is higher than that of *Lb. acidophilus* (82%, at 7.5% NaCl + KCl) and *B. longum* (79%, at 2.5% NaCl + KCl). It has been shown that the cell surface proteins of bacteria can contribute to in vitro adhesion ability to intestinal cells (Kos et al., 2003; Buck et al., 2005). These surface proteins, adhesins, are responsible for adherence of bacteria to intestinal mucus layer. Our previous findings using Fourier transform infrared spectroscopy revealed changes occurring in the surface functional groups of bacteria, particularly in the amide regions, when subjected to varying salt concentrations (Gandhi et al., 2014). These shifts in the Fourier transform infrared spectra are indicative of the changes occurring in cell surface proteins, and could possibly be contributing to the changes in adhesion ability of the bacteria to Caco-2 cells.

CONCLUSIONS

Bacteria respond to environmental stress by altering the nature of the cell wall or by accumulation of compatible solutes within the cell. Different bacteria have different genetic makeup, thus conferring a variation in their tolerance and adaptability to stress. This study revealed that damage to bacterial cell membrane occurred as a result of high level of salt (10.0%), and the effects of these changes were observed in the adhesion ability of the bacteria to human intestinal epithelial-like Caco-2 cells. All 3 bacteria responded to salt stress mainly by increasing the unsaturated FA content. Interestingly, the adhesion ability of stressed bacteria was reduced at high salt concentrations (7.5 and 10.0%), possibly due to changes in the surface functional proteins. The membrane FA and phospholipids were altered in response to salt stress; however, it was revealed that substitution with KCl had a protective effect on the bacterial membrane.

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