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Physiological analysis of *lactobacillus rhamnosus* VTT E-97800

Adaptive response to osmotic stress induced by trehalose

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Abstract

Purpose – This paper aims to describe the physiological analysis of *L. rhamnosus* VTT E-97800 and its adaptive response to osmotic stress induced by trehalose.

Design/methodology/approach – Cells *of L. rhamnosus* E800 in the stationary phase of growth were subjected to osmotic stress induced by trehalose treatments. The effects of osmotic stress on the viability of the study strain were determined by conducting flow cytometric analysis with carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) and by observing the corresponding cells growth on MRS agar plates. Osmotic-induced changes of esterase activity and membrane integrity were monitored. Ability to extrude intracellular accumulated cF (additional vitality marker) was taken into consideration.

Findings – The fluorescence-based approach gave additional insights on osmotic induced changes of cellular events, which could not be explicitly assessed by culture techniques. Trehalose treatments caused a transient membrane permeabilization as revealed by a gradual decrease in esterase activity (a measure of enzyme activity and thus of viability) with increase in trehalose molarity. However, culturability on MRS agar was not significantly affected. Membrane integrity was maintained and there was an improvement in the ability of cells to extrude intracellular accumulated cF.

Originality/value – The paper provides a comparative study of the conventional culture techniques and the flow cytometric viability assessment which showed that esterase activity cannot be relied on to ascertain the culturability and viability status of an organism.

Keywords Food preservation, Metabolic diseases, Micro-organisms, Biotechnology

Paper type Research paper

Introduction

The probiotic strain *Lactobacillus rhamnosus* E-97800 (E800), isolated from human intestines with lactitol enrichment has been characterized and identified by molecular biological typing methods RAPD, automatic ribotyping and PFGE. The probiotic properties such as good tolerance to low pH, bile pancreatic juice, and the ability to adhere to both the intestinal epithelium cell line Caco-2 cells and human ileostomy glycoproteins have been reported (Tynkkynen *et al.*, 1999). Technologically, it adheres well to fibres creating a possibility for cereal-based probiotic products.

Bacteria have evolved stress-sensing systems and defences against stress, which allow them to withstand harsh conditions and sudden environmental changes (Van de

The authors are grateful to the German Academic Exchange Programme for its financial support in the course of this work. Many thanks go to Irene Hemmerich for her technical assistance.

British Food Journal
Vol. 109 No. 9, 2007
pp. 735-748

0007-070X
DOI 10.1108/00070700710780706

Guchte *et al.*, 2002). These stress responses are characterized by the transient induction of general and specific proteins and by physiological changes that generally enhance an organism's ability to withstand more adverse environmental conditions (Ang *et al.*, 1991). In the case of osmotic stress, the significant physiological changes reported in bacteria include the induction of stress proteins as well as intracellular accumulation of compatible solutes (Clark and Parker, 1984; Kets and de Bont, 1994; Kets *et al.*, 1994; Welsh and Herbert, 1999; Prasad *et al.*, 2003), for example, trehalose, amino acids, sucrose, mannitol etc. These solutes restore turgor pressure and membrane tension to low levels similar to those that occur before osmotic shift (Csonka and Hansen, 1999), preserve enzyme activity and protein stability, and maintain the integrity and stability of membranes and nucleic acids (Brown, 1990; Liu *et al.*, 1998).

Trehalose is a disaccharide that is ubiquitous in the biosphere. It consists of two sub-units of glucose bound by an $\alpha:\rightarrow 1$ linkage (α-D-glucopyranosil α-D-glucopyranoside) and is thus non-reducing. Trehalose has been isolated and characterized from a large variety of both prokaryotic and eukaryotic organisms, ranging from bacteria and plants to mammals (Argüelles, 2000). It possesses several unique physical properties, which include nonhygroscopic glass formation and the absence of internal hydrogen bond formation. These features account for the principal role of trehalose as a stress metabolite.

Among the ways to preserve food products, increased osmotic pressure, i.e. lowering of water activity (a_w) is one of the most widely used. Desiccation or addition of high amounts of osmotically active compounds such as salts and sugars lowers the water activity of the food. Most of these compatible solutes are present in significant amounts in foods, thus allowing growth at reduced water activities.

The potential role that trehalose could have as a biotechnological tool include serving as a key element in food preparations subject to drying processes or concentration (Schiraldi *et al.*, 2002). In these cases, the incorporation of probiotics into such foods is practised for consumers' health benefit purposes.

Flow cytometry is a rapid and sensitive method that can be performed by double staining of cells with carboxyfluorescein diacetate (cFDA) and propidium iodide (PI). CFDA is used for the evaluation of cellular enzymatic activity. It is a lipophilic, non-fluorescent precursor that readily diffuses across the cell membrane. Intracellularly, unspecific esterase hydrolyses the diacetate group into a polar, membrane impermeant fluorescent compound, carboxyfluorescein (cF). For cells to be regarded as viable, this probe requires both active intracellular enzymes and intact membranes (Hoefel *et al.*, 2003). PI is a membrane-impermeant, nucleotide-binding probe, which cannot penetrate cells with intact membranes, but once membrane integrity is lost, it diffuses into and stains the nucleic acids.

In this paper, the induction of osmotic stress by the presence of trehalose in a complex rich medium, on a model probiotic bacterium is reported. The study was approached by measuring the fluorescence-related parameters and analysing the plate counts; then making a comparative analysis between both.

Materials and methods

Bacterial strain and growth conditions

The strain of *Lactobacillus rhamnosus* E800 used was obtained from VTT culture collection (Espoo, Finland). The culture which was sent in freeze-dried form in glass

ampoule was later stored as glass beads cultures (Roti(R)_Store, Carl-Roth, Karlsruhe, D) in a −80°C freezer (U101, New Brunswick Scientific, Nürtingen, D) for long-term maintenance. One bead from deep-frozen culture was transferred into MRS broth (Oxoid, Basingstoke, UK) and incubated over-night. This broth was later used to inoculate a final broth (50 ml) at OD_{600} 0.1. Growth was carried out at 37°C over a period of 24 h and monitored spectrophotometrically at 600 nm (Graphicord uV-240, Schimadzu, JPN).

Media preparation

High osmolarity media were obtained by adding trehalose (0.1M, 0.3M, 0.4M, 0.6M, 0.9M, 1.2M and 1.5M) to the MRS broth (5 ml) (Oxford, Basingstoke, UK) at concentrations indicated between the brackets. MRS broth without trehalose served as control. The osmolarities of the solutions were measured (51308 Vapour Pressure Osmometer Wescor Inc.).

Flowcytometry

Stress treatments. Stationary growth phase cultures were harvested, washed twice in Ringer's solution (No. 15525, Merck, Darmstadt, D) and finally resuspended in Ringer's solution to an OD_{600} value of 10 (Ananta *et al.*, 2004). This corresponded to a cell concentration of $3.4x10^9$ $CFU.mL^{-1}$.

Equal volume of the cell concentrate was treated with equal volume of the MRS-trehalose solution at 37°C for 30 mins. Cells treated with MRS broth only served as controls. Heat-treated cells (95°C, 15 mins) served as negative controls. The cells viability was then assessed by flow cytometric methods and plate counts enumeration.

Plate enumeration method

Treated samples and control samples were serially diluted in Ringer's solution (No. 15525, Merck, Darmstadt, D) and cultured on MRS agar plate by drop count technique (Miles and Misra, 1938). The viable cell numbers were determined after 48 h of incubation at 37°C under anaerobic conditions produced by anaerobic kits (Anaerocult® A, Merck, Darmstadt, D).

The impact of osmotic treatment on cell viability, as assessed by plate count method was expressed as the logarithmic value of relative survivor fraction ($\log N/N_o$). N refers to the bacterial count following osmotic exposure, while N_o refers to the initial count before osmotic exposure.

Esterase activity and membrane integrity

Cells were incubated with 50 μm cFDA (Molecular Probes, Inc., Leiden, NL) at 37°C for 10 mins. cFDA is an esterase substrate that yields the fluorescent carboxyfluorescein (cF) upon hydrolysis. Cells were washed to remove excess cFDA and 30 μm PI (Molecular Probes Inc., Leiden, NL) was added. Cells were incubated in ice bath for 10 mins to allow labelling of membrane-compromised cells (Ananta *et al.*, 2004).

cF extrusion activity and kinetics of extrusion

cF stained cells were further incubated with 20mM glucose at 37°C for 40 mins in order to measure the performance of cells in extruding intracellular accumulated cF (Bunthof

et al., 1999). Kinetic measurements were performed by withdrawing samples at intervals over a period of 40 mins to monitor release of cF from glucose energized cells.

Flow cytometric measurement

Analysis was performed on a Coulter® EPICS® XL_MCL flow cytometer (Beckman Coulter Inc., Miami, FL, USA) equipped with a 15mW 488 nm air-cooled argon laser. Cells were delivered at the low flow rate, corresponding to 400-600 events. Forward scatter (FS), side scatter (SS), green (FL1) and red fluorescence (FL3) of each single cell were measured, amplified and converted into digital signals for further analysis. CF emits green fluorescence at 530 nm following excitation with laser light at 488 nm, whereas red fluorescence at 635 nm is emitted by PI-stained cells.

A set of band pass filters of 525 nm (505-545 nm) and 620 nm (605-635 nm) was used to collect green fluorescence (FL1) and red fluorescence (FL3) respectively. All registered signals were logarithmically amplified. A gate created in the density-plot of FS vs. SS was preset to discriminate bacteria from artefacts. Data were analysed with the software package Expo32 ADC (Beckman-Coulter inc., Miami, FL, USA). All detectors were calibrated with FlowCheck^{TM} Flourospheres (Beckman-Coulter Inc., Miami, FL, USA).

Data analysis

Density plot analysis of FL1 vs. FL3 was conducted as described by Ananta *et al.* (2004). Density plot was used to resolve the fluorescence properties of the population measured by flow cytometer. The population was then differentiated and gated.

Residual esterase activity following osmotic treatment was calculated using equation (1):

$$EA(\%) = (\#A4p/\#A4ctrl)x100 \quad (1)$$

Where EA is the residual enzymatic activity in response to a particular osmotic treatment, (A4p is the percentage of population in (A4 following osmotic treatment, (A4ctrl is the percentage of population in (A4 prior to osmotic treatment.

The performance of cells in extruding intracellular accumulated dye is calculated using equation (2):

$$cFA(\%) = (1 - \#A4_{Glu}/\#A4)x100 \quad (2)$$

Where cFA is the measure of performance in extruding cF, ($A4_{Glu}$ is the percentage of population in (A4 following glucose addition and 40 mins incubation, (A4 is the percentage of population in (A4 prior to glucose addition.

The kinetic of relative number of population extruding the intracellular accumulated dye is calculated in equation (3) thus:

$$RcF(\%) = (\#A4_{t-Glu}/\#A4_{t=0})x100 \quad (3)$$

where RcF is the relative number of cells still stained with cF in (A4 following glucose addition, ($A4_{t\text{-}Glu}$ is the percentage of cells still stained with cF in (A4 following glucose addition and incubation t min, ($A4_{t=0}$ is the percentage of cells still stained with cF in (A4 prior to glucose addition.

Statistical analysis

The correlation between the cell viability and osmotic-induced changes on the physiology of LGG was tested by one-way ANOVA test. Differences were considered significant at p(0.05 level of probability. This was performed with Origin7 software package (Origin Lab, Northampton, MA, USA).

Results and discussion

The osmotic strength of MRS broth before trehalose addition was 365mOs. Kg^{-1} H_20. The incorporation of trehalose subsequently generated higher osmotic conditions (Figure 1). The osmolarity of MRS medium containing 1.5M trehalose was greater than 3000 mOs. Kg^{-1} H_20. The influence of these conditions on the viability of *Lactobacillus rhamnosus* E800 was tested, since one of the alternative methods of improving the viability of probiotic cultures is the utilization of their stress adaptation.

Intracellular esterase activity and membrane integrity

Bacterial populations were differentiated into four quadrants (Figures 2, 3 and 4). Populations in: No. 1 have their membranes compromised and they do not possess esterase activity; No. 2 have their membranes minimally damaged and possess esterase activity; No. 3 do not possess esterase activity, membranes are intact; and No. 4 possess esterase activity and their membranes are intact.

Cells possessed residual esterase activity, which reduced with increase in sugar molarity (Figure 5). FL1-FL3 density plots showed the existence of greater parts of cells in No. 4. These cells had intact membranes and possessed esterase activity but some of these moved to No. 3 (Figure 2). The presence of such populations in No. 3 could be taken to indicate a loss of esterase activity. A further study was undertaken, and it was observed that membranes were permeabilized thus allowing intracellular accumulated cF, molecular weight 376, to leak. At physiological pH, cF has predominantly a three-fold negative charge thus making it practically impermeable. The inability of PI (molecular weight 668) to penetrate through the membrane into the cells, as a result of low degree of permeabilization, signifies the ability of the cells to maintain their

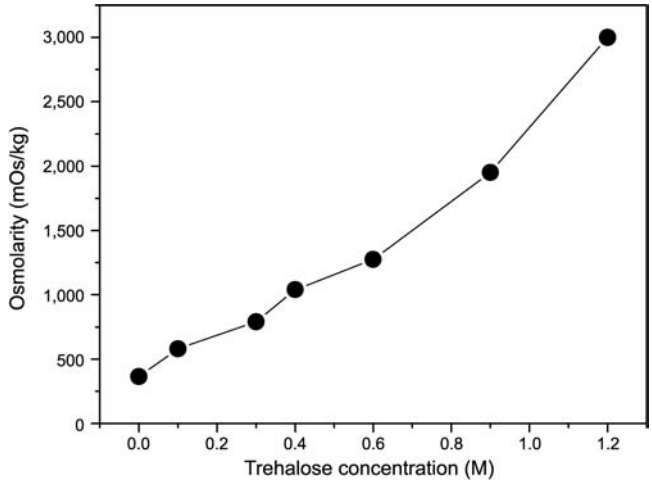
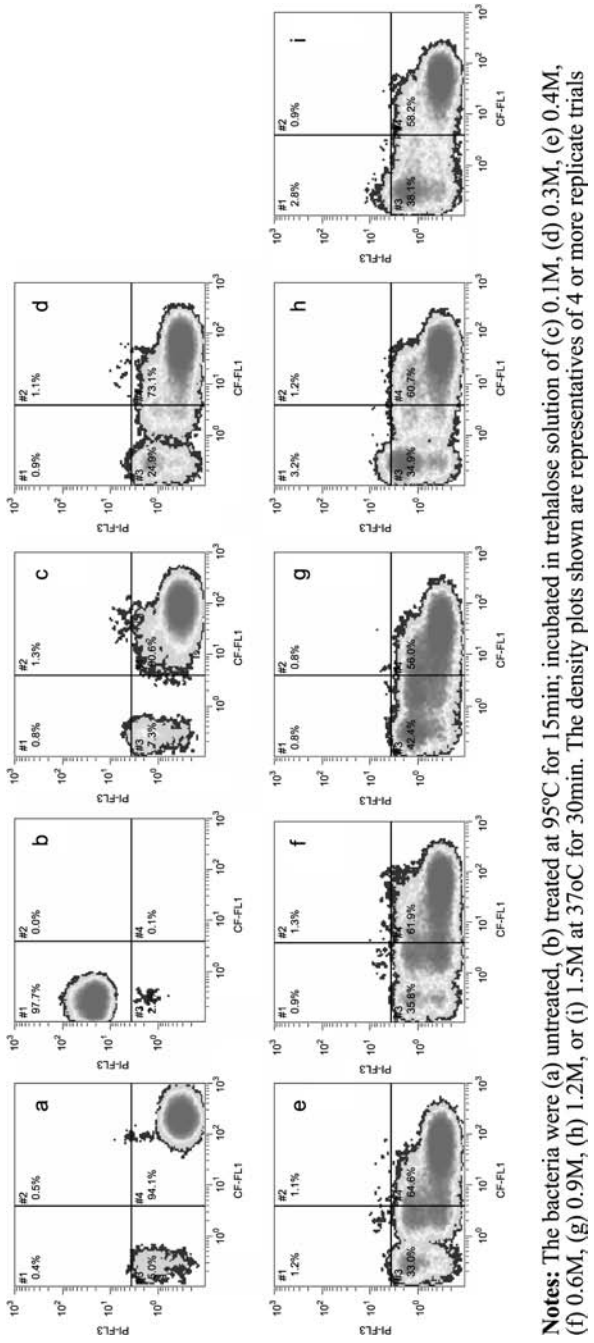
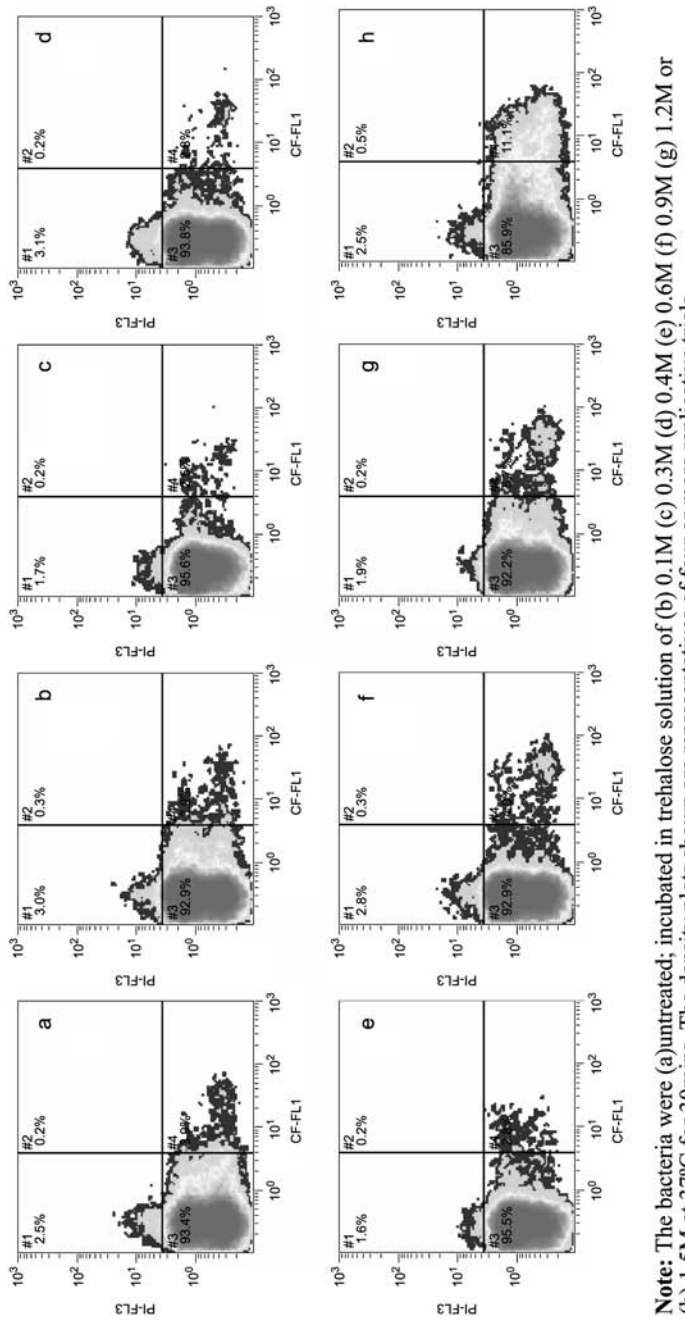


Figure 1.
Graphical representation of the osmolarity of prepared MRS-trehalose solutions



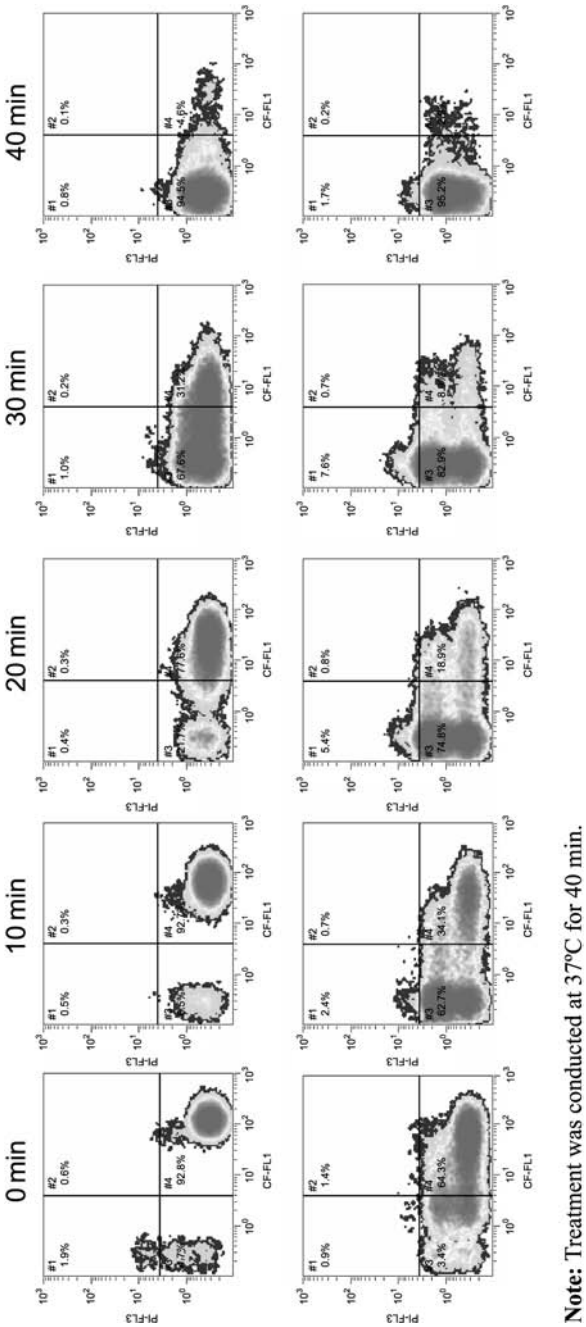
Notes: The bacteria were (a) untreated, (b) treated at 95°C for 15min; incubated in trehalose solution of (c) 0.1M, (d) 0.3M, (e) 0.4M, (f) 0.6M, (g) 0.9M, (h) 1.2M, or (i) 1.5M at 37oC for 30min. The density plots shown are representatives of 4 or more replicate trials

Figure 2.
Flow cytometry density plots of FL1 vs. FL3 of *Lactobacillus rhamnosus* E800 for evaluating the impact of incubation in trehalose solution at different molarities on their membrane integrity and cF accumulation capacity



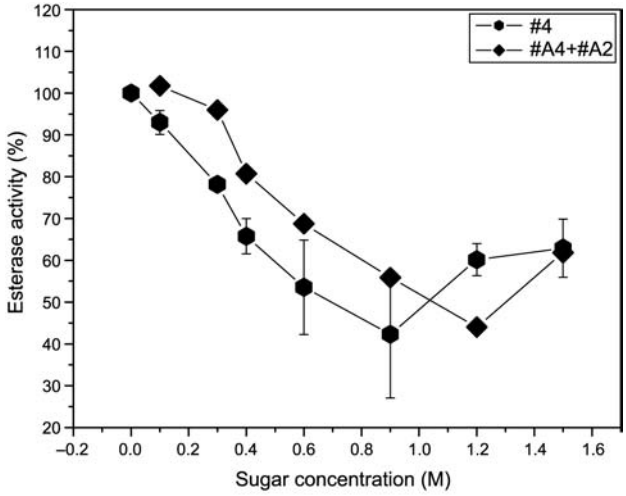
Note: The bacteria were (a)untreated; incubated in trehalose solution of (b) 0.1M (c) 0.3M (d) 0.4M (e) 0.6M (f) 0.9M (g) 1.2M or (h) 1.5M at 37°C for 30mins. The density plots shown are representatives of four or more replicative trials

Figure 3.
Flow cytometry density plots of FL1 vs. FL3 of *Lactobacillus rhamnosus* E800 to assess the impact of incubation in trehalose at different molarities on their cF-extrusion activity



Note: Treatment was conducted at 37°C for 40 min.

Figure 4.
Flow cytometric assessment of the kinetics of cF efflux upon energizing with glucose as shown by density plots flourescent pattern (FL1 vs. FL3) of the untreated (upper row) and 0.6M trehalose treated (lower row) populations



Note: Results were means of at least 3 independent trials

Figure 5.
Impact of trehalose treatment at 37°C for 30 mins on esterase activity at different molarities as derived from fluorescent density plots in Figure 2

membrane integrity. Bunthof *et al.* (1999) observed a gradual decrease of intracellular cF in cF-labelled non-energized *Lactobacillus lactis* cells.

Leslie *et al.* (1995) observed a higher internal trehalose concentration when bacterial cells were resuspended in trehalose at temperatures below the start of the phase transition than those resuspended at 20°C and above. They reported that when cells start to enter their phase transition, the membranes become leaky and sugar flows down its concentration gradient into the cells. Thus, the permeabilization of the membranes of the study strain could have contributed to the accumulation of trehalose inside its cells.

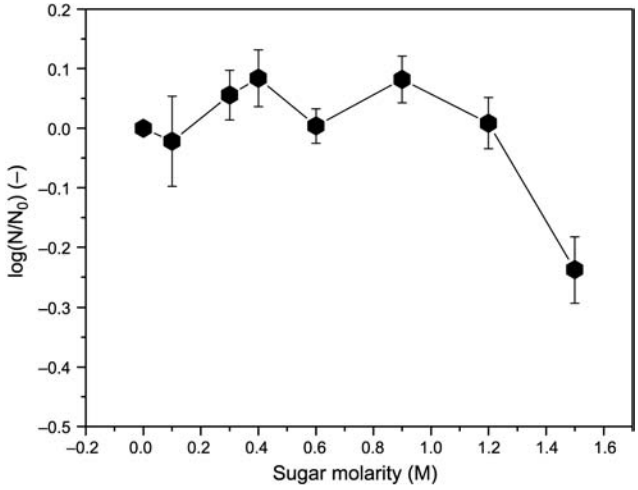
The occurrence of esterase activity (Figure 5) was not in correlation with culturability recorded on MRS agar plates (Figure 6), indicating that membrane permeabilization was transient. *Lactobacillus rhamnosus* E800 must have made use of repair mechanism during cultivation on MRS agar. The intracellular accumulation of cF and the occurrence of esterase activity do not necessarily reflect crucial metabolic activities which are involved in the maintenance of reproductive growth (Vives-Rego *et al.*, 2000).

Extrusion of intracellular accumulated dye

The ability of *L. rhamnosus* E800 to extrude accumulated cF upon glucose energizing was investigated. This was conducted as an additional measure of cell viability to give more information about physiological condition of the cells.

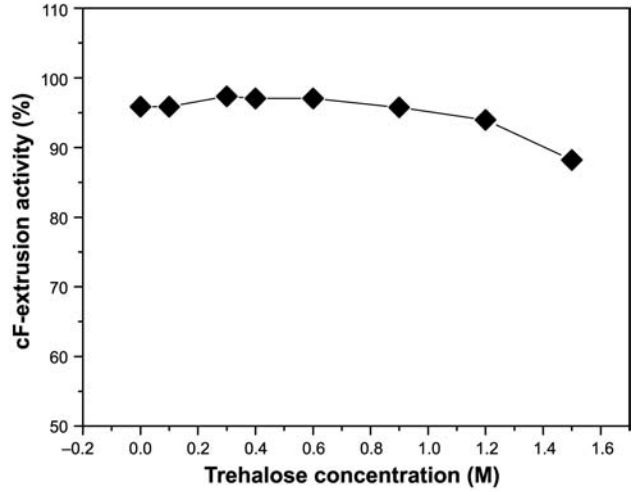
FL1-FL3 density plot analysis showed the extrusion of intracellular cF upon energizing with 20mM glucose as represented by the shift of population in #4 to #3 (Figure 3) due to florescence loss. There was no significant record of the perturbation of the extrusion activity (Figure 7). Cells that exhibited this additional measure of viability were also culturable.

Some bacteria were reported to complete extrusion of accumulated dye in 20 min (Bunthof *et al.*, 1999; Ananta *et al.*, 2004), contrary to a period of 40 min taken by



Notes: Incubation was at 37ºC for 48hr. Results were means of at least 3 independent experiments

Figure 6.
The impact of trehalose treatment on the viability of E800 assessed by plate counting method and exhibited as the logarithmic value of surviving cells (N/N_o)



Note: Results were calculated based on the analysis of fluorescent properties observed in Fig 7. Results were means of at least 3 independent experiments

Figure 7.
Graphical representation of cF-extrusion activity of cells as affected by trehalose treatments at different molarities

L. rhamnosus E800 to completely extrude the dye. Although the mentioned authors prepared cells suspensions in buffer while Ringers solution was used in the present study nevertheless, the duration of extrusion could be strain-specific.

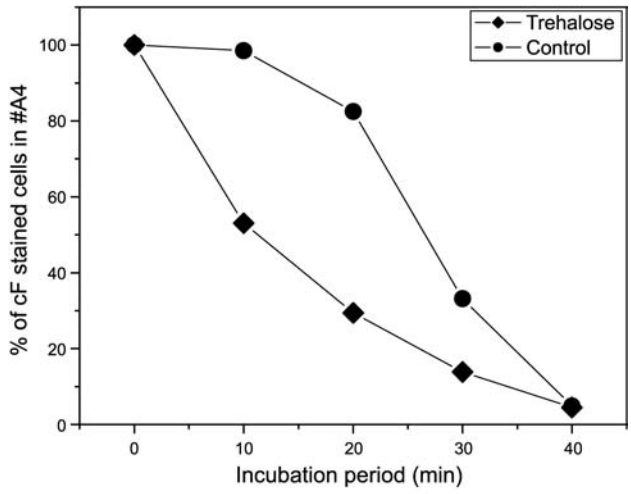
Trehalose improved cells' pump activity, which was most likely mediated by an ATP-driven transport system. ATP production and rapid extrusion of cF upon energizing were observed despite dissipation of proton motive force by addition of

ionophores, valiomycin and nigericin (Molenaar *et al.*, 1992; Bunthof *et al.*, 1999). The level of cF leakage due to the permeabilization of membranes was not as pronounced as the quick extrusion observed upon energizing by glucose addition.

Kinetically, the rate of cF extrusion upon energizing was higher in 0.6M trehalose-treated cells than in control cells, however both the treated and the untreated cells completed extrusion at the end of incubation (Figures 4 and 8).

Trehalose was able to improve cF extrusion rate in *Lactobacillus rhamnosus* E800 therefore, this sugar can be used to enhance the resistance of probiotics to drugs or toxic compounds, for example, antibiotics. Toxic compounds have always been part of the natural habitat of micro-organisms but micro-organisms can reduce the intracellular concentration of drugs by developing drug efflux systems that export lipophilic drugs before these compounds have chance to find their cellular targets (Van Veen and Konings, 1997; Van Veen *et al.*, 1999). Increasing multidrug resistance have been recorded in both gram-positive and gram-negative pathogens, which are responsible for the most devastating and prevalent diseases of humans and domestic animals (Borst and Ouellette, 1995). The use of trehalose in pharmaceuticals should be limited to the manufacture of drugs that are not meant for antimicrobial actions.

Micro-organisms face a sudden change in environmental conditions and most have been recognised to develop mechanisms of adaptation and survival. Under osmotic conditions, they survive by accumulating compatible solutes. HPLC analysis of sugars revealed the presence of trehalose in the cells and also in the stress medium of treated cells only (data not shown in this report). The ability to accumulate trehalose is the result of an elaborate genetic system, which is regulated by osmolarity (Argüelles, 2000). The ability of cells to take up disaccharides so that they are present on both



Note: The performance of this metabolic activity was measured on control population and the cells treated in 0.6M trehalose at 37ºC for 40min

Figure 8.
Graphical representation of the relative numbers of cF stained cells in the presence of 20mM glucose at increasing period of incubation as derived from the density plots in Figure 4

sides of the membrane and in contact with internal, cytosolic proteins increased the tolerance of cells to preservation processes, e.g. drying.

Yeast extract, a component of MRS medium, contains betaine but this osmoprotectant does not protect against non-electrolyte stress in lactic acid bacteria. The uptake of sugar together with the accumulation of glycine betaine may eventually result in the hyper-osmolarity of the cytoplasm, which will then be compensated by net exit of glycine-betaine. This uptake most likely occurs by facilitated diffusion via a system with a very low affinity for the substrates, which is consistent with the inability of the sugars to serve as compatible solutes (at low substrate concentration) and may be subject to osmotic regulation and transport system(s) may be more active under hyper-osmotic conditions (Glaasker *et al.*, 1998).

In a preliminary study (unpublished), it was observed that there was no significant difference between the response of cells in the logarithm-phase of growth and the stationary phase cells to osmotic stress by trehalose despite the fact that the latter are known to develop a general stress resistance to various stresses. Stress responses may be used to enhance the survival of probiotic bacteria in stressful conditions and to improve their technological properties (Van de Gutche *et al.*, 2002). The use of stationary phase cells meets the need of the industrial production of cultures where enough cells densities are needed before harvest.

Conclusions

In this study, the use of FCM and plate counts method for the viability assessment of *L. rhamnosus* E800 subjected to sugar stress was investigated. Cell suspensions were exposed to trehalose treatment at different molarities and fluorescent parameters were compared with plate counts. Trehalose treated cells were vital, having cytoplasmic membranes with selective permeability. Though there was a decrease in the cF fluorescence labelled cells, the exclusion of PI showed that the integrity of membranes was still maintained. Moreover, the experiments showed that the effects of osmotic stress on esterase activity as a result of cF retention did not agree with culturability on MRS agar plates. The esterase activity decreased in all treated samples but it never became a limiting factor for cF extrusion and culturability. The data presented so far showed that the viability of *Lactobacillus rhamnosus* E800 was not affected under the tested hyper- osmotic conditions; therefore, the strain could be a good candidate in the processing of foods containing trehalose. Moreover, any substance, which was expected to increase the tolerance of organisms when dried, should be able to have access into the cells in order to protect the proteins; and also to protect the membranes. Trehalose meets these requirements.

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