CELLULAR DAMAGE AND STORAGE STABILITY OF *LACTOBACILLUS RHAMNOSUS GG* DRIED IN DISACCHARIDE MATRICES UNDER VACUUM

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ABSTRACT

The survival of cell concentrates of Lactobacillus rhamnosus GG (LGG) in selected dissacharides after drying under vacuum was evaluated. Drying is a useful technique for preserving foods, agricultural products and pharmaceuticals. However, biological materials can be irreversibly damaged during this treatment resulting in substantial loss of viability and activity. In this paper, disaccharide systems (trehalose, sucrose and lactose) were used as protectants for L. rhamnosus GG. Among the solutes examined, trehalose improved the recovery of viable cells after drying as well as during storage at 4°C and 25°C. Stored vacuum-dried trehalose systems were found as viscous syrups which indicated that they were not glassy. Damage of cell membrane and reduction in colony sizes occurred as a result of dehydration inactivation. Comparison of the conventional techniques with flow cytometric viability assessment after drying revealed the occurrence of certain cell population which were stressed and lost their ability to grow on agar. The protecting ability of trehalose on bacterial cells was only against lysozyme and pepsin actions. These results could have some relevance, especially in underdeveloped and /or developing countries, for the production of functional confections.

Keywords: Lactobacillus rhamnosus GG, trehalose, storage, viability, flow cytometry.

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Introduction

The industrial preservation of lactobacilli involves processes such as freezing, freeze-drying and air-drying. These processes can result in structural and physiological injury to the bacterial cells, resulting in substantial loss of viability (Prasad et al., 2003). Viability and activity loss occurring on bacteria during drying are related to damages to the cell wall, cytoplasmic membrane and the DNA (Teixeira et al., 1995a, b).

Viability and thermal stability of sensitive strains to dehydration could be improved by the addition of certain amounts of disaccharides to the suspending media (Cerruti et al., 2000) in which the cells are to be dried. The non-reducing disaccharides, sucrose and trehalose have mostly been used as protectants of dry labile biomaterials such as protein, enzymes, liposomes etc. They could be used to preserve intact cells during drying if these sugars are present on both sides of the membrane and in contact with internal, cytosolic proteins. Trehalose is one of the most stable sugars, since it has extremely low disaccharide bond energy (less than 4kJ/mol). It has been described to act as the best stabilizer of structure and function of several macromolecules. This extraordinary effect was attributed to several of its properties such as making hydrogen bonds with membranes or the ability to modify the solvation layer of proteins, however its ability to substitute more water molecules in the solution than other related sugars such as sucrose, maltose (Sola-Penna and Meyer-Fernandes, 1998) have been shown. On the other hand, sucrose has a fructofuranoside disaccharide bond greater than 115kJ/mol and the furanosic ring confers sucrose a relatively unstable disaccharide bond; therefore, the disaccharide splits readily (under adequate conditions) to form glucose and fructose (Schebor et al., 1999).

Two hypotheses have been put forward on the mechanisms by which disaccharides protect the integrity of dry biological systems. The first suggests that the ability to stabilize proteins during drying results from sugars forming hydrogen bonds with the proteins when water is removed, thus preventing protein denaturation by replacing water molecules needed to maintain the protein structure (Schebor et al., 1999). Sugars may also confer protection to liposomes and isolated biological membranes against dehydration damage. This relies

on a direct physical interaction between the hydroxyl groups of the sugars and the polar residues of the phospholipids headgroups in dehydrated state as explained also by the water replacement theory (Crowe and Crowe, 2000; Crowe et al., 1993a,b).

The stabilizing properties of sugars can also be explained by the formation of glassy state by the sugar upon dehydration (Sun and Leopold, 1997). The most important parameter describing the glassy state of amorphous materials is the glass transition temperature (T_g), below which the materials exhibit extremely high viscosities, which gives them a "solid-like" property. Above the glass transition temperature, viscosity drops sharply in the rubbery state and the mobility of the system increases accordingly (Ananta, 2005).

The roles played by probiotic microorganisms in promotion and maintenance of health stimulated significant interests in their incorporation into functional foods and pharmaceutical products and it is recommended that probiotic products contain at least 10^7 live microorganisms per g or ml (Ishibashi and Shimamura, 1993).

For probiotic bacteria in foods to be beneficial in the host, they should be able to survive gastric transit, reach the small intestine in sufficient numbers and persist in this environment to be effective (Charteris et al., 1998).The harsh environment of the gastrointestinal tract is mainly attributed to the low pH conditions of the stomach, in addition to the presence of bile in the stomach (Ananta, 2005).

The application of the commercial available LIVE/DEAD®BacLight™ bacterial viability kit differentiates live and dead bacteria based on plasma membrane permeability. The first component is SYTO9®, membrane permeant, which acts as total cell stain while the second component is PI, membrane impermeant, which penetrates into cells upon cell death and membrane damage thus nullifying the green SYTO9® fluorescence. When used in combination, intact cells are labeled green while cells with damaged membranes are labeled red (Ananta, 2005)

Due to the economic situations in developing and/or underdeveloped countries and the economic importance of probiotic bacteria, this paper investigates the preservation of bacterial cells in commonly available disaccharides using affordable drying facility hoping that the results will be of relevance in the production of confections from under-utilized crops.

The viability of *Lactobacillus rhamnosus GG* cells, a model system, in the presence of sucrose, trehalose and lactose after drying under vacuum at 25°C was evaluated. Storage experiments were conducted at 4°C, 25°C and 37°C over a period of 6-8 weeks. Flow cytometric measurements were conducted to relate the data obtained with that of plate enumeration method.

Materials and Methods.

Culture and growth conditions.

The bacterial strain, *Lactobacillus rhamnosus GG (LGG)* ,was obtained from Valio R and D, Helsinki, FL.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 (50ml) at OD_{600} 0.1. Growth was carried out at 37°C over a period of 24h and monitored spectrophotometrically at 600nm (Graphicord uV-240, Schimadzu, JPN) until the exponential phase ,having $3.0x10^7$ cfu mL^{-1} cell concentration, and stationary phase of growth were reached. 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. This corresponded to a cell concentration of 3.4×10^9 cfu mL^{-1}.

Drying.

Cells were grown in MRS broth at 37°C and harvested at both exponential and stationary phases of growth. Harvested cells were washed in Ringer"s solution (No. 15525, Merck, Darmstadt, Germany). Cells from the exponential phase were mixed with equal volume 20% (w/w) trehalose (Carl Roth GmbH Karlsruhe), 20%(w/w) sucrose (Merck KgaA, Darmstadt) and 20%(w/w) lactose (Merck KgaA, Darmstadt) in order to obtain the sugar that would give the best protection to the cells after drying . Cells from the stationary phase were concentrated to OD_{10} before mixing with equal volumes of 20% w/w, 25% w/w and 30% (w/w) trehalose. 50µl aliquots of each cell-protectant mixture were distributed into 1.5mL Eppendorf tubes and dried in a vacuum oven (Heraeus) above saturated LiCl solution (a_w=0.11) at 25°C for 24h. The final pressure achieved in the vacuum oven was 200Torr.

Enumeration of probiotics after drying.

Determination of the survival of cells in the disaccharide matrices was done by rehydrating the samples with sterile Ringer"s solution (No 15525, Merck, Darmstadt, Germany) to obtain the same solids concentration as the original feed solution. Mixing was done vigorously for about 15min using a vortex mixer to dissolve the samples. Serial dilution was performed and drop plating of the appropriate dilution was in duplicate on MRS agar

(Oxoid, Basingstoke, UK). Plates were placed in an anaerobic jar (Anaerocult®, A, Merck, Darmstadt Germany) and incubated at 37^{o}C for 48h. Survival rates were calculated as % survival =N/N_ox100, where N_o represented the number of bacteria before drying and N was the number of bacteria after drying.

Storage tests.

Dried samples were stored at 4^{o}C, 25^{o}C and 37^{o}C in closed Eppendorf tubes. Storage experiments were performed in three replicate trials for 6-8 weeks. Storage inactivation data were expressed as logarithmic value of relative survival fraction ($logN/N_o$). N refers to the bacterial count at a particular storage period while N_o refers to the bacterial count at the beginning of storage.

Salt tolerance test.

In order to study the potential cellular damage arising from the drying method, the sensitivity of cultures to NaCl before and after processing was determined (Gardiner et al., 2000). Fresh cultures and dried ones were plated on MRS agar plates supplemented with NaCl (5%, Merck KGaA, Darmstadt, Germany)). The plates were examined after 5-6 days incubation in anaerobic jar (Anaerocult® A, Merck, Darmstadt; Germany) and viable numbers were compared with numbers obtained on MRS plates without NaCl.

Staining procedure with LIVE/DEAD® BacLight^{TM} bacterial viability kit

The LIVE/DEAD® BacLight bacterial viability kit (Molecular Probes Europe BV, Leiden, NL) consisting of two separate stock solutions, SYTO9 and PI, were prepared according to manufacturer‟s instructions by dissolving both in dimethyl sulfoxide at 3.34mM and 20mM respectively. 20 to 60µl of the dissolved samples were then added to 1mL sterilized distilled water. The mixtures were incubated on ice for 10min with 1. 5µl of SYTO9® and 20µl of PI in the dark before fluocytometric measurements.

Flow cytometric measurement and data analysis

Analysis was performed on a Coulter$^{®}$EPICS$^{®}$XL-MCL flow cytometer (BeckmanCoulter Inc., Miami-FL, USA) equipped with a 488 nm laser. Cell was delivered at the low flow rate, corresponding to 400 to 600 events per s. Forward scatter (FS), sideward scatter (SS), green (FL1) and red fluorescence (FL3) of each single cell were measured, amplified, and converted into digital signals for further analysis. SYTO9$^{®}$ 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 ± 20 nm and 620 ± 15 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 (BeckmanCoulter Inc., Miami-FL, USA).

Density plot analysis of FL1 vs FL3 was applied to resolve the fluorescence properties of the population measured by flow cytometer (Fig. 1&2). With this graph the population was able to be graphically differentiated and gated according to their fluorescence behaviours.

Two regions were created in this plot for gating cells with intact membrane and the ones with ruptured membrane.

The designation of gates according to the properties of cellular membranes was performed by means of measuring fluorescence density plot signals of untreated cells, which were located in gate LIVE. On the other hand, cells heat treated at 95°C for 15 min were entirely encountered in the area surrounded by gate DEAD (Ananta, 2005)

Screening of acid, bile, lysozyme and pepsin tolerances.

Equal volume of dried bacterial samples was treated separately with equal volumes of PBS Buffer adjusted to pH 2.0 with HCl and 1.5% Bile (Sigma- Aldrich, Munich, Germany) (Saarela et al., 2004). Treatment was also conducted in 5mg mL^{-1} Lysozyme (Sigma Aldrich, Steinheim, Germany) and 3mg mL^{-1} Pepsin (Sigma- Aldrich, Steinheim, Germany).Samples were incubated at 37^{o}C in water-bath for 3h and only 2h for acid treatment. Appropriate dilutions of samples were drop plated on MRS agar and incubation was conducted for 48h at 37^{o}C.

Statistical Analyses

Statistical analyses were performed by the ANOVA methodology. Differences were considered significant at $p< 0.05$. All statistical analyses were performed with Origin7 software package (OriginLab, Northhampton, MA, USA).

Results and Discussion

Survival of *Lactobacillus rhamnosus GG,* in the exponential phase, vacuum-dried in the presence of sucrose, trehalose and lactose over a period of 24h revealed that about 60% of the cells survived the drying process in trehalose while no survivor was found in sucrose and lactose. Extending the drying period to 48h showed a reduction of the surviving cells to 10% due to a longer exposure to osmotic stress. Trehalose, a naturally occurring disaccharide of glucose is known as an effective protectant for bacteria and yeast cells in the absence of water (Eleutherio et al., 1993; Leslie et al., 1995; Linders et al., 1997). Also the accumulation, of

trehalose, with drying in fungal spores, nematodes and cysts of the brine shrimp Artemia ; or of sucrose in pollens and seeds of higher plants (Hoekstra et al., 1992; Chapman, 1994) improves the tolerance to drying.

Trehalose and sucrose samples were found as viscous syrups irrespective of the period of drying. Vacuum drying of enzyme solution in the presence of trehalose and sucrose were not found in a glassy state but rather yielded a paste or very viscous fluid (Rossi et al., 1997), which did not become solid even after extensive desiccation (Uritani et al., 1995). Enzyme activity was retained both after drying and during storage irrespective of the physical state of the dried samples, thus it was proposed that the stabilization of the biological structure was through a preferential interaction with trehalose rather than to a modification of the properties of water (Rossi et al., 1997). The absence of solid-like properties was an indication that the dried systems, in this study, were not in the glassy state. Therefore the protective ability of trehalose could be explained as a result of the hydroxyl groups of the sugar making a direct interaction with the polar residues of the phospholipids head groups as described by the water replacement hypothesis (Crowe et al., 1993a,b, Crowe and Crowe, 2000). The replacement leads to maintenance of phospholipids bilayers at their hydrated spacing, which in turn preserves the structure of the membrane, thereby preventing damage during freezing or drying (Crowe et al., 1987). The property of trehalose as an effective glass former has been used to partly explain its superior protective ability (Miller et al., 1997; Miller at al., 1998). There is literature evidence that sucrose may be hydrolysed during freezing, dehydration and storage (Karel and Labuza, 1968; Flink, 1983). There could be a possibility of sucrose presenting same effectiveness as trehalose if the concentration of sucrose was corrected by the percentage of the occupied volume; but the improvement of tolerance to desiccation by accumulation of sucrose has mostly been reported in plants (Hoekstra, 1992).

Lactose could not protect the bacterial cells against dehydration though the samples were in glassy form. Lactose serves as the major constituent (ca. 52%) of skim milk, a commonly used drying medium but researches have proved that the protecting ability of skim milk was due to the milk components other than lactose (Ananta, 2005; Daemen and van der Stege, 1982; Abadias et al., 2001).

Cells in the stationary phase of growth are of higher densities compared to cells in the exponential phase thus a higher concentration of trehalose was needed to improve the viability status of cells in the stationary phase (Table 1). The exhibition of tolerance to drying showed that cells of *L. rhamnosus GG* were able to take up trehalose in order to protect the functional proteins embedded in the bacterial membranes besides the membranes.

Bacterial cells were sensitive to NaCl following drying. Prior to drying, there were no differences between the colony counts of this strain on MRS containing 5% NaCl and MRS alone but after drying, cells numbers decreased in the presence of NaCl. The strain exhibited 52% sensitivity to NaCl after drying. This indicated that cell membrane damage occurred as a result of long period of drying process. At low drying temperatures, thermal inactivation is negligible but dehydration inactivation may impose serious problems and cell membrane damage is associated with this dehydration inactivation (Linders et al., 1997). Colony sizes were also reduced (data not shown).

The flow cytometric measurements of osmotically treated cells showed the presence of majority of non-dried cells in „LIVE" gates (Fig 1 & 2). After drying, some of these cells moved towards the „DEAD" gates. The protection provided by compatible solute, such as trehalose, to cells during osmotic stress can be reported to differ from the one given when cells are subjected to drying processes which bring much reduction in available cell water.

The results of flow cytometric measurements differ significantly ($p<0.05$) from the values obtained by plate counts as revealed in Table 1&Fig 1. This could be an indication that some cells occurring in the „LIVE" gate, as shown by FL1-FL3 density plots, must have been injured to the extent of not having the ability to resume growth on agar plates. The presence of such non-culturable bacteria with a high degree of membrane intactness in food might be critical as they may be active in excreting toxic or food spoilage metabolites. However, many possible probiotic effects of bacteria depend on activity rather than culturability, and even dead cells can have some probiotic effect such as immunomodulation (Pessi et al., 1999; Ouwehand et al., 2000). In contrast, the flow cytometric density plots of cells at the exponential phase revealed a lower percentage of viable cells after drying in trehalose medium (Fig. 2) compared to 60% surviving cells earlier reported on MRS agar plates. This significant difference ($p<0.05$) could be as a result of the ability of these sublethally injured bacteria in making use of a repair mechanism for survival when cultured on a fresh growth medium; cells at the exponential stage are at the peak of their metabolic activity. For further studies, incubating cells for a longer time than 10mins in PI might bring about a distinct differentiation of cells into" LIVE" and „DEAD" gates.

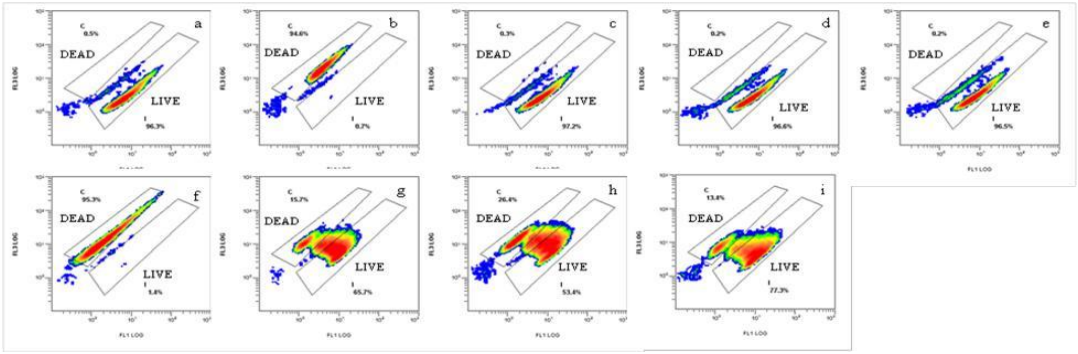

Fig 1: Flow cytometry density plots of FL1 (fluorescence collected at 525nm) vs FL3 (fluoresecence collected at 620nm) of *Lactobacillus rhamnosus GG* to assess the impact of drying on the integrity of cellular membranes of stationary phase cells. Two gates were fixed for discrimination of the two extreme states of membrane conditions i.e. intact and completely ruptured represented by 'LIVE' and 'DEAD' respectively. Upper figures represent non-dried (a) control cells; (b) cells treated at 95^{0}C for 15 min (negative control); fresh cells suspended in (c) 20% w/w (d) 25% w/w, and (e) 30% w/w trehalose : lower figures represent (f)control cells dried without sugars; cells dried under vacuum after treatments with (g) 20%, (h) 25% and (i) 30% w/w trehalose respectively

Table 1: The impact of trehalose concentration during drying of *L. rhamnosus GG* under vacuum on the % surviving cells (N/N_0) on MRS agar and % viable cells encountered in 'LIVE' gates. An initial cell ^{9} cfu mL^{-1}. Data are means of 3 drying experiments.countofbacterialsolutionwas~10
***The FL1-FL3 density plots are as shown in Fig 1.**

Parameters	%Trehalose (w/w)		
	20	25	30
% surviving cells (N/N_0)	1.03±0.2	7.46±0.6	16.58±3.5
*% SYTO9 stained cells indicated by „LIVE" gate	59.55±8.7	57±5.0	73.7±5.1

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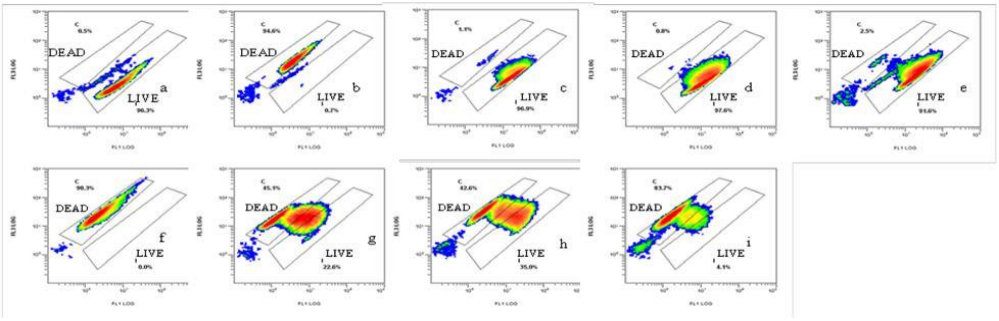

Fig 2: Flow cytometry density plots of FL1 (fluorescence collected at 525nm) vs FL3 (fluoresecence collected at 620nm) of *Lactobacillus rhamnosus GG* to assess the impact of drying on the integrity of cellular membranes of exponential phase cells.
Two gates were fixed for discrimination of the two extreme states of membrane conditions i.e. intact and completely ruptured represented by' LIVE' and 'DEAD' respectively.
Upper figures represent non-dried (a) control cells; (b) cells treated at 95^{0}C for 15 min (negative control); cells treated with 20% w/w (c) trehalose; (d) sucrose, and (e) lactose : lower figures represent (f)control cells dried without sugars; cells dried under vacuum after treatments in 20% w/w (g) trehalose; (h) sucrose and (i) lactose.

The weight of vacuum dried samples was estimated to be ca.5mg which might contain some residual water. The moisture content could not be determined accurately by the conventional oven gravimetric method due to the obtained small mass. The storage stability of vacuum dried *L. rhamnosus GG* in 30% (w/w) trehalose during prolonged storage at 4°C, 25°C and 37^{o}C was investigated.

The decline of bacterial load was represented by the logarithmic values of the survival fractions after different storage periods. At 4°C, survival was higher ($p<0.05$) than at other storage temperatures. For a shelf life of 4 weeks of storage, there was a reduction of 2 log units at 25°C (Fig. 3) which was equated to be a level ~ 10^6 cfu mL^{-1} . In general, a level of 10^6 cfu/g , by the time of consumption, is

required (Boylston et al, 2004) although in some cases a minimal level of 10^5 cfu/g till the expiry date was considered as sufficient (Shah and Lankaputhra, 1997; Schillinger, 1999). *Lactobacillus acidophilus* cells vacuum dried in 20% trehalose exhibited 8.7% recovery rate after 16 days of storage at room temperature (Conrad et al., 2000). At 37^{o}C, LGG exhibited increased fluidity and within a week, viability reduced drastically by more than 4 log cycles (data not shown). Temperature is a critical factor for microbial survivability during storage and higher survival rates have been obtained at lower storage temperatures (Gardiner et al, 2000). Storage at refrigeration temperature, though impractical from a commercial point of view, is optimal for culture viability in vacuum-dried samples. The viscous nature of the dried samples portrays the limitation of the use of these probiotics samples. However, their use could be of significance in the production of functional confections since high viability (~10^8 cfu mL^{-1}) syrup containing probiotic lactobacilli was obtained.

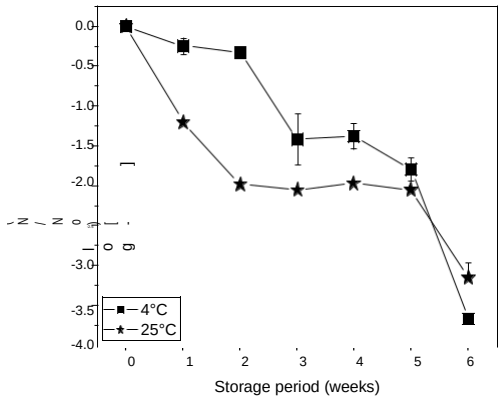

Fig 3: Viability loss of dried *Lactobacillus rhamnosus GG* expressed as the logarithmic values of relative survival fraction (log N/N_o) as described in Materials and Methods during storage at 4°C and 25°C.

Bacterial samples treated with lysozyme showed log reductions of ~ -0.4 after drying. This sensitivity, though minimal, could be a manifestation of damage to the cell wall component. Log reduction of ~ -0.8 was observed with pepsin treatment. Trehalose could not protect samples against the actions of acid and bile; resistance to bile and acid being recognized as an important feature of LAB used as probiotics. Reducing the period of drying under vacumm may help to overcome this challenge since irrespective of the drying period, only syrups were obtained. An earlier study revealed the ability of the strain of study to resist bile action after its subjection to osmotic stress by trehalose treatment (unpublished). Therefore in this study, drying must have brought about much reduction in available cell water thus leading to additional stress. Microorganisms that have been damaged, but not killed, by exposure to stress often become more sensitive to other types of agents (Teixeria et al., 1995c).

Conclusions

The results of this study suggested that since solid-like properties were absent, the protective ability of trehalose was as a result its hydroxyl groups making a direct interaction with the polar residues of the phospholipids head groups as described by the water replacement hypothesis. The growth phase of an organism determines the amount of sugars needed for drying processes in order to protect the organisms against drying damages. Drying in the presence of sugars can result in the occurrence of stressed and non-culturable cells but viable as indicated by flow cytometric methods. The protection conferred on membranes by sugars during osmotic stress and drying processes were seen to be different from one another. In both under-developed and developing countries, the use of available non-sophisticated equipment and processes can be employed in the production of syrup- containing probiotic bacteria which can be tailored for use in functional food applications such as chocolates, candies, etc.

References

Abadias, M., A. Benabarre , N. Teixido, J. Usall, and I. Vinas , 2001. Effect of freeze-drying and protectants on viability of the biocontrol yeast *Candida sake.* Int. J. Food Microbiol. 65(3):173-182

Ananta, E. ,2005. Identification of environmental factors involved in viability and stability of probiotic bacteria *Lactobacillus rhamnosus GG (ATCC 53103)* during spray drying and application of high pressure pre-treatment for the improvement of stress tolerance. PhD Thesis University of Technology Berlin. 123-129pp

Boylston, T.D., C.G Vinderola, H.B. Ghodussi and J.A. Reinheimer, 2004. Incorporation of bifidobacteria into cheeses: challenges and rewards. Int. Dairy J. 14(5): 375-387

Cerrutti, P., M.S. de Huergo, M. Galvagno, C. Schebor and M. del Pilar Buera, 2000. Commercial baker‟s yeast stability as affected by intracellular content of trehalose, dehydration procedure and the physical properties of external matrices.

Appl. Microbiol. Biotechnol. 54(4):575-580

Chapman, D., 1994. The role of water in biomembrane structures. J. Food Eng. 22(3): 367-380

Charteris, W.P., P.M. Kelly, L. Morelli and J.K. Collins, 1998. Development and application of an in vitro methodology to determine to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. J. Appl. Microbiol. 84(5):759-768

Conrad, P.B., D.P Miller, P.R. Cielenski and J.J. de Pablo, 2000. Stabilization and preservation of *Lactobacillus acidophilus* in saccharide matrices. Cryobiology 41:17-24

Crowe, J.H. and L.M. Crowe, 2000. Preservation of mammalians cells-learning nature‟s tricks. Nat. America 18:145-146

Crowe, J.H., L.M. Crowe and J.F. Carpenter, 1993a Preserving dry biomaterials. The water replacement hypothesis. Part 2. BioPharm 6:40-43

Crowe, J.H., L.M. Crowe and J.F. Carpenter, 1993b. Preserving dry biomaterials: The water replacement hypothesis. Part 1. Biopharm 6:28-33

Daemen, A.L.H. and H.J. van der Stege, 1982. The destruction of enzymes and bacteria during spray drying of milk and whey 2. The effect of drying conditions. Neth. Milk Dairy J. 36:211-229

Eleutherio, E:C:A., P.S. de Araujo and A.D. Panek,1993. The role of trehalose carrier in dehydration resistance of saccharomyces cerevisiae. Biochim. Biophys Acta 1156: 263-266

Flink, M.J.,1983. Non enzymatic browning of freeze-dried sucrose. J. Food Sci. 48 (2):539-542

Gardiner, G.E., E. O‟Sullivan, J. Kelly, M.A.E. Auty, G.F. Fitzgerald, J.K.Collins, R.P. Ross and C. Stanton,2000. Comparative survival rates of human-derived probiotic *Lactobacillus paracasei* and *L. salivarius* strains during heat treatment and spray drying. Appl. Environ. Microbiol. 66 (6):2605-2612

Hoekstra, F.A., J.H. Crowe, L.M. Crowe, T. van Roekel and E. Vermeer, 1992. Do phospholipids and sucrose determine membrane phase transitions in dehydrating pollen species? Plant Cell Environ. 15(5): 601-606

Ishibashi, N. and S. Shimamura, 1993. Bifidobacteria: research and development in Japan. Food Technol. 46:126-135

Karel, M. and T. Labuza, 1968. Non enzymatic browning in model systems containing sucrose. J. Agric. Food Chem. 16:717

Leslie, S., E. Isreali, B. Lightart, J. Crowe and L. Crowe, 1995. Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. Appl. Environ. Microbiol. 61(10):3592-3597

Linders, L.J.M., G. Meerdink and K. Van‟t Riet, 1997. Carbohydrates and the dehydration inactivation of *Lactobacillus plantarum*-The importance of moisture distribution and water activity. J. Food Eng. 31(2): 237-250

Miller, D.P., R.E. Anderson and J.J. de Pablo, 1998. The stabilization of lactate dehydrogenase following freeze-thawing and vacuum-drying of trehalose and borate. Pharm. Res. 15(8): 1215-1221

Miller, D.P., J.J. de Pablo and H.R. Corti, 1997. Thermo-physical properties of trehalose and its concentrated aqueous solutions. Pharm. Res. 14(5):578-590

Ouwehand, A.C. , S. Tölkkö, J. Kulmala, S. Salminen and E. Salminen. 2000, Adhesion of inactivated probiotic strains to intestinal mucus. Lett. Microbiol. 31: 82-86

Pessi, T., Y. Sütas, M. Saxelin, H. Kallioinen, and E. Isolauri, 1999. Antiproliferative effects of homogenates derived from five strains of candidate probiotic bacteria. Appl. Environ. Microbiol. 65: 4725-4728

Prasad, J., P McJarrow and P. Gopal, 2003. Heat and osmotic stress responses of probiotic *Lactobacillus rhamnosus HN001 (DR 20)* in relation to viability after drying. Appl. Environ. Microbiol. 69(2):917-925

Rossi, S., M.P. Buera, S. Moreno and J. Chirife, 1997. Stabilization of the restriction enzyme EcoRI dried with trehalose and other selected glass forming solutes. Biotechnol. Prog. 13(5):609-616

Saarela, M., M. Rantala, K. Hallama, L. Nohynek, I. Virkajärvi and J. Mätto, 2004. Stationary-phase acid and heat treatments for improvement of the viability of probiotic lactobacilli and bifidobacteria. J. Appl. Microbiol. 96(6):1205-1214

Schebor, C., L. Burin, M. del Pilar Beura and J. Chirife, 1999. Stability to hydrolysis and browning of trehalose and raffinose in low moisture systems in relation to their use as protectants of dry biomaterials. Lebens. Wiss. U.-Technol. 32:481-485

Schillinger, U. ,1999. Isolation and identification of lactobacilli from novel-type probiotic and mild yoghurts and their stability during

refrigerated storage. Int. J. Food Microbiol. 47(1-2): 79-87

Shah, N.P. and W.E.V. Lankaputhra, 1997. Improving viability of *Lactobacillus acidophilus* and *Bifidobacterium spp*. in yoghurt. Int. Dairy J. 7(5): 349-356

Sola-Penna, M. and J.R. Meyer-Fernandes, 1998. Stabilization against thermal inactivation promoted by sugars on enzyme structure and function: Why is trehalose more effective than other sugars? Arch. Biochem. Biophys. 360(1):10-14

Sun, W.Q. and C. Leopold, 1997. Cytoplasmic vitrification and survival of anhydrobiotic organisms. Comp. Biochem. Physiol. 117A:327-333

Teixeira, P.M., H.P. Castro and R. Kirby, 1995a Death kinetics of Lactobacillus *bulgaricus* in a spray-drying process. J. Food Prot. 58(8):934-936

Teixeira, P.M., H.P. Castro and R. Kirby, 1995b Spray dying as a method for preparing concentrated cultures of *Lactobacillus bulgaricus.* J. Appl. Bacteriol. 78: 456-462

Teixeria, P.C, M.H. Castro, F.X. Malcata and R.M. Kirby, 1995c. Survival of *Lactobacillus delbrueckii ssp. bulgaricus* following spray drying. J. Dairy Sci. 78(5):1025-1031

Uritani, M., M. Takani, and K. Yishinaga, 1995. Protective effect of disaccharides on restriction endonucleases during drying under vacuum. J. Biochem. 117:774-779

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