



Effect of salt on cell viability and membrane integrity of *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium longum* as observed by flow cytometry



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ABSTRACT

The aim of the current study was to investigate the effect of varying sodium chloride concentrations (0–5%) on viability and membrane integrity of three probiotic bacteria, *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium longum*, using conventional technique and flow cytometry. Double staining of cells by carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) enabled to evaluate the effect of NaCl on cell esterase activity and membrane integrity. Observations from conventional culture technique were compared with findings from flow cytometric analysis on the metabolic activities of the cells and a correlation was observed between culturability and dye extrusion ability of *L. casei* and *B. longum*. However, a certain population of *L. acidophilus* was viable as per the plate count method but its efflux activity was compromised. Esterase activity of most bacteria reduced significantly ($P < 0.05$) during one week storage at NaCl concentrations greater than 3.5%. The study revealed that *L. casei* was least affected by higher NaCl concentrations among the three probiotic bacteria, as opposed to *B. longum* where the cF extrusion performance was greatly reduced during 1 wk storage. The metabolic activity and salt resistance of *L. casei* was found to be highest among the bacteria studied.

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1. Introduction

With the increasing awareness about the food–health relationship, functional food and probiotic food products have gained popularity. Some of the health benefits of probiotics have been well established, while other benefits need to be confirmed by human studies (Shah, 2007). For bacteria to confer their functionality as a probiotic, it is important to maintain their viability in the product during production, processing and storage. The inclusion of probiotics poses several technological challenges (Shah, 2000). A variety of stress factors like osmotic pressure, reduced water activity, increased concentration of certain ions or nutrient depletion affect the survivability of bacteria, in turn lowering their functionalities as probiotics.

Sodium chloride (commonly named as table salt) is the most common food additive, which contributes to the functional

properties of food products apart from enhancing their texture and flavor (Albarracin et al., 2011). However, owing to the increased awareness about the risks associated with increased intake of salt, there have been efforts to lower salt consumption (Buemi et al., 2002; Heaney, 2006; Kotchen, 2005; Massey, 2005). Dairy products, like cheeses contain varying levels of salt and there has been increasing interest to particularly lower the salt consumption through these products. However, a major challenge in reducing the salt content in cheeses is to maintain the right texture and taste of the cheese. Studies have evaluated the effect of NaCl reduction and its substitution on the taste and texture of various cheeses (Ayyash and Shah, 2011a,b). The varying salt concentrations in dairy products like Lebneh and cheeses also affect cell membrane of the bacteria, which in turn, reduces their growth and activity. Several studies have shown the effect of osmotic stress on the viability of lactic acid bacteria. Flow cytometric studies on the effect of osmotic stress have been performed on some lactobacilli exposed to varying levels of sugar concentrations (Sunny-Roberts et al., 2007). However, limited studies have monitored the injury induced by sodium chloride stress to the probiotic bacteria. It is

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therefore important to evaluate the degree of injury sustained by probiotic bacteria when subjected to salt stress.

The traditional plate enumeration technique to study the reproducibility of cells is commonly used, however, it may not provide detailed information about the degree of injury and the metabolic activity of the cells caused due to salt stress. Furthermore, cells with partial or minimal damage to the cell membrane may recover and still appear as colonies on agar plates. In contrast, fluorescence based methods provide a better technique to study the structural and functional properties with improved sensitivity, and enable to monitor single-cell responses (Bunthof et al., 2000). Flow cytometric analysis allows evaluation of cell membrane integrity, enzymatic activities of cell and membrane potential at a single cell level (Steen, 2000). Use of functional dyes which target the enzyme activities leads to a better understanding of the state of the cell, by providing sensitive measurement of physiological characteristics, metabolic activity and the reproducibility of cells. The use of appropriate dyes enables differentiation of bacterial population in four categories: metabolically active cells with intact membrane, metabolically active cells with minimally damaged membrane, reproductively viable cells with compromised metabolic activity, and dead cells with no metabolic activity and compromised membrane (Hewitt and Nebe-Von-Caron, 2001).

The objectives of this study were to investigate the effects of varying NaCl concentrations on the cell viability and degree of injury in three probiotic bacteria, *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium longum* using conventional technique and flow cytometry. In flow cytometry, double staining of cells using carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) enables detection of live, damaged or dead cells simultaneously.

2. Materials and methods

2.1. Bacterial cultivation

L. acidophilus (CSCC 2400), *L. casei* (ASCC 290) and *B. longum* (CSCC 5089) were obtained from the Australian Starter Culture Collection (Dairy Innovation Australia Limited, Werribee, Australia) and were stored at -80°C . The organisms were activated in sterile deMann Rogosa and Sharpe (MRS) broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) by inoculating 1% of each organism followed by incubation at 37°C for 24 h. The activated organisms were used after 3 successive transfers in sterile MRS.

2.2. Salt treatment

The activated bacteria were grown in MRS broth to attain an initial concentration of approximately $8 \log \text{CFU/mL}$. The cells were then washed twice with potassium phosphate buffer, and resuspended to an optical density of 0.6–0.7 at 620 nm (Ben Amor et al., 2002). The cells were then subjected to varying NaCl concentrations (0 (control), 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5%) for 1 h at 37°C .

To monitor the effect of storage with NaCl on the cell viability, the control (0% NaCl) and the NaCl treated cells (as described above) were stored at room temperature ($18\text{--}20^{\circ}\text{C}$) for 1 wk. In order to ensure that the cell death is not due to lack of nutrients, lactose was added (2% final volume) to the cell suspension.

2.3. Determination of cell viability

Cell viability as affected by varying NaCl treatment for different time of exposure (1 h and 1 wk) was enumerated after serially diluting of each sample in sterile peptone water (0.15% w/v) and

spread plating on MRS agar plates for *L. acidophilus* and *L. casei*, and MRS-cysteine (0.05%) agar plates 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.

2.4. Esterase activity and membrane integrity

A 10 mM stock solution of cFDA (Life Technologies Inc., Carlsbad, CA, USA) was prepared by dissolving 4.6 mg cFDA in 1 mL acetone and stored at -20°C in dark, and it was diluted to 1 mM before use. Stock solution of PI (Sigma Aldrich, St. Louis, MO, USA) was prepared by dissolving 1 mg PI in 1 mL distilled water and was used as the working solution (Ben Amor et al., 2002).

Cells subjected to varying NaCl concentrations were incubated with 50 mM cFDA at 37°C for 10 min for intracellular enzymatic conversion of cFDA to carboxyfluorescein (cF). Cells were washed with potassium phosphate buffer to remove excess cFDA and 30 mM PI was added to the cells. Cells were incubated in ice bath for 10 min to label the cells with damaged membrane (Ben Amor et al., 2002; Bunthof and Abee, 2002; Bunthof et al., 2001).

2.5. Carboxyfluorescein extrusion activity and kinetics of extrusion

The intracellular cF extrusion activity of the treated cells was measured by addition of 20 mM glucose to the cF stained cells and incubating for 20 min at 37°C (Bunthof et al., 1999). The kinetics of cF-extrusion from glucose-energized cells was measured by incubating the cells with 20 mM glucose and progress of dye extrusion was monitored every 5 min.

2.6. Flow cytometric measurement

Flow cytometric measurement was performed on a BD FACS Aria III flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) equipped with 488 nm air-cooled argon laser. The cells were delivered at a flow rate corresponding to 500–700 events per second. For each measurement, forward scatter (FSC), side scatter (SSC), green (cF) and red (PI) fluorescence were recorded, amplified, and converted into digital signals for further analysis. The cells remain stained with cFDA if the membrane was intact and/or the intracellular enzymes are active, while the cells stained with PI indicated compromised membrane (Hoefel et al., 2003). Carboxyfluorescein emits green fluorescence at 530 nm following excitation with laser light at 488 nm, whereas PI-stained cells emit red fluorescence at 635 nm. The recorded signals were amplified logarithmically and a quad-gate was created in the dot plot to discriminate differentially populated bacteria. The data were analyzed using FlowJo version 7.6.2 (TreeStar Inc., Ashland, OR, USA).

2.7. Flow cytometric data analysis

Flow cytometric dot-plot of cF vs. PI of the NaCl treated cells was used to resolve the fluorescence properties of the population measured by flow cytometer (Ananta et al., 2004). The bacterial population as reflected in the dot-plot was gated and differentiated into four quadrants based on their fluorescence performances.

The esterase activity of the cells as affected by NaCl treatment was calculated using equation (1). The cells in quadrant Q3, stained only with cF were considered to monitor the esterase activity.

$$EA(\%) = \left(\frac{Q3}{Q3_{ctrl}} \right) \times 100, \quad (1)$$

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