INTERPRETIVE SUMMARY

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. Gandhi. Sodium chloride induced stress responses in potential probiotic bacteria were evaluated. Effect of substitution of sodium chloride with potassium chloride was determined on the structure and function of the bacteria. The findings provide insights to bacterial responses to stress and their ability to adhere to human intestinal cells.

SALT INDUCED STRESS RESPONSES IN BACTERIA

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 sodium chloride (NaCl) reduction (10.0%, 7.5%, 5.0%, 2.5% and 0% NaCl) and its substitution with potassium chloride (KCl; 50% substitution at each given concentration) on morphology of *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium longum* was investigated using transmission electron microscopy (TEM). 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 TEM. Fatty acid content was altered with an increase in the ratio of unsaturated to saturated fatty acid content on increasing the sodium chloride induced stress. Among the phospholipids, phosphatidylglycerol was reduced, whereas phosphatidylinositol and cardioplipin 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.

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

INTRODUCTION

Sodium chloride (table salt) being one of the most important food additives contributes to flavor, texture and functional properties of food. With increasing awareness about diet and health, there have been numerous attempts to reduce the salt intake 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 like KCl, $MgCl_2$ and $CaCl_2$ 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, there is limited literature 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 phenotypical 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 **(FAs)** 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 as 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 to intestinal mucus layer. Furthermore, exposure to environmental stress and/or any change on the surface membrane of the bacteria may alter the adhesion of bacteria to the intestinal cells (Buck et al., 2005). However, prior to investigation of their functional properties in 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, there is no study evaluating the effect of salt stress on adhesion ability of these potential probiotic bacteria. The objectives of this 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 three potential probiotic bacteria, *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium longum*.

MATERIALS AND METHODS

Bacterial Cultivation and Experimental Design

Lb. acidophilus (CSCC 2400; **LA**), *Lb. casei* (ASCC 290; **LC**) and *B. longum* (CSCC 5089; **BL**) 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 Mann Rogosa and Sharpe **(MRS)** broth (Becton Dickinson and Company, NJ, USA) by 1% (v/v) 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% NaCl, 7.5% NaCl, 5.0% NaCl 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% w/v) 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 **(CFU)** were enumerated.

Transmission Electron Microscopy (TEM)

The morphological changes in all three bacteria were evaluated in the highest NaCl concentration (10.0% w/v) 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 three 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 and the cell pellet was re-suspended 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 4-8°C. Second fixation was performed using 1% osmium tetroxide (OsO_4) in cacodylate buffer for 30 min at room temperature (~ 22°C). The cell pellet was immersed in 0.5 mL of pre-warmed agar solution. The cell pellets were then subjected to successive dehydration on a rotary shaker as follows: 50% ethanol - 10 min, 70% ethanol - 10 min, 90% ethanol - 10 min, 100% ethanol - 3 times, 20 min each, and propylene oxide - 2 times, 10 min each. The samples were infiltrated with epoxy resin/propylene oxide mixture (1:1) for 1 hour 30 min at 37°C, followed by infiltration in epoxy resin/propylene oxide mixture (2:1) for 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, Netherlands).

Extraction and Analysis of Membrane Fatty Acids by Gas-Chromatography (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 (0.5g in 0.5 mL). Proteinase K (25 µg/mL, Sigma) 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 to collect the cell pellet (0.5 g wet weight) for extraction of fatty acids (FAs). Membrane FAs were extracted and converted to methyl esters **(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 extracted FAs were concentrated by drying under nitrogen and re-dissolved in GC-grade hexane (Fisher Scientific, Pittsburgh, PA, USA) before analysis.

Separation and Identification of Fatty Acids by Gas Chromatography – Mass Spectroscopy (GC-MS)

The separation and identification of fatty acids was performed on a gas chromatograph (Agilent 6590N- 5973N GC–MS system; Agilent, Atlanta, GA, USA) equipped with and

Agilent 7694E auto-sampler and a capillary DB-wax column (30 m Å~ 0.25 mm id, 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA). The injection volume was 1 µL (split-less 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 to saturated FAs was also calculated (Zhao and Shah, 2014).

Extraction and Profiling of Membrane Phospholipids by Thin Layer Chromatography (TLC)

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) 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 **(PLs)**: Two step extraction method was used for efficient extraction of major PLs (Ozbalci et al., 2013). Neutral extraction was carried out by dissolving the cell pellet (collected after digestion of cell wall proteins) in 1800 µL chloroform/methanol (1:2, v/v) 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 × g for 5 min at 4°C, and the supernatant 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 × 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, v/v) 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 cold chloroform and 450 µL 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 × 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 N_2 stream. The weight of dried lipids was determined and stored in chloroform/methanol (90:10, v/v; 50 mg/mL) at -20°C until analysis.

Separation of Phospholipids by High Performance Thin Layer Chromatography (HPTLC)

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 prior to spotting in order 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 TLC plates using chloroform/methanol/acetic acid/water (71:20:6.25:2.5, v/v) as the mobile phase. The bands were visualized by spraying with the following reagents: (i) 0.5% (w/v) vanillin in ethanol/conc. H_2SO_4 (97:3, v/v) solution for all polar lipids (Rakhuba et al., 2009) and (ii) 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 (Biorad, Hercules, CA, USA), and analysed in Image Lab (version 4.0, Biorad). 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 (R_f) of phospholipid

201 standards and the lipid bands detected in samples. The following standard phospholipids
202 (Sigma) were prepared by dissolving in chloroform/methanol (90:10, v/v) and used for
203 identification: cardiolipin **(CL)**, phosphatidylcholine **(PC)**, lyso-phosphatidylcholine **(LPC)**,
204 phosphatidylethanolamine **(PE)**, phosphatidylglycerol **(PG)**, and phosphatidylinositol **(PI)**.
205
206 ***Cell Line***
207 The human intestinal epithelial-like Caco-2 cell line was obtained from the American
208 Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained and
209 cultured in Difco's minimum essential medium (**DMEM**; Gibco BRL, Life Technologies,
210 Grand Island, NY, USA), supplemented with 10% fetal bovine serum **(FBS)** and 1% mixture
211 of penicillin-streptomycin solution (Gibco BRL). The cells were incubated at 37°C in a
212 humidified incubator (US Air Flow-NuAire, Plymouth, MN, USA), in an atmosphere of 5%
213 CO_2 and 95% air. All cells used in this study were between 32 and 37 passages. Cells were
214 sub-cultured at 80% confluence by trypsinization (0.25% trypsin-EDTA, Gibco BRL).
215
216 ***Determination of Adhesion Ability***
217 The adhesion ability of bacteria to Caco-2 cells was measured by the cell adhesion assay
218 as per the method of Parkar et al. (2008) with some modifications. Briefly, Caco-2 cells were
219 seeded at approximately 1 x 10^4 cells per well in 12-well plates and incubated to obtain
220 confluence prior to the assay (10-12 days). Bacteria were grown in MRS broth supplemented
221 with varying salt concentrations as described earlier. The bacterial cell pellet was washed
222 with PBS buffer (pH 7.2) and resuspended in antibiotic-free DMEM at a cell density of
223 approximately 10^9 CFU/mL. The cell line monolayer was washed twice with PBS buffer to
224 remove interference of antibiotics. The bacterial cell suspension was added to each well
225 (100:1 bacteria: Caco-2 cells) and incubated at 37°C for 2 h. After the incubation period, the
226 supernatant was removed and wells were gently washed with PBS to remove any non-

specifically 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 to the control using the following equation:

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

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 analysis of variance **(ANOVA)** at 95% level of significance using SPSS statistics software v 20.0 (IBM, IL, USA). Post-hoc analysis **(LSD)** 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 (log_{10} cfu/mL) of bacteria is shown in Figure 1. An increase in NaCl concentration was inversely associated with the cell viability for all the three bacteria. Among the three bacteria, *B. longum* was the most affected by salt stress. The lowest viable cell count of *B. longum* was observed to be about 3 log_{10} CFU/mL when exposed to 10% NaCl, whereas *Lb. acidophilus* and *Lb. casei* showed significant ($P < 0.05$) reduction only after exposure to higher NaCl concentrations (7.5% and 10%). At a given salt concentration, substitution with KCl (50%) increased the viable cell count as compared to that of the bacteria grown in media 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 level 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 2-b) 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 TEM, 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 2-c), these morphological changes were less distinct as compared to the control (0% NaCl/KCl; Figure 2-a) possibly owing to the protection offered by KCl.

Morphological changes in microorganisms are visible indicators of their adaptation to environmental stress and transmission electron microscopy is a powerful technique to observe such changes in bacterial cells. At high NaCl concentration (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 bacterial cell that is caused due to 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 possibly be due to the weaker bonding of potassium ions to the membrane as compared to the sodium ions (Gurtovenko and Vattulainen, 2008), leading to reduced alterations in the cellular membrane.

Fatty Acid Composition by Gas Chromatography

The relative percentages of major fatty acids (FAs) and the ratios of unsaturated to saturated (U/S) FA are presented in Figure 3 and 4, respectively. The fatty acid content of the bacteria was altered owing to the exposure to salt stress. In all the three bacteria, a decrease in C14:0 was observed on increasing the NaCl concentration, however, substitution with KCl increased the relative percentage of C14:0. On the other hand, C16:0, the most abundant fatty acid, was higher in bacteria subjected to high salt concentration. In general, the U/S FA 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. *Lb. casei* showed the least difference (almost one 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 unsaturated to saturated fatty acid (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 FA ratio for *Lb. casei* was slightly different as compared to the other two 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 and saturated fatty acids, and the fatty acyl chain conformation affects the membrane fluidity. The physical properties of bacteria, for instance membrane fluidity, are directly correlated with the level of unsaturated fatty acids in the membrane (Szalontai et al., 2000). Bacterial cells regulate the membrane fatty acids to adapt to the environmental stress; change in the ratio of unsaturated to saturated fatty acids 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 like acid stress (Wu et al., 2012), bile stress (Kimoto-Nira et al., 2009) and cold stress (Wang et al., 2005) increases the ratio of unsaturated to saturated fatty acids. Studies have also correlated an increase in presence of unsaturated fatty acids 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 fatty acid unsaturation may be linked with the stress response proteins in the bacteria (Torok et al., 1997). The findings from this study present further support the hypothesis of Chatterjee et al. (2000) and Guerzoni et al. (2001) that fatty acid unsaturation is possibly involved in stress signal transduction, thereby affecting the bacterial stress response mechanisms.

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). Phosphatidylglyercol (PG) was the major phospholipid present in the bacterial cells. Cardiolipin (CL) and phosphatidylinositol (PI) contents increased in *Lb. acidophilus* and *Lb. casei* when subjected to 10.0% NaCl concentration. These findings are similar to those observed by (Lopez et al., 2000) where PG content decreased and CL increased when *Bacillus subtilis* was grown in hypertonic medium, suggesting that increase in CL acts like a barrier against high ionic level. An increase in PI

329 was observed in *B. longum* when subjected to higher salt concentrations (10.0%); however,
330 CL content was not affected by substitution in *Bifidobacterium*. The most significant ($P <$
331 0.05) change in PI was observed in *Lb. casei* on increasing the salt concentration. In general,
332 no significant changes were observed in less abundant phospholipids
333 (phosphatdylethanolamine (PE), lyso-phosphatidylcholine (LPC), and phosphatidylcholine
334 (PC)).

335 Owing to the closely related cross-regulation between the different membrane
336 components, dramatic alteration in individual constituents may affect the cell membrane
337 thereby causing cell death. The membrane phospholipids are the most adaptable molecules in
338 response to environmental stress. Stability of membrane depends on the stability of the lipid
339 bilayer conformation that depends on the geometrical shape of the lipids. When exposed to
340 salt stress, bacteria respond by alterations in the phospholipid composition. In all the three
341 bacteria the most abundant phospholipid was PG, and thus the lipid bilayer was more
342 negative due to higher concentration of PG (anionic) as compared to PC (zwitterionic).
343 However, when subjected to salt stress the content of PG was reduced in all bacteria whereas
344 PC remained unaffected and, PI and CL were increased in all bacteria. This shift in the
345 membrane lipid metabolism towards the reduced synthesis of anionic phospholipids may
346 reduce the electrostatic repulsion between the lipid bilayer and outer media environment
347 (Lewis and McElhaney, 2000), which could be attributed to the adaptive response of the
348 bacteria to salt stress. An important component of osmotic tolerance is restoration and
349 stabilization of the bacterial membrane lipid bilayer phase, which is attained by an increase in
350 anionic lipids and a decrease in zwitterionic lipids (Beales, 2004). The negative charge on the
351 surface of the membrane increases and thus, there would be an increase of positively charged
352 molecules near the surface (Lee, 2004). This change is important for osmotic adaptation of
353 bacterial cells, since the first stress response in bacteria is the uptake of K^+ into the cytoplasm
354 (Mclaggan et al., 1994).

355

356 ***Adhesion Ability to Caco-2 Cell Line***

357 The bacteria were examined for their adhesion ability to human intestinal epithelial-like
358 Caco-2 cells, as affected by varying NaCl/KCl concentrations (Figure 6). The adhesion
359 ability of stressed bacteria was expressed as a percentage of the control (bacteria grown in
360 0% NaCl). In general, the adhesion ability of all three bacteria decreased when subjected to
361 salt stress. The least adhesion was observed at 10.0% NaCl, which was observed to be 52%
362 for *Lb. casei*, and approximately 40% for *Lb. acidophilus* and *B. longum*. However, at a
363 particular total salt concentration, substitution with KCl increased the adhesion ability of all
364 bacteria. The increased effect of substitution was observed at higher total salt concentration
365 (10.0%); highest adhesion ability was at 7.5% total salt (3.75% of each NaCl and KCl) for
366 *Lb. casei* and *Lb. acidophilus*, which was almost double of the percentage adhesion of
367 bacteria grown in 7.5% NaCl only. Significant ($P < 0.05$) reduction in adhesion ability of *Lb.*
368 *acidophilus* and *B. longum* at all salt concentration, whereas in *Lb. casei*, the adhesion ability
369 was significantly ($P < 0.05$) reduced when subjected to salt concentrations higher than 5.0%.
370 Among the three bacteria, the adhesion ability of *Lb. casei* was the highest at all NaCl/KCl
371 concentrations.

372 Human intestinal epithelial-like Caco-2 cell line is a well-characterized colon carcinoma
373 cell line that has been extensively used to study the organization and function of human
374 intestinal cells *in vitro*. With proper cultivation, spontaneous differentiation and formation of
375 polarized epithelial cell monolayer, Caco-2 cells mimic the mature enterocyte lining of the
376 small intestine functionally and morphologically (Sambuy et al., 2005). Bacterial adhesion to
377 epithelial intestinal cells is important to prevent immediate washout of bacteria by peristalsis
378 and thus for colonization by microorganisms (Falkow et al., 1992). Bacterial adhesion is also
379 likely to be involved in competitive exclusion of enteropathogens and immuno-modulation of
380 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 to only with NaCl. This may be due to weaker bonding of KCl ions to the cell membrane as compared to the stronger bonding of NaCl ions (Gurtovenko and Vattulainen, 2008). *Lb. casei* 290 was found to be the most adherent bacteria at all NaCl/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). *Lb. casei* showed the highest adhesion ability of 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), respectively. 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 (FT-IR) spectroscopy have 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 FT-IR 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.

CONCLUSION

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 was observed in the adhesion ability of the bacteria

to human intestinal epithelial-like Caco-2 cells. All the three bacteria responded to salt stress mainly by increasing the unsaturated fatty acid 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 fatty acids 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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FIGURE CAPTIONS

Figure 1: Effect of NaCl reduction and substitution with KCl on viable cell count (log_{10} CFU/mL)

Values are means ± standard error of at least three replicates ($n \geq 3$)

*Values are significantly different ($P < 0.05$) from control (0%)

LA: *Lb. acidophilus*; LC: *Lb. casei*; BL: *B. longum*

Figure 2: Transmission electron micrographs (TEM) of thin sections of bacteria grown in (a) MRS, (b) MRS+10.0% NaCl and (c) MRS+10.0% total salt (5.0% each of NaCl and KCl). Magnification of micrographs is 21000 fold (black bars below are 200 nm)

(Representative micrographs of bacteria in different conditions)

LA: *Lb. acidophilus*; LC: *Lb. casei*; BL: *B. longum*

Figure 3: Relative membrane fatty acid composition as affected by NaCl reduction and substitution with KCl

Values are means ± standard error of three replicates (n = 3)

*Values are significantly different ($P < 0.05$) from control (0%)

LA: *Lb. acidophilus*; LC: *Lb. casei*; BL: *B. longum*

Figure 4: Profiles of unsaturated fatty acid: saturated fatty acid ratio (U/S) as affected by NaCl reduction and substitution with KCl

Values are means ± standard error of three replicates (n = 3)

*Values are significantly different ($P < 0.05$) from control (0%)

LA: *Lb. acidophilus*; LC: *Lb. casei*; BL: *B. longum*

Figure 5: Phospholipid profile of bacteria as affected by NaCl reduction and substitution with KCl

Values are means ± standard error of three replicates (n = 3)

*Values are significantly different ($P < 0.05$) from control (0%)

LA: *Lb. acidophilus*; LC: *Lb. casei*; BL: *B. longum*

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 ± standard error of three replicates (n = 3)

*Values are significantly different ($P < 0.05$) from control (0%)

LA: *Lb. acidophilus*; LC: *Lb. casei*; BL: *B. longum*