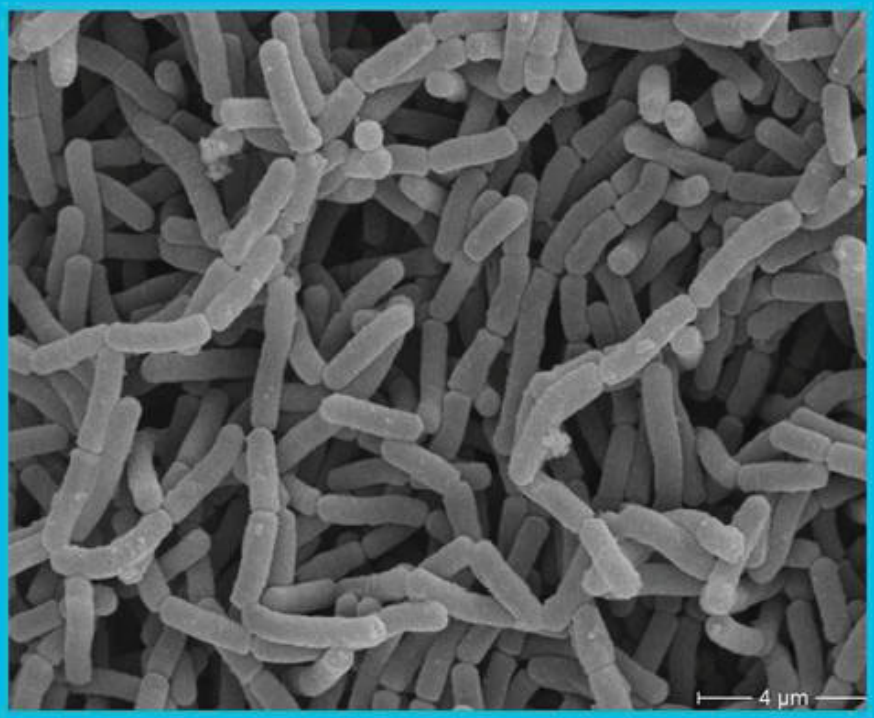
Elizabeth O. Sunny-Roberts

Application of disaccharides pre-treatment in improving tolerances of *Lactobacillus rhamnosus* strains to environmental stresses or during vacuum- and spray drying processes



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Application of disaccharides pre-treatment in improving tolerances of *Lactobacillus rhamnosus* strains to environmental stresses or during vacuum- and spray drying processes

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Zusammenfassung

Probiotische Mikroorganismen sind während Fermentation, Einsatz im Produkt und Verzehr schwankenden Umweltbedingungen ausgesetzt, die sich durch die Entwicklung von Stresserkennungs und –anpassungsmechanismen teilweise ausgleichen können. Da der gesundheitsfördernde Effekt von Probiotika durch den Konsum einer täglichen Dosis von mindestens 10^8 lebenden Zellen erreicht werden kann, sollten diese umweltbedingten Stressfaktoren genutzt werden, um das Überleben der Mikroorganismen sowohl während der Prozessierung als auch im Lebensmittel bis zum Ende der Mindesthaltbarkeit zu garantieren.
Die vorliegende Arbeit untersucht die Anwendung von Trehalose und Sucrose zur Induktion von osmotischem Stress bei *Lactobacillus rhamnosus* GG *(LGG)* und *Lactobacillus rhamnosus E-97800 (E800)* und die Eignung dieser Substanzen als Trägermedium bei der nachfolgenden Sprüh- und Vakuumtrocknung.
Durchflusszytometrie, Keimzahlbestimmung und die Anwendung der Rasterelektronenmikroskopie wurden als wesentliche analytische Methoden zur Charakterisierung von *Lb. rhamnosus* eingesetzt. Ein Verlust der Membranintegrität wurde bei *LGG* nur nach Behandlung in einer 1.5 M Sucrose Lösung festgestellt. Permeabilisierung der Zellmembranen war verstärkt, wenn *E800* in Trehalose-Medium behandelt wurde. Es wurde eine Reparatur von Membranschäden nach Re-kultivierung in Wachstumsmedium beobachtet. Trehalose war im Vergleich zu Sucrose besser in der Lage, die Ausschleusungsfähigkeit der Zellen zu verbessern, die als ein Schutzmechanismus gegen toxische Metaboliten verstanden werden kann. *E800* war dabei hinsichtlich der Dauer des Ausschleusungsvorganges weniger aktiv als *LGG*. Morphologische Veränderungen in Richtung kapselähnlicher Formen wurden bei *LGG* beobachtet. Die Reaktion auf osmotischen Stress war stammspezifisch.
Eine osmotische Vorbehandlung im sub-lethalen Bereich wurde eingesetzt um den Einfluss auf die Zellstabilität unter anderem Stressbedingungen wie lethale Salzkonzentrationen, Hitze oder lethaler osmotischer Stress, die während der Verarbeitung bzw. im Intestinaltrakt auftreten können, zu untersuchen. Trehalose verbesserte die Salztoleranz von *LGG* während es die Hitzetoleranz von *E800* drastisch reduzierte.
Die Überlebensrate der *Lb. rhamnosus* Stämme in ausgewählten Disacchariden nach Vakuumtrocknung bei 25°C und anschließender Lagerung wurde untersucht. Durchflusszytometrische Analysen und die Bewertung der Toleranz der Mikroorganismen gegenüber Selektivmedien dienten der Charakterisierung der Zellschädigung während des Trocknungsprozesses. Trehalose verbesserte dabei die Erholung lebender Zellen nach der Trocknung und während der Lagerung bei 4°C und 25°C. Membranschäden und eine Verringerung der Koloniegrößen wurden als Resultate der Schädigung während der Trocknung beobachtet. Der Vergleich konventioneller Kultivierungstechniken mit den Ergebnissen der Durchflusszytometrie zeigte eine bestimmte Zellfraktion, die durch Stressung die Fähigkeit zur Vermehrung auf dem Nährmedium verloren hatte.
Die Verwendung von Trehalose und der Einfluss von enthaltenem Mononatriumglutamat auf die Überlebensrate wurde während der Sprühtrocknung und der Lagerung untersucht. Mittels Durchflusszytometrie und der Bewertung der Toleranz der Bakterien gegenüber Selektivmedien erfolgte die Charakterisierung und Lokalisierung auftretender Zellschäden während der Sprühtrocknung. Sprühgetrockneter *LGG* wurde in Lebensmittel eingebracht und die Lagerstabilität des Keims untersucht. Während der Sprühtrocknung wurde eine Luftaustrittstemperatur von 65-70°C als optimale Prozesstemperatur ermittelt und die resultierenden Wassergehalte des Pulvers aus Trehalose und *E800* bzw. *LGG* betrugen 4.1% (w/w) bzw. 3.79 % (w/w) mit Lebendzellzahlen von 3.65×10^8 KBE/ml bzw. 1.8×10^9 KBE/ml im resuspendierten Produkt. Dabei wurden Zellmembranschäden und Schäden an der Zellwand festgestellt. Die aus der Gefriertrocknung bekannten stabilisierenden Eingenschaften der Trehalose ließen sich auf die Sprühtrocknung übertragen, aber nicht auf die Lagerung. Jedoch das Hinzugeben von Mononatriumglutamat verbesserte den Schützenden Effeckt von Trehalose. Schutz vor oxidativem Stress durch Mononatriumglutamat konnte bei der Lagerung festgestellt wer-

den und eine Überlebensrate von mindestens 10^8 KBE/ml wurde nach 6 Wochen bei einer Lagertemperatur von 25°C erreicht.
Die Nicht-Sichtbarkeit der getrockneten Bakterien in Trehalose pulver deutet deren Verkapselung an. Aber die verbesserten Überlebensraten bei Mononatriumglutamat waren Anzeichen von Schutz vor oxidativem Stress neben anderen Mechanismen, deren Aufklärung weiterer wissenschaftlicher Arbeit bedarf.
Die Keimzahl von *LGG* nach Einbringen in Apfelsaft und einer Lagerung von 12 Tagen bei 4°C betrug 10^5 KBE/ml, in Instant-Kakao-Mischgetränk wurde nach 10 Wochen Lagerung bei 25°C eine Keimzahl von 10^7 KBE/g detektiert, wobei in beiden Fällen eine Ausgangskeimzahl von 10^9 KBE/ml bzw. g eingestellt wurde.
Die Überlebensrate und Stabilität der *Lb rhamnosus* Stämme während der Verarbeitung und Lagerung wurden durch die Anwendung osmotischer Stressbedingungen verbessert.

Abstract

Probiotics bacteria are naturally exposed to environmental changes but they withstand such stress by evolving stress-sensing systems and defence mechanisms. Since the health promoting effects of probiotics can achieved by consuming at least 10^8 living cells, these environmental stresses should be used to guarantee microbial survival during processing as well as in food up to the end of the shelf life.

This work deals with the use of trehalose and sucrose in the induction of osmotic stress on *Lactobacillus rhamnosus GG* (*LGG*) and *Lactobacillus rhamnosus E-97800* (*E800)* ; and these osmotic agents were further employed as carrier media in spray-drying or vacuum drying processes.

Responses of the *L. rhamnosus* strains to osmotic stress were investigated by means of flow cytometric (FCM) analysis, plate count enumeration method and scanning electron microscopy (SEM). It was observed that there was no loss of membrane integrity except when *LGG* was treated in 1.5M sucrose. Permeabilization of cells' membranes was pronounced when E800 was subjected to trehalose treatments. Membranes were repaired upon re-culturing on growth media. Trehalose performed better than sucrose in improving cells' extrusion ability, a defence mechanism against toxic metabolites, but *E800* was not as active as *LGG* in respect to duration for completion of efflux. Morphological changes in form of capsules formation were observed in *LGG*. Responses to osmotic stress were strain specific.

Sub-lethal osmotic pre-treatment was applied on cells to evaluate their impact on the cells' stability when exposed to other stressful conditions such as lethal bile, heat and/or osmotic; which are hurdles they may encounter during drying processes and in the intestinal tracts. Trehalose improved the tolerance of *LGG* to bile while it reduced the thermo-tolerance of *E800* drastically.

The survival of *L. rhamnosus* strains in selected disaccharides after drying under vacuum at 25°C and during storage was evaluated. FCM analysis and bacterial tolerances to selective agents were conducted in order to characterize the sites of cellular damages during drying process. Among the solutes examined, trehalose improved the recovery of viable cells after drying as well as on storage at 4°C and 25°C. Damage of cell membrane and reduction in colony sizes occurred as a result of dehydration inactivation. Comparison of the conventional techniques with FCM viability assessment after drying revealed the occurrence of certain cell population which were stressed and lost their ability to grow on agar.

The use of trehalose and the effects of incorporated monosodium glutamate (MSG) on the survival rates during spray-drying and storage were examined. FCM analysis and bacterial tolerances to selective agents were conducted in order to characterize the sites of cellular damages during drying process. Spray-dried *LGG* was formulated into food products and its stability at storage time intervals was also monitored. An air outlet temperature of 65–70°C was taken as optimal for the drying process, as the resultant moisture levels in trehalose containing these bacteria, *E800* and *LGG,* were 4.1% (w/w) and 3.79% (w/w) with corresponding viable counts of 3.65×10^8 cfu mL^{-1} and 1.80×10^9 cfu mL^{-1}, respectively. Damages to cell membranes and cell wall components were recorded. Although the well known protective effect of trehalose during freeze drying was observed during spray drying but it could not be transferred during storage periods. However, addition of MSG improved the protective effect of trehalose. During storage at 25°C survival rates at constant minimum level of ~10^8 cfu mL^{-1} after 6 weeks was achieved due to pronounced protection against oxidative stress.

The non-visibility of bacteria in trehalose powders indicated encapsulation but the improved survival rates in the presence of MSG further proved oxidative stress prevention, amongst other mechanisms which may still be investigated.

LGG, at initial count of ~10^9 cfu mL^{-1} or g^{-1} was recovered at minimum level of ~10^5 cfu mL^{-1} in apple juice after 12 days and ~10^7 CFU g^{-1} in chocolate beverages after 10 weeks.

In summary, the application of osmotic stress improved the survival rates and stability *of L. rhamnosus* strains during processing and storage.

Dedication

To my dearest mother Mrs F.A. Adeleke;
to every one who stood by me throughout the ‘dramatic’ period of my life, and those that are delivered from shame.

Acknowledgements

My profound gratitude goes to my able doctor father, Prof. D. Knorr for accepting me in his laboratory and also for his guidance, support and open-mindedness towards the completion and success of this work. I also appreciate the roles played by Frau Hemmerich, I H., Herr Luscher, C., Herr Mathys, A., Herr Bunzeit, Herr Boguslawski., Frau Martens, G., Frau Riedel, H., Prof. Dr. Smetanska, I., and Frau Katinka Niederstrasser in making my stay in the department a memorable one. The meaningful contributions of Dr. Edwin Ananta are appreciated. Herr Henry Jäger thanks for your kindness and to Frau Antje Schulz –thank you.

I appreciate the love, understanding, endurance and good temperament demonstrated by my lovely daughters, Faith and Fidelia throughout the period of my study. You are wonderful.

My sincere appreciation is also due to German Academic Exchange Service (DAAD) for her financial magnanimity towards the research programme and the living expenses incurred on my children and I.

I will not fail to mention the spiritual and physical support of Elder and Deaconess S.A. Adeleke, Emmanuel Adeleke, Levi Jones, Dr. Simeon Jekayinfa, Bimbo and Deji Okegbile, Pastor Joshua Ojo, Lola and Peter Adetunji, Zieclinde and Klaus Ziegler, Lola and Tunde Adenmosun, Olulere Adewale, Princess and Kelly Sanusi, Janet and Lambert Fandegla and all my models and mentors in the Lord.

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The Divinity and Supremacy of JEHOVAH, the fourth man in the midst of the burning fiery furnace, were fully expressed from the beginning till the end of this programme. Despite all crises, He made all things beautiful for me in His own time. I give HIM praise.

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List of Media and Reagents

Man, Rogosa and Sharpe (MRS) agar	Oxoid, Basingstoke, UK
Man, Rogosa and Sharpe (MRS) broth	Oxoid, Basingstoke, UK
Sucrose	Merck kgaA, Darmstadt, Germany
Trehalose	Carl Roth, GmbH, Karlsruhe D
Lactose	Carl Roth, GmbH, Karlsruhe D
Glucose	Carl Roth, GmbH, Karlsruhe D
Sodium chloride (NaCl)	Merck kgaA, Darmstadt, Germany
Lysozyme	Sigma Aldrich, Steinheim Germany
Pepsin	Sigma Aldrich, Steinheim Germany
Monosodium glutamate (MSG)	Sigma Aldrich Steinheim Germany
Bile	Sigma Aldrich Steinheim Germany
Ringer's solution	Merck kgaA, Darmstadt Germany
Anaerobic kits	Anaerocult ® A, Merck Darmstadt, D
5 (6) - Carboxyfluorescein diacetate (cFDA)	Molecular probes, Inc. Leiden, NL
Fluorochrome 5 (6)-carboxyfluorescein (cF)	Molecular probes, Inc. Leiden, NL
Propidium iodide (PI)	Molecular probes, Inc. Leiden, NL
SYTO 9	Molecular probes, Inc. Leiden, NL
Lithium chloride (LiCl)	Merck kgaA, Darmstadt, Germany

1 Introduction and Objective of Work

Lactic acid bacteria (LAB) are the most important group of bacteria encountered in the food industry. They are used as starter cultures for fermentation of milk, vegetables, and meat. They are also used as probiotics and as silage inoculants. The reproduction of LAB and the activities of starter (cultures) containing LAB are important for the success of these fermentations. The production, storage and use of LAB impose environmental stresses on the bacterial cells, such as freezing and drying of starter cultures, low pH during fermentations, and low temperatures and high salt concentrations during cheese ripening (Sandine, 1996; Marteau et al., 1997; Bunthof et al., 1999).

The use of microorganisms as probiotic products is of increasing economic importance. The microorganisms are commonly defined as “live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbalance (Fuller, 1989). Their main use is the treatment of intestinal disorders; however, they may be subjected to various physical and chemical stresses before ingestion by the human host. Moreover bacteria that are used as probiotics have to survive the low pH of the stomach and the high bile salt concentrations in the intestine to be effective in the gastrointestinal tract (Holzapfel et al., 1998; Leverrier et al., 2003).

Bifidobacterium and *Lactobacillus* species have been the focus of probiotic interest since a large population of these bacteria in the intestinal tract is generally considered to be indicative of a healthy microbiota (Ballongue, 1998). And they are increasingly being included as functional ingredients, particularly in dairy products such as yoghurts and fermented milks, as evidence accumulates that they have beneficial effects on human health (Crittenden et al., 2001).

Many beneficial effects with the use of live microbial cells have been related to the release of bioactive molecules either directly produced by the probiotic bacteria or resulting from their enzymatic activities. Numerous modulating antihypertensive, opioid, antimutagenic, and immunomodulating peptides derived from caseins and plant proteins have been already isolated and characterized (Matar et al., 2000). Probiotics are known to aid digestion by producing enzymes such as galactosidase, bile hydrolase, protease, and lipase. They also prevent the adherence of pathogenic microorganisms either directly, through a barrier effect, or indirectly, through the stimulation of mucin production. Therefore, it is obvious that their ability to reproduce in the GI tract is an important factor for their overall efficacy (Suita-Cruce and Goulet, 2001).

Although probiotics can elicit beneficial effects but little is known about the molecular mechanisms of the reported benefits (Andersson et al., 2001). The mechanisms may vary from one probiotic to another (for the same benefit via different means) and the mechanisms may be a

combination of events, thus making this a very difficult and complex area. It could involve the production of a specific enzyme(s) or metabolite(s) that act directly on the microorganism(s) or the probiotic could also cause the body to produce the beneficial actions. According to FAO/WHO (2001), the possible mechanisms of action include antimicrobial substances production, modulation of the immune system, competitive exclusion of pathogen binding, competition for nutrients etc.

It is more obvious that the probiotic properties of microbial cells often require a complex substrate to be fully expressed. This has been shown extensively over the past few years with the appearance of the "prebiotic" concept (Suita-Cruce & Goulet, 2001). A prebiotic can be defined as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and or activity of one or a limited number of bacterial species in the colon, thus improves the host health" (Gibson and Roberfroid, 1995).

Prebiotic substances are not hydrolyzed or absorbed in the small intestine but are available as substrates for the indigenous bacteria in the large intestine. The most commonly used prebiotics are non-digestible fructooligosacscharide although research is being conducted on the use of galactooligosaccharides and soy-bean oligosaccharides (Berg, 1998). When in a single mixture, both probiotic bacteria and prebiotic compounds exist as a "symbiotic" product. This is defined as a mixture of probiotic and prebiotic that beneficially affects the host by improving the survival and the implementation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, including the ones in the symbiotic mixture (Roberfroid, 1998).

The importance of ingesting "live" bacteria to have a significant impact on the composition of the intestinal flora within animals is now well known. The use of germ-free animals, whose intestinal microflora was gradually implanted through controlled inoculation, has clearly demonstrated the value of live bacteria (Fuller, 1992). Recently, the use of antibiotics in animal health has been seriously questioned; probiotic supplements have been found to be highly efficient and desirable alternatives (Abe et al., 1995).

In their natural environments or during industrial processes, bacterial cells are often subjected to a variety of abiotic stresses. In order to survive, bacteria have developed a set of mechanisms leading to protection against severe injury after an unfavourable environmental factor has been sensed (Segal and Ron, 1998).

Initial investigations carried out on bacteria such as *Escherichia coli* have demonstrated that they possess an inherent ability to adapt to unfavourable environments by the induction of various general and specific stress responses. The survival of these bacteria under adverse conditions is frequently enhanced by these mechanisms (Prasad et al., 2003). 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; Prasad et al., 2003). Organisms subjected to a longer period of time to reduced water activity (a_w) respond by accumulating low-molecular-weight organic and/or inorganic compounds known as compatible solutes. Under osmotic stress, the significant physiological changes reported in bacteria include the induction of stress proteins as well as the accumulation of compatible solutes such as betaine, carnitine, and trehalose (Clark and Parker, 1984; Kets and de Bont, 1994; Welsh and Herbert, 1999). These solutes are accumulated by de novo biosynthesis in many organisms subjected to an elevated osmolarity and their intracellular content remains at a high level as long as the stressing conditions are maintained. After a sudden decrease in osmolarity, or cell decay, accumulated compatible compounds may be liberated into the surrounding environment and subsequently taken up, via an active transport process, by other organisms under osmotic stress. Such organic compounds taken up and accumulated by organisms unable to synthesize them de novo and able to improve growth under inhibitory osmolarities are called osmoprotectants (Gouffi et al., 1998). Since the term osmoprotectant is vague and not well defined, it is preferable to only use compatible solute for any compound that offers protection to high osmolarity, by accumulating to high cytoplasmic concentration, either by uptake from the medium or by de novo synthesis (Poolman and Glaasker, 2009).

These compatible solutes are not inhibitory to vital cell functions even at high cytoplasmic concentrations. These functions that were assessed for accumulation of compatible solutes in cells cultivated under osmotic stress conditions include;

a) maintaining the integrity of biological membranes during periods of desiccation (Crowe et al., 1987)

b) retaining a positive turgor pressure, which contributes towards osmotic balance with extracellular environments (Csonka, 1989);

c) enhancement of stability of enzymes (Lippert and Galinski, 1992).

The diverse group of compatible solutes include sugars and polyols like trehalose, glycerol, arabitol and manitol accumulated by a range of yeasts, fungi and bacteria (Van Laere, 1989; Van Eck et al., 1993). Amino acids and amino derivatives such as proline and betaine are accumulated up to high intracellular concentrations in *Escherichia coli* (Larsen et al., 1987; Dinnbier et al., 1988; Welsh et al., 1991), *Pseudomonas aeruginosa* (D'Souza-Ault et al., 1993), *Staphylococcus aureus* (Miller et al., 1991) and several other microorganisms (Csonka, 1989).

The role of compatible solutes in lactic acid bacteria has been under investigations. The role of betaine and proline in *Lactococcus lactis* (Molenaar et al., 1993); carnitine in *Lactobacillus plantarum* (Kets et al., 1994) and glycine betaine in *Lactobacillus plantarum* (Glaasker et al.,

1998) have been reported. It was noted in most of these reported studies that the induction of osmotic stress in the presence of salts e.g. sodium chloride was majorly investigated and the response of microorganisms towards these stresses were extensively studied. Glaasker et al. (1998) reported that KCl and NaCl inhibited the growth of lactic acid bacteria much more than equi-osmolar concentrations of sucrose. Growth stimulation of osmotically stressed cells by exogeneous glycine betaine was frequently observed. The effects of glycine betaine on the growth of salt-and sugar-stressed cells are not always the same. Unlike in the *Enterobacteriaceae*, a stimulatory effect of glycine betaine in the lactic acid bacteria was only observed when a salt (KCl or NaCl) stress was applied (Glaasker et al., 1996; Molenaar et al., 1993).

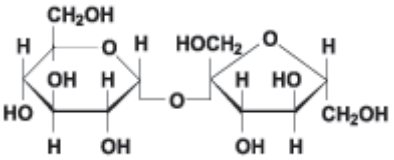
The disaccharide trehalose is widely distributed in nature and can be found in many organisms, including bacteria, fungi, plants, invertebrates and mammals. It consists of two subunits of glucose bound by a α: $1 \rightarrow 1$ linkage (α-D-glucopyranosil α-D-glucopyranoside) and is thus non-reducing. It is the most stable known sugar since it has an extremely low disaccharide bond (less than 4kJ/mol). Due to its particular physical features, trehalose is able to protect the integrity of the cell against a variety of environmental injuries and nutritional limitations. Bacteria can use exogeneous trehalose as the sole sources of carbon and energy as well as synthesize enormous amounts of the disaccharide as compatible solute. This ability to accumulate trehalose is the result of an elaborate genetic system, which is regulated by osmolarity (Argüelles, 2000).

Trehalose is involved in several physiological stress responses including osmotolerance (Larsen et al., 1987; Hounsa et al., 1998) and anhydrobiosis (Madin and Crowe, 1975; Gadd et al., 1987). Both of these phenomena are responses to loss of cellular water, either through hypertonicity of the environment or through desiccation respectively.

The chief role of trehalose in osmotolerance is as a compatible solute, acting to counterbalance extracellular osmotic pressure (Brown, 1976) and to stabilize biomolecules by preferential exclusion within the cell (Xie and Timasheff, 1997). In contrast, in anhydrobiosis trehalose has been proposed to act as a water replacement molecule (Clegg et al., 1982), as a means of avoiding damaging phase transitions in biomembranes (Crowe et al., 1984a) and as a glass former, including biological molecules in protective matrix. Since the early stages of dessication can involve exposure to hypertonic environments, trehalose may also play a role as compatible solute in anhydrobiosis (Garcia de Castro and Tunnacliffe, 2000).

Sucrose is a disaccharide with the molecular formula $C_{12}H_{22}O_{11}$. Its systematic name is α D-glucopyranosyl-$(1 \rightarrow 2)$-β-D-fructofuranose. It consists of two monosaccharides, α-glucose and fructose, joined by a glycosidic bond between carbon atoms 2 of the fructose unit. This fructofuranoside disaccharide bond energy is greater than 115KJ/mol and this renders it unstable in the presence of reactive chemical groups such as amino groups of protein. Unlike

trehalose, sucrose splits readily (under adequate conditions) to form glucose and fructose which are strong reducing sugars (Schebor et al., 1999). The uses of sucrose as protectants of dry biomaterials (Leslie et al., 1995, Schebor et al., 1999) and as compatible solutes (Glassker et al., 1998, Sunny-Roberts et al., 2007) have been reported.

(a) 

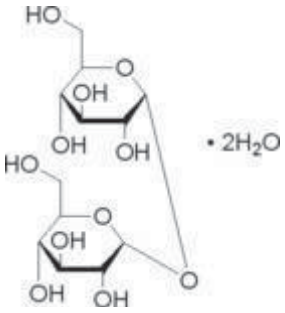
(b) 

Figure 1: The molecular structure of (a) sucrose and (b) trehalose.

The application of physical stress to micro-organisms is the most widely used method to induce cell inactivation and promote food stability. Determination of the impact of treatment on bacterial strains have been made mainly by the use of classical plate count methods, however, this method bears a major draw back in the sense that it only indicates how many cells replicate under the conditions provided for growth and its long term determination (Ritz et al., 2001; Ben Amor et al., 2002). Moreover, bacteria may occur in chains and clumps, resulting in underestimation of bacterial numbers. In addition cell injury and dormancy may result in low viable counts (Barer and Harwood, 1999; Kell et al., 1998).

However, flow cytometry is a rapid and sensitive technique that can determine cell numbers and measure various physiological characteristics of each individual cell using appropriate probes. The differentiation of the viable states of cultures are made possible by the use of specific fluorescent probes into four classes viz a viz reproductively viable, metabolically active, intact and permeabilized (Hewitt and Nebe – von – Caron, 2001). The applied probes include nucleic acid probes such as propidium iodide (PI), SYTO9, carboxyfluorescein diacetate (cFDA) and bis-(1,3-dibuty(barbituric acid) trimethine oxonol ($DiBAC_4^{(3)}$) (Auty et al., 2001; Ananta et al., 2004; Alakomi et al., 2005).

Flow Cytometry permits cell-by-cell multiparameter analysis that is often used in combination with fluorescent labelling (Shapiro, 1995). Cells are analyzed at rates of 100-1,000 per second as they are carried within a fast-flowing fluid stream that passes a focused light beam. The forward-angle light scatter (FSC), the side angle light scatter (SSC), and the fluorescence at selected wave-lengths are measured. The analyses are done on large populations of cells, typically 5,000 to 10,000. Subpopulations can be identified and distinguished when they differ in light scatter or fluorescence characteristics. Subpopulations can also be selected for further study (Bunthof et al., 2001).

For use in foods, probiotic micro-organisms should not only be capable of surviving passage through the digestive tract but also have the capability to proliferate in the gut. This means they must be resistant to gastric juices and be able to grow in the presence of bile under conditions in the intestines, or be consumed in a food vehicle that allows them to survive passage through the stomach and exposure to bile. The commonly used probiotics in foods are gram- positive bacteria included primarily in two genera, *Lactobacillus* and *Bifidobacterium* (Holzapfel et al., 1998; Klein et al., 1998).

Given that probiotic micro-organisms play a role in promoting and maintaining health (Salminen, et al., 1998) has stimulated considerable interest in incorporating these into functional foods and pharmaceutical products and it is recommended that probiotic products contain at least 10^7 live micro-organisms per g or per ml (Ishibashi and Shimamura, 1993). Therefore, from a commercial point of view, an inexpensive method for large-scale production of cultures containing high levels of viable probiotic cells in a form suitable for product application is highly desirable (Gardiner et al., 2000).

Lyophilization and vacuum – or spray – drying is some of the most useful techniques for preserving foods, agricultural products and pharmaceuticals. Biological materials, however, can be irreversibly damaged during these treatments. These processes can result in structural and physiological injury to the bacterial cells, resulting in substantial loss of viability. Cell membranes are involved in solute transport, cellular attachment and many other essential functions. It therefore follows that stabilization of the membrane and its integral proteins is key to cell recovery (Conrad et al., 2000) after dying processes.

In previous studies researchers investigated the production of freeze-dried powders and frozen concentrates of probiotic *Bifidobacterium* and *Lactobacillus* spp. (Gilliland and Lara, 1988; Misra and Kuila, 1991; Carvalho et al., 2003; Saarela et al., 2005). However, there are many disadvantages associated with this drying method viz a viz time – consuming and expensive, high transport and storage costs associated with frozen concentrated cultures, and the freeze – thaw process is associated with a loss of culture viability.

Spray drying, one of the predominant processing tools used in the dairy industry, can be used to produce large amounts of dairy ingredients relatively inexpensively; it has been estimated that the cost of spray drying is six times lower per kilogram of water removed than the cost of freeze-drying (Knorr, 1998). Spray-dried powders can be transported at low cost and can be stored in a stable form for prolonged periods but there are obvious challenges associated with using spray drying to produce viable cultures, including the requirement that the micro-organisms survive the relatively high temperatures used (Daemen and van der Stege, 1982). Nevertheless, researchers have found that there is no difference in microbial viability between these methods (Teixeira et al., 1995a,b).

Viability loss during dying was related to damage to the cell wall and cytoplasmic membrane, so that the dried cells became more sensitive to NaCl (Teixera et al., 1995b; Gardiner et al; 2000). Protective compounds, primarily saccharides protect membrane and proteins from dehydration damage, most likely by hydrogen bonding to polar residues in the dry macromolecules, as described by the water replacement hypothesis (Crowe et al., 1993 a,b) or by the ability of the sugars to form a high viscous glassy matrix during dehydration (Crowe et al., 1996).

The large commercial interest in bacterial cultures explains continued research on the drying of these cultures. The disadvantages of dried cultures undermine their application, but the advantages can outweigh the disadvantages if the inactivation during drying and storage can be more clearly understood and consequently reduced (Teixeira et al., 1995b).

Therefore the objectives of this work were as follows:

- the assessment of the effect of osmotic or high pressure pre-adaptation on the tolerance of bacterial strains to selected physical and chemical conditions e.g. heat , osmotic and bile,
- application of flow cytometry analysis to evaluate the impact of osmotic stress induced by trehalose and sucrose on the physiological and morphological conditions of *Lactobacillus rhamnosus* strains,
- investigation of vacuum – and spray drying methods in the production of sugars media containing probiotic cultures, and
- characterization of dried probiotic cultures obtained by vacuum- and spray drying methods.

The probiotic cultures used in this study were *Lactobacillus rhamnosus GG* and *Lactobacillus rhamnosus E-97800 (E800)*.

2 Literature Review

2.1 Probiotic products

2.1.1 Definition of probiotics

The works of Metchnikoff and Tissier were the first to make scientific suggestions about the probiotic use of bacteria, even if the word “probiotic” was not coined until 1960, to name substances produced by micro-organisms which promoted the growth of other micro-organisms (Lilly and Stillwell, 1965). Fuller (1989), in order to point out the microbial nature of probiotics, redefined the word as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance”. A quite similar definition was proposed by Havenaar and Huis in’t Veld (1992) “a viable mono or mixed culture of bacteria which when applied to animal or man, beneficially affects the host by improving the properties of the indigeneous flora”. Another definition is “live micro-organisms, which when consumed in adequate amounts, confer a health effect on the host” (Guarner and Schaafsma, 1998).
The World Health Organization has defined probiotic bacteria as “live microorganisms which when ministered in adequate amounts confer a health benefit on the host (FAO/WHO, 2001). The most recent definition, but probably not the last is “a probiotic should benefit the host either nutritionally or by changing its immediate environment (Kesarcodi-Watson et al., 2008).

2.1.2 Important factors for the development of probiotic cultures

Traditionally, probiotics have been added to yoghurt and other fermented foods, but they have also recently been incorporated into drinks, as well as marketed as supplements in the form of tablets, capsules, and freeze-dried preparations. There are more than 70 bifidus- and acidophilus – containing products produced worldwide, including sour cream, butter milk, yogurt, produced milk, and frozen deserts (Shah, 2001).
Some trials conducted in incorporating probiotic lactic acid bacteria in food products are as shown in Table 2.

Table 2: An overview of some studies that dealt with the incorporation of probiotic lactic acid bacteria in food products.

Organism	Food products	References
Bifidobacterium	Skim milk with prebiotics	Shin et al. (2000)
Bifidobacterium lactis Bb12	Oat-based cereal bar	Ouwehand et al. (2004)
L. rhamnosus, L. salivarius, L. paracasei	Spray-dried skim milk powder with or without prebiotics	Gardiner et al. (2000) Corcoran et al. (2004)
B. bifidum, B. infantis	Mayonnaise	Khalil and Mansour (1998)
L. acidophilus, L. paracasei, B. animalis, L. rhamnosus	Cheese-based dip	Tharmaraj and Shah (2004)
B. bifidum, L. acidophilus	Ice cream	Hekmat and McMahon (1992); Christiansen et al. (1996)
L. casei	Dried fruits	Betoret et al. (2003)
L. delbrueckii ssp. bulgaricus	Spray-dried skim milk with antioxidants	Teixeira et al. (1995b)
L. sakei	Spray-dried and freeze dried skim milk	Ferreira et al. (2005)
L. rhamnosus R101	Semisweet biscuits, Frozen cranberry, vegetable juice	Reid et al. (2007)
L. paracasei, L. salivarius	Cheddar cheese	Gardiner et al. (1998)

In developing a probiotic product, the viability of the organisms is of paramount importance.

2.1.2.1 Viability of Probiotic Organisms

To realize health benefits, probiotic bacteria must be viable and available at a minimal concentration, typically 10^6-10^7 cfu/g of product (Ishibashi and Shimamura, 1993). Several factors have been claimed to be responsible for the loss of viability of probiotic organisms. These include the acidity of the products, level of oxygen in the products, oxygen permeation through the package, sensitivity to antimicrobial substances and lack of nutrients in the products (Dave and Shah, 1997).

As reviewed by Shah (2001), some of the important criteria to consider for proper strain selection include:

Acid and Bile Tolerance

One of the most important criteria for selection of probiotic organisms is their ability to survive in the acidic environment of the product and in the stomach, where the pH can reach as low as 1.5. Similarly, the organisms must be able to survive in the bile concentration usually encountered in the intestine. Reports from Lankaputhra and Shah (1995) showed that it cannot be generalized that all probiotic strains are acid and bile tolerant. Acid and bile tolerance is strain dependent and care should be taken to select strains on the basis of these attributes.

Antagonism among Bacteria

Probiotic organisms produce lactic acid, citric acid and hippuric acid. Hydrogen peroxide, diacetyl and bacteriocin can be produced as antimicrobial substances. These inhibitory substances create hostile environments for food-borne pathogens and spoilage organisms. For example, yoghurt bacteria are reported to produce bacteriocin against probiotic bacteria and vice versa (Dave and Shah, 1997). Since a probiotic product such as yoghurt may contain one or more groups of the organisms and the production of bacteriocins may affect the viability of one bacteria or the other, therefore, before any combination of bacteria is used in a product, their antagonism should be checked.

Adherence Properties

Adherence is one of the most important selection criteria for probiotic bacteria. The desired effects of probiotic micro-organisms are produced only if they are able to adhere, colonize, and multiply in the intestine. The ability of probiotic bacteria to adhere to the intestine will improve their chances of winning the competition against “unfriendly bacteria” to occupy the intestinal “niches”. Adherence to the intestinal cell wall is an important prerequisite for colonization in the gastrointestinal tract. A few *Lactobacillus* species, such as*, Lactobacillus gasseri ADH*, *L. acidophilus BG2F04*, *L. rhamnosus GG* and *L. rhamnosus E800* have been studied for their adherence properties. Among bifidobacteria, *B.breve*, *B. longum*, *B. bifidum* and *B. infantis* have been studied (Bernet et al., 1993). However, not all probiotic bacteria adhere to the intestinal cells adequately.

2.1.3 Beneficial Effects of Probiotics

The beneficial effects of food with added live microbes (probiotics) on human health, and in particular of milk products on children and other high-risk populations, are being increasingly promoted by health professionals. It has been reported that these probiotics can play an important role in immunological, digestive and respiratory functions and could have a significant effect in alleviating infectious disease in children.

There is sufficient evidence to support the view that oral administration of lactobacilli and bifidobacteria is able to restore the normal balance of microbial populations in the intestine. In addition to their established role in gastrointestinal therapy, the probiotic organisms are claimed to offer several nutritional and therapeutic benefits. Probiotics have been successfully employed to treat antibiotic-associated diarrhoea. They also have other various functional properties such as:

2.1.3.1 Antimicrobial Properties

With the emergence of antibiotic-resistant bacteria and natural ways of suppressing pathogens, the concept of probiotics has attracted much attention. Probiotic bacteria produce organic acids such as lactic and acetic acids, hydrogen peroxide and bacteriocins. Lactic acids and acetic acids account for more than 90% of the acids produced. Other acids produced in small quantities include citric, hippuric, orotic and uric (Lankaputhra and Shah, 1998a). Lowering of pH due to lactic acid or acetic acid produced by these bacteria in the gut has a bactericidal or bacteriostatic effect.

The antimicrobial substances suppress the multiplication of pathogenic and putrefying bacteria. Because of these virtues, probiotic bacteria show strong antimicrobial properties against Gram-positive bacteria such as *S. aureus* and *Cl. perfringes* rather than against Gram-negative bacteria such as *Salmonella typhimurium* and *E. coli*. Hydrogen peroxide in the presence of organic acids such as lactic acid is inhibitorier to bacteria than hydrogen peroxide or lactic acid alone (Lankaputhra and Shah, 1998b).

2.1.3.2 Antimutagenic Properties

The antimutagenic activity of acetic, lactic, pyruvic and butyric acids against eight mutagens and promutagens have been reported using the Ames Salmonella test (Lankaputhra and Shah 1998b). While acetic acid showed higher antimutagenic activity than lactic or pyruvic acids, butyric acid showed a broad spectrum antimutagenic activity against all mutagens or promutagens studies. Live bacteria cells showed higher antimutagenicity than killed cells suggesting that live bacterial cells may metabolize or bind the mutagens. Inhibition of mutagens and promutagens by probiotic bacteria appeared to be permanent for live cells and temporary for killed cells. This emphasized the importance of consuming live probiotic bacteria and of maintaining their viability in the intestine to provide efficient inhibition of mutagens.

2.1.3.3 Anticarcinogenic Properties

Lactic acid bacteria and fermented products made from them have potential anticarcinogenic activity (Mitsuoka, 1989). Probiotic bacteria may remove the sources of procarcinogens or the enzymes that lead to the formation of carcinogens. Direct removal of procarcinogens by probiotic bacteria might involve a reduction in the rate at which nitrosamines are produced. It has been shown that probiotic bacteria can greatly reduce the mutagenicity of nitrosamines. The antitumor effects of probiotics such as B. longum, B. infantis and L. acidophilus have been reported (Goldin & Gorbach, 1984, Shah, 2001).

2.1.3.4 Improvement in Lactose Metabolism

Lactose malabsorption is a condition in which lactose, the principal carbohydrate of milk, is not completely digested into its component monosaccharide, glucose and galactose. Since lactose is cleaved into its constituent monosaccharide by β-D-galactosidase, lactose malabsorption results from a deficiency of this enzyme. Lactose mal-absorbers often complain of "gastric distress" after consuming fresh, unfermented milk or milk products (Onwulata et al., 1989).

Several studies have reported that yoghurt or probiotic yoghurt is tolerated well by lactose malabsorbers; however, the mechanisms are not very clear. Some lactose is hydrolyzed by yoghurt bacteria during fermentation; bacterial enzyme autodigests lactose intracellularly before reaching the intestine, and a slower oral-caecal transit time could all account for better tolerance.

2.1.3.5 Reduction in Serum Cholesterol

Studies have shown that consuming certain cultured dairy products can help reduce serum cholesterol level. Feeding of fermented milks containing very large numbers of probiotics bacteria (~10^9/g) to hypercholesterolemic human subjects has lowered cholesterol levels from 3.0g/L to 1.5g/L (Homma, 1988). Mann and Spoerry (1974) observed a decrease in serum cholesterol levels in men fed large quantities of milk fermented with *Lactobacillus*. This may have been due to the production of hydroxymethyl glutarate by lactic acid bacteria, which inhibit hydroxymethyl –glutaryl-CoA reductases required for the synthesis of cholesterol. The effects of lactic acid bacteria on cholesterol levels have been reported by several authors (Gilliland et al., 1985; Klaver and Meer, 1993).

2.2 Responses of Microorganisms to Environmental Stresses

2.2.1 General Introduction

Lactic acid bacteria (LAB) constitute a heterogeneous group of bacteria which are found in diverse environments from the human and the animal body to plants. These bacteria have been used for long to produce various fermented foods from products derived from animals (milk, meat, fish etc.) or plants (vegetables, wine olives etc.) .The industrialization of food bio-transformations increased the economical importance of LAB. Although LAB are low cost ingredients of the food transformation processes, they play a crucial role in the development of the organoleptic and hygienic quality of fermented products. However, bacteria are subjected to potentially stressful environmental changes in industrial processes and also in nature where the ability to quickly respond to stress is essential for survival. It is now well established that LAB, like other bacteria, evolved stress-sensing systems and defences against stress which allow them to withstand harsh conditions and sudden environmental changes (van de Guchte, et al., 2002).

Stress-sensing system and defence mechanism of microorganisms were utilized to prepare themselves in withstanding either harsh conditions or sudden environmental changes. In response to these external disturbances specific metabolic processes of the treated cells (transcription rates, translocation products, metabolic pathway, etc.) are altered, resulting in increased production of certain stress metabolites, which are involved in counteracting such abnormalities in their environment; thus help the bacteria survive the deleterious conditions. These survival mechanisms exhibited by bacteria when confronted to stress are generally referred to as the stress response. The exploration of bacterial stress response to adverse environmental conditions is motivated by basic scientific reasons but also by industrial and safety aspect in food microbiology (Abee and Wouters, 1999; van de Guchte et al., 2002; Beales, 2004; Ananta, 2005).

Regarding the type of external stimuli applied to induce stress response, one survival mechanism is the adaptive response, that is, when cells are exposed to a moderate level of stress, they acquire increased resistance to a subsequent exposure to a more severe level of the same stress at lethal dose (homologous agents). When cells are exposed to one stress they develop resistance, not only to that stress, but to other unrelated stresses (heterologous agents). This is known as cross-protection (Kim et al., 2001). The cell membrane plays an important role in stress resistance. The membrane can change in adaptation to environmental conditions and these changes contribute to the protection of bacteria e.g. acid stress, thermal shocks and oxidative stress. Adaptive responses appear to be a usual mode of

stress protection in lactic acid bacteria. The cross protections often induced by the expression of a given adaptive response can be advantageous when cells are exposed to a combi nation of stresses. However, the cross protections associated to the adaptive response to a given stress appear to vary between species suggesting that the molecular bases of adaptive responses are, at least in part, species or subspecies specific. The induction of adaptive responses can increase bacterial tolerance to stress and can be used to prepare strains to harsh conditions (van de Guchte et al., 2002). For example the heat adaptation of *O. oeni* leads to higher pH and ethanol tolerance and improves the efficiency of its direct inoculation in wine (Guzzo et al., 1994). Desmond et al. (2001) applied sublethal treatments of heat and salt on *L. paracasei* NFBC 338; Kim et al. (2001) pre-treated *L. acidophilus* with bile and NaCl; Park et al. (1995) also pretreated *B. breve* cells with acid at pH 5.2. These few studies reported the possible protective effects of these sublethal treatments on microbial viability during lethal treatments.

2.2.1.1 Bacterial cell structure

Micro-organisms consist basically of procaryotic and microscopic eukaryotic cells. Procaryotes are cells that lack a membrane-bound nucleus and they have few internal structures that are distinguishable under a microscope. They include bacteria, mycoplasmas, rickettsiae, chlamydiae, and algae (Olutiola et al., 1991).

Procaryotic structural components consist of macromolecules such as DNA, RNA, proteins, polysaccharides, phospholipids, or some combination thereof. The macromolecules are made up of primary subunits such as nucleotides, amino acids and sugars. A prokaryotic cell has five essential structural components: a nucleotide (DNA), ribosomes, cell membrane, cell wall and some sorts of surface layer which may or may not be an inherent part of the wall. A typical prokaryotic cell may be represented by a bacterial cell shown in Figure 2.1 and only three components, cell envelope, cell wall and plasma membrane, are briefly discussed.

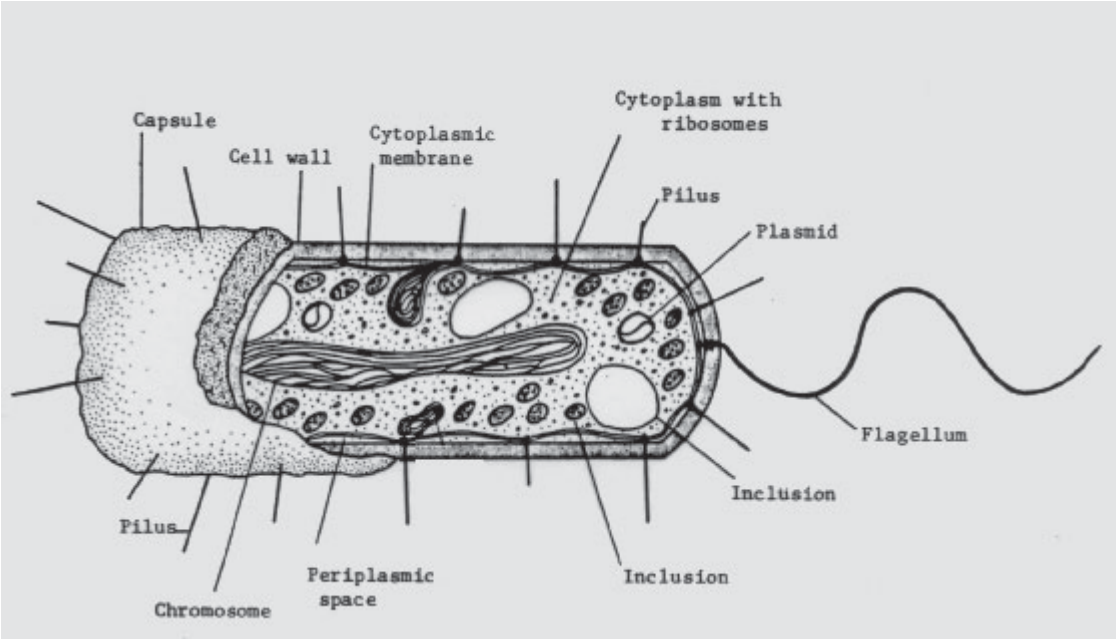


Figure 2.1: A typical bacterial cell showing structural components (Todar, 2009).

The Cell Envelope

The procaryotic cells are bound by layers referred to as cell envelope. These layers include the plasma membrane, the over-lying cell wall, polysaccharides or specialized proteins and other outer structures. All cells have a membrane, which is the essential and definitive characterisitic of a 'cell'. Almost all prokaryotes have a cell wall to prevent damage to the underlying protoplasm. Outside the cell wall, foremost as a surface structure may be a polysaccharide capsule or glycocalyx. The cell envelope serves as or contains transport sites for nutrients and receptor sites for bacteriophages. It influences the host parasite interactions. The cell envelope in gram-negative bacteria is highly complex than in gram-positive bacteria, with multilayered structures. The cytoplasmic membrane, also known as the inner membrane, is surrounded by a single layer of peptidoglycan to which the complex outer membrane is attached. There occurs a space between the inner and outer membrane called the periplasmic space. On the other hand, the cell envelope of the gram-positive bacteria is relatively simple. It consists of three layers of cytoplasmic memrane, a thick peptidoglycan layer and like in some gram-negative bacteria, an outer capsular layer which is not found in all gram-positive bacteria (Olutiola et al., 1991).

Cell Wall

The cell wall is an essential structure that protects the cell protoplast from mechanical damage and from osmotic rupture or lysis. Procaryotes usually live in relatively dilute environments such that the accumulation of solutes inside the procaryotic cell cytoplasm greatly exceeds the total solute concentration in the outside environment. Thus, the osmotic pressure

against the inside of the plasma membrane may be the equivalent of 10-25 atm. Since the membrane is a delicate, plastic structure, it must be restrained by an outside wall made of porous, rigid material that has a high tensile strength. Such a material is murein, a ubuquitos component of bacterial cell walls.

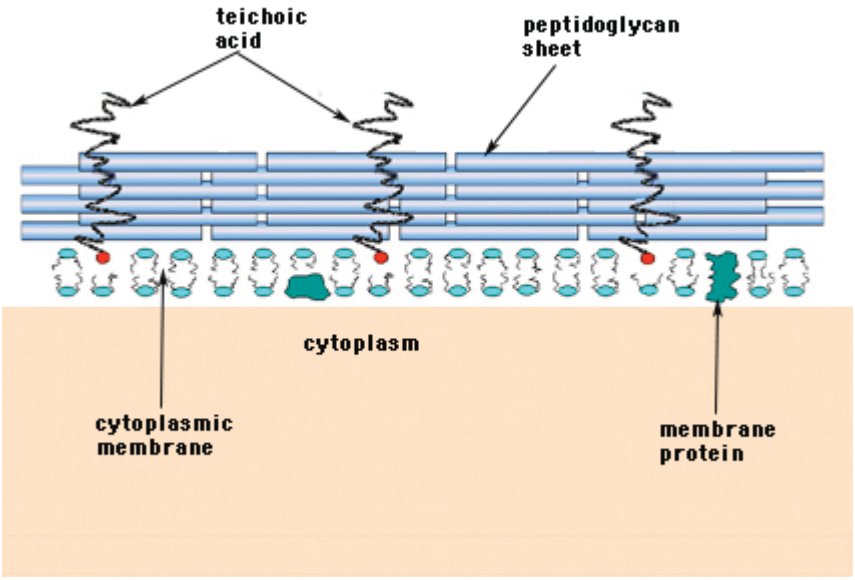


Figure 2.2: Structure of Gram-positive bacterial cell wall.
The wall is relatively thick and consists of many layers of peptidoglycan interspersed with teichoic acids that run perpendicular to the peptidoglycan sheets (Todar, 2009).

In the Gram-positive bacteria, the cell wall consists of several layers of peptidoglycan. The glycan backbone of the peptidoglycan molecule can be cleaved by an enzyme called lysozyme that is present in animal serum, tissues and secretions and in phagocytic lysozyme. The function of lysozyme is to lyse bacterial cells as a constitutive defense against bacterial pathogens. Some gram positive bacteria are very sensitive to lysozyme while gram negative bacteria are less vulnerable because their peptidoglycan is shielded by the outer membrane (Todar, 2009). Bacterial cell wall also plays an important role in cell division.

Plasma Membrane

The plasma membrane, also known as cytoplasmic membrane, is the most dynamic structure of a prokaryotic cell. Its main function is a selective permeability barrier that regulates the passage of substances into and out of the cell. The plasma membrane is the definitive structure of a cell since it sequesters the molecules of life in a unit, separating it from the environment. The membranes are composed of 40% phospholipids and 60% protein. The arrangement of proteins and lipids to form a membrane is called the fluid mosaic model as illustrated in Figure 2.3. The membranes of bacteria are structurally similar to the cell mem-

brane of eukaryotes, except that bacterial membranes consist of saturated or monounsaturated fatty acids and do not contain sterols.

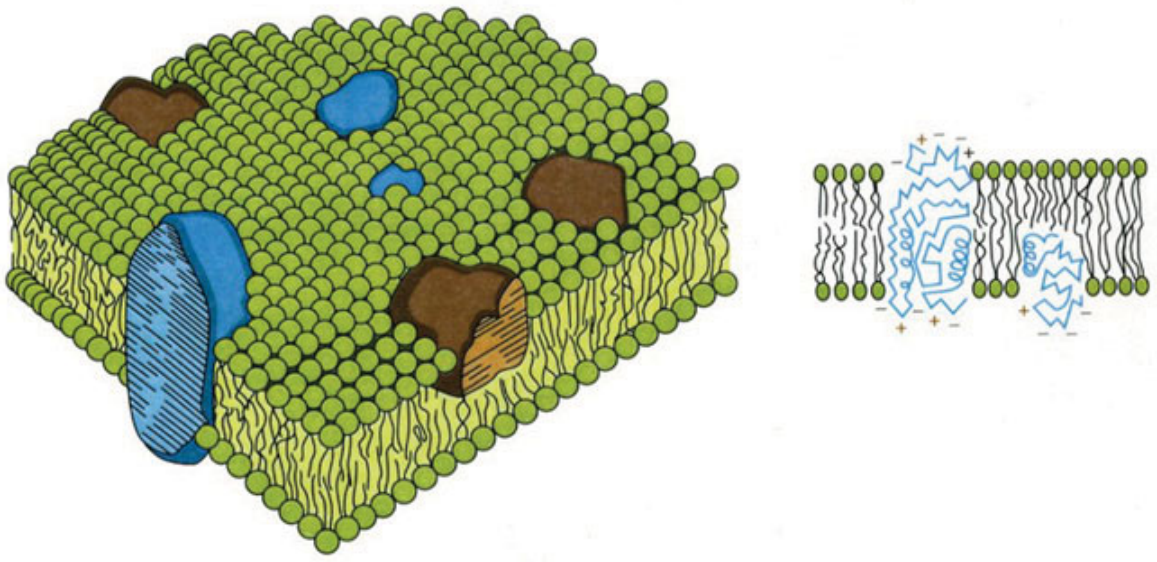


Figure 2.3: Fluid mosaic model of a biological membrane (Todar, 2009).

In aqeous environments, membrane phospholipids arrange themselves in such a way that they spontaneously form a fluid bilayer. Membrane proteins which may be structural or functional may be permanently or transiently associated with one side or the other of the membrane or even permanently built into the bilayer, while the other proteins span the bilayer and may form transport channels through the membrane. Functions of prokaryotic cell membrane include synthesis of membrane lipids and cell wall peptidoglycan (murein); assembly of secretion of extracytoplasmic proteins; coordination of DNA replication and segregation with septum formation and cell division etc. (Todar, 2009).

2.2.2 Environmental stressful conditions

2.2.2.1 Osmotic Stress

Among the many ways to preserve food products, increased osmotic pressure i.e. lowering of water activity (a_w), is one of the most widely used method. Desiccation or addition of high amounts of osmotically active components such as salts and sugars lowers the water activity of food (Abee and Wouters, 1999). In their various applications in the food and feed industry, lactic acid bacteria can be exposed to osmotic stress when important quantities of salt or sugar are added to the products (Poolman and Glaasker, 1998).

Biological membranes are readily permeable to water molecules, but they present an effective barrier to most other solutes. Under conditions of high external osmolarity, water will move out of the cell, causing changes in cell volume, intracellular solute concentration, and turgor pressure. For active metabolism to occur, the intracellular conditions must remain relatively constant with respect to ionic composition, pH and metabolite levels (Csonka and Hanson, 1991). In addition, the maintenance of constant positive turgor is generally considered as the driving force for cell expansion. As the bacterial cytoplasmic membrane is permeable to water but forms an effective barrier for most solutes, a change in the osmolality of the environment could, therefore, rapidly compromise essential functions, and bacteria need to adapt to such a change in their environment in order to survive (van de Guchte et al., 2002).

A universal response to the temporary loss of turgor following a hyperosmotic shock is the cytoplasmic accumulation of a certain class of solutes that do not interfere too seriously with the functioning of cytoplasmic enzymes These are “compatible solutes”, which are small organic molecules that have a number of common properties: they are soluble to high concentrations and can be accumulated to very high levels in the cytoplasm of osmotically-stressed cells. They are usually either neutral or zwitterionic molecules; specific transport systems are present in the cytoplasmic membrane allowing the controlled accumulation of these compounds, and they do not alter enzyme activity and may even protect enzymes from denaturation by salts or protect them against freezing and drying.(Csonka, 1989; Csonka and Epstein, 1996; Booth et al., 1994). Bacteria can, under hypoosmotic conditions, release these compatible solutes, which apart from protection against osmotic stress, also protect against high temperatures (Kets et al., 1996; Poolman and Glaasker, 1998; Panoff et al., 2000).

Unlike the enteric bacteria and *B. subtilis*, lactic acid bacteria have limited or no possibilities to synthesize compatible solutes and rely primarily on the uptake of such compounds from the culture medium. The compatible solutes accumulated or synthesized when bacteria are exposed to stressful osmotic conditions include amino acids (glutamate, glutamine and proline), amino acid derivatives (betaines, peptides and N - acetylated amino acids), polyols and sugars (trehalose and sucrose) (Csonka, 1989; Csonka and Hanson, 1991). A wide range of halophilic bacteria synthesize tetrahydropyrimidines (ectoines) which are accumulated to high intracellular levels in response to osmotic stress (Severin et al., 1992). Ectoines do not only provide direct protection against osmotic stress but also act as chemical mediators that affect the synthesis of other osmolytes (Jebbar et al., 1992; Talibart et al., 1994). The responses of microorganisms to growth-inhibiting salt and sugar concentrations have been reported and the effects of these stresses on the accumulation of compatible solutes have also been determined (Csonka, 1989; Glaasker et al., 1996; Molenaar et al., 1993).

Studies on enteric bacteria showed that upon an osmotic shock, K^+ uptake is activated and K^+ ions accumulate to high levels also with an uptake of anions (e.g. protons) (Csonka, 1989)

however, *L. plantarum* was unable to respond adequately to osmotic stress by accumulation of K^+ or Na^+ to levels beyond those already existing in the unstressed cells (Glaasker et al., 1998) but by the accumulation of an exogeneous organic osmolyte (e.g. glycine betaine) in reversing the decrease in tugor pressure upon salt stress. Upon osmotic downshift an efflux of glycine betaine was observed at steady state conditions.

The uptake and efflux of compatible solutes are mediated by separate systems, for example, in *L. plantarum* the uptake of glycine betaine, carnitine, and proline are mediated by a single semiconstitutive ATP-dependent uptake system, QacT while the efflux is probably by a mechanosensitive channel protein. However, in contrast, glycine betaine uptake in *L. lactis* is subject to osmoregulation at two levels i.e. gene expression and transport activity which have been identified (Obis et al., 1999, Bouvier et al., 2000).

Few studies have also compared the osmolytes accumulated in salt-stressed cells with those in sugar-stressed cells (D'Souza-Ault et al., 1993; Pocard et al., 1994). It was shown that salt-stressed cells do not contain detectable amounts of organic osmolytes while sugar-stressed cells contain sugar (and some sugar – derived) compounds. Hyperosmotic conditions imposed by sugar stress are much less detrimental and only transient, because the cells are able to equilibrate the extra-and intracellular concentrations of lactose (and sucrose) (Glaasker et al., 1998). It was observed that KCl and NaCl inhibited the growth of *L. plantarum* and *L. lactis* much more than equiosmolar concentrations of sucrose or lactose.

There could be an induction of some proteins, which are similar to that of heat shock, after salt stress, although the induction factors varied. Two-dimensional electrophoresis were used to identify proteins induced upon osmotic upshift in *L. lactis* and were identified as general stress proteins GroES, GroEL and Dnak (Kilstrup et al., 1997). Apart from effects on growth rate, growth medium osmolarity was reported to affect the membrane fatty acid composition (van de Guchte et al., 2002); exopolysaccharide (Liu et al., 1998) and bacteriocin (Uguen et al., 1999) production in *L. lactis*.

In brief trehalose and sucrose which can impose osmotic stress on micro-organisms, either in nature or in food applications, are discussed below:

2.2.2.1.1 Trehalose

The name trehalose was introduced in 1958 to describe a main constituent of shells that are secreted by various insects on tree leaves in the Middle East. These shells were often dried and used as sweetening agents, and are believed to be related to biblical Manna, the food supplied to the Israelites in their journey through the wilderness of Arabia. One variety of these shells, produced by the beetles *Larinus maculates* and *Larinus nidificans* was called

"trehala manna" and the sugar extracted from it was named trehalique glucose, or trehalose (Richards et al., 2002).

Trehalose is a disaccharide that is ubiquitous in the biosphere. It consists of two subunits of glucose bound by an α: 1 → 1 linkage (α-D-glucopyranosil α-D- glucopyranoside) and is thus nonreducing. It has been isolated and characterized from a large variety of both prokaryotic and eukaryotic organisms (Thevelein, 1984, 1996; Strom and Kaasen, 1993). It possesses several unique physical properties which include high hydrophilicity and chemical stability; non-hygroscopic glass formation. As reviewed by Argüelles (2000) trehalose can be used as external carbon source or stored as compatible solute in prokaryotic organisms; or serve as a structural component of the cord factor in mycobacteria.

Physiological roles of trehalose in bacteria

Trehalose as carbon source

Several gram-positive and gram-negative bacteria use exogeneous trehalose as sole source of carbon and energy. The metabolism of trehalose has been studied in different species of Bacillus especially *Bacillus subtilis* in which trehalose serves exclusively as carbon source (Whatmore and Reed, 1990). The genetic system for trehalose utilization is well characterized in *B. subtilis* and *E. coli*; trehalose role as a carbon source occurs in an osmolarity – dependent fashion (Horlacher and Boos, 1997). The accumulation of trehalose in *E.coli* in response to high osmotic strength clearly fits well with its role as compatible solute in other bacteria (Argüelles, 2000).

Trehalose as compatible solute

Trehalose is also accumulated as an osmotically active compound in cyanobacteria (Reed et al; 1984), in the moderately halophilic sulphate-reducing bacterium *Desulfovibrio halophilus* and in several purple sulphur and non-sulfur bacteria (Welsh et al., 1998). The net storage of trehalose is dependent on the medium composition and is balanced with respect to the content of other organic solutes. In *D. halophilus*, the level of intracellular trehalose increases in the absence of exogenous glycine betaine whereas in the halophilic phototropic bacterium *Ectothiorhodospira halochloris*, nitrogen depletion in the culture medium causes both a rapid betaine mobilization and an increase in trehalose content (Galinski and Herzog, 1990).

Other roles of trehalose

In addition to the above-stated roles, trehalose is located as a cell wall glycolipid called "cord factor" in bacteria such as *Mycobacterium, Nocardia, and Rhodococcus* etc. Cord factor elicits immunogenic responses in the host and acts as a toxic element responsible for some

pathogenic lesions caused by mycobacteria. It has been pointed out that the cord factor inhibits fusion between phagosomes and lysosomes, thus preventing efficient phagocytosis and resulting in persistence of the bacteria within the host cell (Spargo et al., 1991).
In nodules of the common bean (*Phaseolus vulgaris*), the level of stored trehalose seems to be correlated with resistance to drought stress (Farias – Rodriguez et al., 1998) and the protective role of trehalose has been postulated in dormant spores under dehydration conditions, where it acts as a substitute for water (Martin et al., 1986).
Trehalose can also function as a major reserve compounds and stress protectant in yeast cells (Argüelles, 2000).

2.2.2.1.2 Sucrose

Sucrose is generally isolated from natural sources; however, its chemical synthesis was first achieved in 1953 by Raymond Lernieux. Like other carbohydrates, sucrose has hydrogen to oxygen ratio 2:1. It consists of two monosaccharides α- glucose and fructose, joined by a glycosidic bond between carbon atom 1 of the glucose unit and carbon 2 of the fructose unit. What is notable about sucrose is that unlike most disaccharides, a glycosidic bond is formed between the reducing ends of both glucose and fructose and not between the reducing end of one and the non-reducing end of the other. The effect of this inhibits further bonding to other saccharide units. Water breaks down sucrose by hydrolysis; however the process is so gradual that it could sit in solution for years with negligible change. Sucrose is the most important sugar in plants and can be found in the phloem sap. It is extracted from sugar cane or sugar beet and then purified and crystallized. It is ubiquitos in food preparations due to both its sweetness and its functional properties; it is important to the structure of many foods including biscuits and cookies, cakes and pies, candy canes, ice cream and also assists in preservation of food (www.en.wikipedia.org/wiki/sucrose). Sucrose has been proved to act as a powerful osmoprotectant for *S. meliloti* in media of inhibitory osmolarity (Gouffi et al., 1998), by alleviating the osmotic inhibition of growth. Other few examples of sucrose accumulation in response to water and salt stresses have been reported for dessication-tolerant (Bianchi et al., 1993) and salt-stressed (Pilon-Smits et al., 1995) plants and for photosynthetic eubacteria (Welsh and Herbert, 1993) in which sucrose is accumulated by de novo biosyntheis. Sucrose can also be used as a carbon source by many microorganisms.

2.2.2.2 Heat Stress

The knowledge of physiological adaptation of *Lactobacillus* to different stresses is essential for the understanding of bacterial behaviour when facing industrial processes and during starter elaboration.

One of the industrial processes that cause a large loss of viability is bacterial conservation by freezing, freeze-drying or by spray drying. During these processes bacteria are subjected to adverse conditions, one of the most encountered and drastic being the heat stress (Gouesbet et al., 2001). The best described effects induced by high temperatures concern the protein denaturation (Visick and Clark, 1995), and their subsequent aggregation (Somero, 1995) but membranes, nucleic acids and certain enzymes have been equally identified as cellular sites of heat injury when *Lactobacillus bulgaricus* was heat treated at temperatures in the vicinity of 60^{o}C and above. The structural changes in cellular components may lead to cell death (Teixeira et al., 1997). Heat stress is also responsible for a disturbance of the transmembrane proton gradient, leading to decrease of the intracellular pH (Piper, 1993; Weitzel et. al., 1987 and Hansen et al., 2001).

Most of the studies about heat stress in gram positive bacteria describe the synthesis of a protein family (HSP), a phenomenon attributed to a universal response after a heat shock (Hecker et al., 1996). Mild heat treatments can lead to adaptation of the cell membrane by increasing the saturation and the length of the fatty acids in order to maintain optimal fluidity of the membrane and activity of intrinsic proteins (Russell and Fukanaga, 1990). Teixera et al. (1997) demonstrated that *Lactobacillus bulgaricus NCFB 1489* shows an inducible thermotolerance at 64^{o}C after being pre-treated at 52^{o}C. Gouesbet et al. (2001) also reported increased thermotolerance of *Lactobacillus delbruekii ssp. bulgaricus* at 65^{o}C after heat pretreatment at 50^{o}C.

Apart from exposure to sublethal heating temperatures, bacterial thermotolerance was shown to increase upon exposure to viral infections and chemical compounds such as ethanol, methylating agents, antibiotics (such as kanamycin, streptomycin) and amino acid restrictors (Neidhardt et al., 1984). Protection against heat may be achieved by the accumulation of osmolytes that may enhance protein stability and protect enzymes against heat activation (Taneja and Ahmed, 1994; Earnshaw et al., 1995). Another successful adaptation of certain microorganisms, like the members of the genera *Bacillus* and *Clostridium*, to resist exposure to heat is their ability to produce spores (Gould et al., 1995).

2.2.2.3 High Pressure

With increasing consumer demand for high-quality products with fresh characteristics, there has been an increasing interest in new mild methods of food preservation. One of the most promising is high hydrostatic pressure, and this is now being increasingly applied commercially for the processing of foodstuffs such as sauces, fruit juices, oysters and meat products. The main advantage of this technology is that it can better preserve the taste, colour, and original texture of the product (Cheftel, 1995; Patterson, 1999). The combinations of high pressure with other methods such as heat, low pH or antimicrobial peptides were investigated (Garcia-Graells et al., 1999; Pagan et al., 2001; Manas and Mackey, 2004). Moreover, the potential of high pressure technology as an alternative tool on modifying macromolecules as well as to assist and/or to substitute conventional freezing methods has been reviewed (Stute et al. 1996; Knorr et al., 1998).

Hydrostatic pressure has a great influence on the biological functions and viability of living organisms (Zimmerman, 1971). Life and death of microorganisms are strongly affected by pressure. This is the case for piezophilic and piezotolerant microorganisms (respectively requiring or tolerating high pressure during growth) living in the deep sea and the deep subsurface (Newman and Banfield, 2002) and also for non-piezophiles that are subjected to pascalization, an emerging process for preserving foods by treatment with ultrahigh pressure (100 to 1,000 MPa) (Cheftel, 1995).

Some effects of pressure on biomolecules and biological systems that have been well studied in vitro and explained on the basis of thermodynamic principles are protein denaturation and phase transition in membranes (Balny et al., 2002). Therefore, most pressure effects on microorganisms observed in vivo, such as inhibition of key enzymes (Simpson and Gilmour, 1997) and processes (Erijman and Clegg, 1998) and distruption of cellular structures (Kobori et al., 1995) and membranes (Rosin and Zimmerman, 1977; Gänzle and Vogel, 2001) are believed to stem from these primary events (Aertsen et al., 2004).

Elevated pressure can exert detrimental effects on microbial physiology and viability. Growth of microorganisms is generally inhibited at pressures in the range of 20 to 130MPa, while higher pressures of between 130 and 800 MPa, may result in cell death; the maximum pressure allowing for growth or survival depends on the species and medium composition. The exact nature of cellular damage responsible for pressure killing has been suggested to be membranes and ribosomes (Welch et al., 1993; Patterson et al., 1995). Differential Scanning Calorimetry allowed the detection of in vivo changes in ribosomes conformation (Niven et al., 1999).

Since ribosomes have been implicated as temperature sensors, cold-schock protein (CSPs and heat schock proteins HSPs may also play a role in stress response under inactivation conditions such as UHP. Welch and Bartlett (1998) reported an increase in the relative rates

of a set of heat shock proteins on cells grown at elevated pressure. Proteins synthesized at increased rate with higher pressure are defined as pressure induced proteins (PIP). A decrease in growth rate and protein synthesis occurred when *E.coli* cells were isothermally shifted from atmospheric pressure to 53MPa. Many of the identified PIPs were heat shock (GroEL, Dnak), cold shock or ribosomal proteins, while others corresponded to proteins of unknown function (Welch et al., 1993).

Cytoskeleton and enzyme complexes have also been shown to be very sensitive to high pressure. This may be due to dissociation reactions favoured under pressure or pressure mediated loss of ions. The vacuolar compartment of yeast cells contains a large quantity of hydrolytic enzymes, and plays an important role in the degradation of cellular proteins and storage of amino acids, carbohydrates, polyphosphates and ions. Acidification of these vacuoles is essential for the activity of vacuolar enzymes, protein transport, and cytosolic homeostatic (Sato et al., 1984; Klionsky et al., 1992). Application of hydrostatic pressure promoted the acidification of the vacuoles in strain IFO 2347 and also caused a delay or cessation of cell growth in the strain (Abe and Horikoshi, 1995).

Hydrostatic pressure was reported to affect the intracellular pH of microorganisms by enhancing the dissociation of weak organic acid, thus increasing the permeability of the cytoplasmic membrane and inactivation of enzymes required for pH homeostasis (Ulmer et al., 2000; De Angelis and Gobetti, 2004). The regulation of a fairly constant internal pH (pH_{in}) was considered crucial for maintaining microbial viability and a substantial pressure-induced loss of this transport functionality would reduce the ability of microorganisms to survive harsh environment during and after pressure treatments (Molina- Gutierrez et al. 2002). Damage of membrane bound H^+-ATPase, which is responsible for pH homeostasis in acidic environment by discharging H^+ from the cell can reduce its ability to tolerate acidic conditions. (Poolman, 1993; Ananta, 2005).

Other major constituents located in the cellular membrane but which are responsible for crucial cellular function include multidrug resistance transport system, HorA, Lmr P etc. These have been equally reported to be negatively affected by high pressure treatments (Gänzle et. al., 2001; Chang, 2003).

As earlier stated, the majority of bacterial stress responses to osmotic conditions were performed with ionic agents and responses to other lethal environmental conditions were also mostly investigated after ionic pre-treatments (Kim et al., 2001; Gouesbet et al., 2001; Desmond et al., 2001). It is therefore important to make use of non-ionic agents in the induction of osmotic stress on bacterial cells and also to evaluate the impact of non-ionic pre-treatment/ high pressure pre-treatment on the viability/stability of bacterial cells under stressful environmental conditions.

Development of rapid and reliable methods for measuring viability is of the utmost importance for studies on bacterial physiology. In concept, bacterial viability is the reproductive capacity, and survival is the maintenance of the viability (Barer and Harwood, 1999). The conventional method for quantitative survival studies is the plate count technique, in which replication on an appropriate agar medium is tested. Although this is the only direct proof of viability (Kell et al., 1998) the plate count method has major draw backs (Breeuwer and Abee, 2000). It could not give insights about population heterogeneities or the physiology of individual organisms (Shapiro, 2001). Besides, long term determination time which principally arises from the application of this method leads to significant limitations in anticipating abnormalities in growth or surviving behaviour of bacteria during industrial processes. Moreover, culturing technique may underestimate the numbers of truly viable bacteria. It is known that under stress conditions or limitations of nutrient availability or due to imposed sub-lethal injury, some cells can enter a non-culturable state, yet they can still exhibit metabolic activity (Barer and Harwood, 1999; Hewitt and Nebe-von Caron, 2001). The use of flow cytometry allows an extended description of bacterial viability beyond the one based on reproductive capacity as well as identification of heterogeneities within population with regard to structure and function (Ananta, 2005; Kell et al., 1991).

2.2.3 Flow Cytometry

General Introduction

Flow cytometry (FC) is a very useful technique for cell studies. It is a technique based on the measurement of fluorescence in probes having structural and/or functional specificities, using or not using specific stains. Thus, structural properties, such as cell size, granularity and pigment contents can be measured by using specific stains producing fluorescence, and some macromolecule content or intracellular ion concentration can be measured without stained samples. Functional specificites, such as redox potential, cell viability and DNA synthesis can be followed with specific stains, while membrane integrity, membrane potential and even cellular transport can be evaluated without the use of fluorescence stains (Maftah et al., 1993; Alcon et al., 2004).

Flow cytometry has usually been employed with animal cells (Crissman et al., 1985) but also flow cytometry can be used for intracellular compound measurements in microbial systems. If quantitative values can be determined, evolution of intracellular compounds can be measured. This can be of interest in bioprocess design and development, mainly for structured kinetic model development and also for cell damage description (Garcia-Ochoa et al., 1998; Pinder et al., 1990)

Many studies have described cell cycle of different microorganisms (Steen et al., 1982; Seo and Bailey, 1987); metabolic studies (Stockall and Edwards, 1985); cell characterization (Donnelly and Baigent., 1986; Allman et al., 1990 and Pinder et al., 1990) and evolution of the intracellular compounds during fermentation processes (Bailey et al., 1978; Agar and Bailey, 1982). Throughout the course of any microbial process, it is essential to monitor cell proliferation and viability. Accurate measurements relating to biomass concentrations are an important consideration if informed decisions on process control are to be made because process performance, at large, will depend on cell number and individual cell physiology states (Hewitt and Nebe-von Caron, 2001).

Most studies on probiotics only use plate counts to assess the viability of the microorganisms. Cell viability is more complex than whether cells are culturable or not. Cells that are active but not culturable might contribute to many of the proposed health effects. For actions such as lactose conversion, production of antibacterial compounds, assimilation of cholesterol and antioxidants effects, the microorganisms need to be active but not necessarily culturable. Cells in this viable but not culturable (VBNC) state undergo metabolic changes leading to the production of cells that no longer actively form colonies on solid media, but retain other indicators of cell viability such as active membrane potential, maintenance of cellular integrity and the capacity for metabolic activity. However, for some probiotics effects the microorganisms do not have to perform metabolic activities. It has been reported that non-viable forms of probiotic bacteria can adhere to intestinal mucus, and have immunomodulatory effects (Ouwehand et al., 2000).

Bogosian et al., (2000) reported that resuscitation of non-culturable cells of *Vibrio vulnificus* as a result of temperature upshift were attributable to a previously unrecognized population of hydrogen peroxide-sensitive culturable cells. The AODC and SYTOPI staining results of the non-culturable cells had been interpreted to indicate that such cells were still viable. The residual metabolic activities of such undetected bacteria by conventional culture techniques could lead to food spoilage or accumulation of toxins due to retention of gene encoding virulence and resuscitation of nonculturable cells, so that non-culturable pathogens may still pose a hazard to public health (Nebe- von Caron et al., 2000; Keer and Birch, 2003).

2.2.3.1 Principles of the flow cytometer

One of the fundamentals of flow cytometry is the ability to measure the properties of individual particles. When a sample in solution is injected into a flow cytometer, the particles are randomly distributed in three-dimensional space. The fluidics system consists of a central channel through which the sample is injected, enclosed by an outer sheath that contains faster flowing fluid. Each particle from single file of particle (created as a result of hydrody-

namic focusing) passes through one or more beam light. Light scattering or fluorescence emission (if the particle is labelled with fluorochrome) provides information about the particle's properties. Light that is scattered in the forward direction, typically up to 20^{o} offset from the laser beam's axis, is collected by a lens known as the forward scatter channel (FSC). The FSC intensity roughly equates to the particle's size and can also be used to distinguish between cellular debris and living cells. Light measured approximately at a 90^{o} angle to the excitation line is called side scatter. The side scatter channel (SSC) provides information about the granular content within a particle. Both FSC and SSC parameters are unique for every particle, and a combination of the two may be used to differentiate different cell types in a heterogeneous sample. The data acquired in each parameter are known as 'events' and refer to the number of cells displaying the physical feature of marker of interest (Rahman, 2005).

Fluorescent probes are useful in a wide range of applications including: identifying and quantifying distinct populations of cells; cell-surface receptors or intracellular organelles; cell sorting; immunophenotyping; calcium influx experiments; determining nucleic acid content; measuring enzyme activity, and for apoptosis studies (Rahman, 2005).

Various fluorescent probes are used for viability assessment (Porter et al., 1996; Haugland, 1999; Bunthof et al., 2001). These include

- redox probes such as tetrazolium salts that are reduced by the electron transfer chain
- membrane potential probes such as anionic oxonol dyes and the cationic dye rhodamine 123.
- esterase substrates such as fluorescein diacetate and calcein AM
- dye exclusion probe especially DNA binding compounds.

For visualization purposes, data are displayed either as a frequency distribution where the magnitude of the measured parameter (SS, FS, of fluorescence) is plotted against the number of cells. Alternatively, the data can be represented by two – or three – parameter density plots of light scattering versus fluorescence or – in case of dual staining procedure – the fluorescence from a DNA stain versus the fluorescence owing to microbial enzyme activity (Ananta, 2005).

2.2.3.2 Assessment of physiological attributes of microorganisms

Given below is an overview of the various microbial assets that can be measured with fluorescent probes and flow cytometry; these are also used to study the physiological state of microbes.

2.2.3.2.1 Membrane integrity

An intact membrane protects the cell constituents and is a prerequisite to generate gradients between the cytoplasm and the environment. Cells need electrochemical gradients to remain functional. Cells can recover from a transient permeabilization, but if the membrane is irreversibly compromised the cell is doomed to die. Without an intact membrane a cell cannot maintain electrochemical gradients so it will loose its membrane potential and pH gradients. As the intracellular compartment is no longer separated from the environment, components leak out of the cell and potentially toxic chemicals from the environment diffuse freely into the cell. This will lead to breakdown of cell components and finally the degradation of the whole cell (Bunthof, 2002). Based on the fact that intact cells have a cytoplasmic membrane with selective permeability, this membrane integrity can be determined by dye exclusion.
Two groups of dye exclusion probes are of importance-phenanthridium nucleic acid dyes and cyanine nucleic acid dyes. The phenanthridium nucleic acid dyes include ethidium bromide, propidium iodide (PI), and ethidium homodimer-1. These probes have been used most extensively to evaluate cell membrane integrity of bacterial as well as eukaryotic cells (Bunthof et al. 1999; Haugland, 1999). Cyanine nucleic acid dyes are compounds that also have the chemical characteristics necessary for a viability assay based on dye exclusion. These are the compounds of monomeric TO-PRO series, the dimeric TOTO series and the SYTOX series (Molecular Probes Inc., Eugene, Oreg). (Hirons et al.,1994; Haugland, 1999). Propidium iodide (PI) is an impermeant dye with a molecular weight of 668. Following loss of membrane integrity, PI diffuses into the cells and intercalates into the double stranded helical structure of nucleic acids (DNA of RNA), forming a red-flourescent complex. The fluorescence is enhanced 20 – to 30 – fold above the unbound PI (Bunthof, 2002).

2.2.3.2.2 Esterase activity

Esterases are present in all living organisms and assays that measure esterase activity are commonly used to assess metabolic activity (Haugland, 1999). cFDA (carboxyfluorescein-diacetate) is used primarily for the evaluation of cellular enzymatic activity. It is a lipopholic, non-fluorescent precursor that readily diffuses across the cell membranes. In the intracellular compartment it undergoes hydrolysis of diacetate groups by unspecific esterases into a polar, membrane-impermeant fluorescent compound cF (carboxyfluorecein). cF is a derivative of fluorescein which are more negatively charged at physiological pH, thus less likely to leak out of the cells (Breeuwer and Abee, 2000; Haugland, 2002; Ananta, 2005). Though the synthesis of the enzyme requires energy, the enzyme-substrate reaction itself is energy-independent and esterase functionality is found to be quite persistent (Bunthof, 2002). Con-

sequently the cells only remain fluorescent if their membranes are intact and cF are unable to diffuse out, indicating that for viable cells, this probe requires both active intracellular enzymes and intact membranes.

Some conducted researches revealed the major drawback in using esterase substrate in identification of bacterial viability status. It was reported that H_20_2-killed cells, as well as У – radiated cells maintained detectable esterase activity for over a week, and also 70^O C-heat killed cells had considerable remaining esterase functionality (Bunthof et al., 1999; Vives-Rego et al; 2000). There could also be a possibility of cells' inability to retain cF, irrespective of active esterase, due to membrane permeabilization. Sunny-Roberts et al. (2007) reported reversible membrane permeabilization in *LGG* as a result of osmotic stress induced by trehalose. Although the cells were not stained by PI as a result of maintenance of membrane integrity but cF leakage into the surrounding media was observed.

Several researches had been performed to enumerate viable bacteria such as *Listeria monocytogenes* (Nexmann Jacobsen et al., 1997); *Saccharomyces cerevisiae* (Attfield et al; 2000); *Lactobacillus plantarum* (Bunthof and Abee, 2002); *Bifidobacterium spp*. (Ben Amor et al, 2002); and *Lactobacillus rhamnosus spp*. (Sunny-Roberts et al, 2007; Sunny-Roberts and Knorr, 2007; Sunny-Roberts and Knorr, 2008) with cFDA. A number of other fluorescein derivatives have also been used and compared for various applications and species. These include fluorescein diacetate (FDA) (Chrzanowski et al, 1984; Diaper et al, 1992); Calcein AM (Comas and Vives-Rego, 1998; Comas-Riu and Vives-Rego, 1999); cDFDA (Brul et al; 1997) and Bis-carboxyethyl-carboxyfluorescein (BCECF-AM) (Diaper, et al., 1992). Reported limitations of fluorogenic esterase substrates are poor dye uptake, low labelling efficiency of some species and active extrusion (Molenaar et al., 1992; Bunthof et al; 1999). Also, the outer membrane of Gram-negative bacteria is generally impermeable to the lipophylic probes, and the permeabilization of the outer membrane is required (Haugland 1999; Molenaar et al., 1992; Bunthof et al., 2001).

2.2.3.2.3 Pump Activity

When cells are labelled by cF a subsequent lactose-energized efflux assay can be used as an additional measure of cell viability. The combined methods could be used to assess multiple aspects of cell viability: enzyme activity, membrane integrity, and metabolic activity upon energizing. More information is therefore given about the physiological condition than cF labelling alone does. cF labelling of *L. lactis* and the ATP-driven extrusion of cF gave the best indication of the reproduction and acidification capacities of stressed cells (Bunthof et al., 1999). Extrusion of cF from intact cells of *Lactobacillus rhamnosus GG* (Ananta and Knorr, 2004; Sunny-Roberts et al., 2007) and *Lactobacillus rhamnosus E800* (Sunny-Roberts and

Knorr, 2007) have also been reported. The extrusion of cF by lactic acid bacteria can also help in detecting heterogeneity among cells.

Efflux may be used to investigate drug efflux transport. Fluorescent probe efflux is commonly used to test the drug efflux capacity of mammalian carcinoma cells (Haugland, 1999). Active dye extrusion from energized cells as described for rhodamine 123 and/or fluorescein may be linked to existing multidrug resistant pumps (Ueckert et al., 1995). Proton gradient could be detected by observing the efflux of ethidium bromide (EB). This nucleic acid dye can cross the intact cytoplasmic membrane but is actively pumped out of the cells via a non-specific proton antiport transport system (Midgley, 1987). Active exclusion of EB was reported to correlate with metabolic activity (Nebe- von Caron et al., 1998; Ananta, 2005).

2.2.3.2.4 Membrane Potential

Cells build up potential energy in metabolic process by the unequal distribution of protons and of electric charges (outside more H^+ ions and positively charged ions than inside).The proton distribution determines the pH-gradient and the charge distribution determines the membrane potential. Together they form the proton motive force, which plays a critical role in bacterial physiology. Proton motive force is needed for various energy – requiring processes such as uptake of amino acids, sugars and rotation of flagella. In addition, respiring bacteria use proton motive force which they generate by the electron transfer chain, for synthesis of ATP. Fermentative bacteria, on the other hand, may need to maintain proton motive force at the expense of ATP by extrusion of H^+ ions by H^+ATPase (Kaprelyants and Kell, 1993; Kaprelyants et al., 1996; Lopez-Amoros et al., 1997; Turner et al., 2000; Bunthof, 2002).

The membrane potential of active bacteria is typically in the order of -150mV. The magnitude of membrane potential is reduced to zero in dead cells, particularly when the integrity of membrane is destroyed by physical or chemical agents or by certain classes of antimicrobial drugs (Novo et al., 1999). Alternatively, collapse in the membrane potential can be due to treatment with proton ionophores by eliminating the proton gradient across membrane. Any treatment that reduces the magnitude of membrane potential is said to depolarize the cell (Ananta, 2005).

The analysis of membrane potential can be conducted by the uneven distribution of ionic probe molecules, which are either positively charged or negatively charged dyes that can readily pass and accumulate in the cell membrane according to their charge. For example, positively charged dyes will concentrate in cells with a membrane potential while negatively charged dyes on the other hand, are excluded from cells that maintain a membrane potential. Cationic dyes applied to microbes are rhodamine 123 (Kaprelyants and Kell, 1992) and the carbocyanine dyes $DiOC_2(3)$ and $DiOC_6(3)$ (Novo et al. 1999, Ratinaud and Revidon, 1996).

$DiOC_6$ was used in detecting decrease of membrane potential in *Listeria* cells subjected to the mode of action of bacteriocin leading to the induction of pore formation and ionic leakage (Ratinaud and Revidon, 1996) while rhodamine 123 has been applied for mitochondrial membrane potential measurement in yeasts (Ludovico et al., 2001). $DiBAC_4(3)$, an oxonol dye, is a negatively charged molecule which enters depolarized and dead cells and binds to lipid-rich compounds, resulting in bright green fluorescence. Anionic dyes only enter the cell once the active transport system ceases and the membrane potential is lost (Hewitt and Nebe-von Caron, 2001) unlike the cationic membrane potential probe which requires the presence of active transport system, which pumps out the fluorescence stain (Ueckert et al., 1995; Nebe-von Caron and Badley, 1995).

In addition, membrane potential sensitive probes as $DiBAC_4(3)$ have been extensively used to assess bacterial susceptibility to antibiotics (Mason et al., 1994; Jepras et al., 1997) and cell viability (Lopez-Amoros et al., 1995; Mason et al., 1995; Lopez-Amoros et al., 1997) by FCM. Examining the membrane potential provided an additional means of characterizing the physiological status of bile – salt stressed *Bacillus lactis* and *Bacillus adolescent* cells. $DiBAC_4$ (3) fluorescence for bacterial size variation allowed for better discrimination between viable cells and cells that were depolarized (dead) (Ben Amor et al., 2002).

The potential of using multiple probes in supplying information on the physiological conditions of single cell in a bacterium population have been revealed in the above-mentioned examples. These probes have also been found useful in monitoring the effects of imposed environmental changes on microbial cells. Therefore the characterization of the physiological and metabolic behaviour of *Lactobacillus rhamnosus* strains, following exposures to osmotic conditions induced by non-ionic agents such as trehalose and sucrose, using some of these dyes and flow cytometric analysis necessitate investigation.

2.3 Drying of Biological Products

2.3.1 Introduction

Drying, consisting of lowering water activity, is an efficient method of preservation of biological products, since it does not require severe heat treatment and allows storage at ambient temperature. The stabilization of enzymes and other biomaterials (e.g. microorganisms) by their incorporation into aqueous carbohydrate or polymer solution followed by drying to low moisture to immobilize such labile material in a solid matrix is a known preservation. Compared to frozen concentrated bacterial preparation, the use of dried bacterial population does not require cryogenic shipment and storage. Owing to the removal of water the weight of the

product can be markedly reduced. However, it was reported that the time lag before acidification begins is longer for the dried than for the frozen cultures (Champagne et al., 1991).

Lactobacillus and *Bifidobacterium* spp. have been developed, and while less attention has focused on spray drying as a means of probiotic culture preparation, recent studies have demonstrated the potential of this approach for some strains including *Lactobacillus acidophilus* cultures (Espina and Packard, 1979), *Lactobacillus paracasei* (Desmond et al., 2001, 2002), *Lactobacillus rhamnosus GG and Lactobacillus rhamnosus E800* (Corcoran et al. 2004; Ananta et al.,2005), *Lactobacillus paracasei* and *L. salivarius* (Gardiner et al., 2000) and *Lactobacillus sakei* (Ferreira et al., 2005).

For successful delivery in foods, probiotics must survive food processing and storage during product maturation and shelf-life. It is recommended that the probiotics culture must be present in the product at minimum numbers of 10^7 CFU ml^{-1} and even higher numbers have been recommended. In addition, the probiotic food product should be regularly consumed in sufficient quantities to deliver the relevant “dose” of live bacteria to the gut, keeping in mind the losses in cell viability typically encountered during gastric transit. Consequently, the technological issues relating to the development of foods containing these bacteria in sufficient numbers throughout shelf-life need to be overcome, as well as means of stabilization following ingestion, i.e. during exposure to the adverse conditions of the human gastrointestinal tract (GIT) (Ishibashi and Shimamura 1993; Lee and Salminen, 1995 ;Ross et al., 2005).

Most probiotic lactobacilli do not survive well due to the temperature and osmotic extremes to which they are exposed during the spray-drying process. When used for the preservation of potential probiotic cultures much of their activity is typically lost after a few weeks of storage at room temperature. This is associated with stress that is induced by temperature changes and drying, a combination of which tend to damage cell membranes and proteins (Teixeira et al., 1995a,b; Gardiner et al. 2000, Ross et al., 2005).

A number of researchers were able to improve the performance of probiotics in food systems by the addition of protectants to the media prior to drying. These include the incorporation of thermoprotectants such as trehalose (Conrad et. al. 2000); non-fat milk solids (Concoran et al., 2004); various probiotic/prebiotic combinations (Desmond et al., 2002; Ananta et al., 2004; Corcoran et al., 2004) and soluble fibre/gum acacia (Desmond et al., 2002). Protective roles of trehalose and sucrose on both membranes and proteins in intact bacteria during drying have been further reported (Leslie et al., 1995; Conrad et al., 2000) and it was demonstrated that α,α-trehalose was a reasonable protectant for preserving lactic acid producing bacteria during freeze-drying and vacuum-drying. However, the protective abilities of trehalose can be enhanced significantly by the addition of borate ions which cross link the trehalose, thereby raising the glass transition temperature of the dry matrix (Miller et al., 1998). Webb (1965) showed that vegetative bacterial cells that are not desiccation resistant are pro-

tected from irreversible damage if they are dried in the presence of inositol, and he suggested that the inositol was acting as a water substitute at macro-molecular surfaces.
A variety of animals have acquired the ability to lose reversibly essentially all of their cellular water at ordinary temperatures but yet remain viable (Crowe and Clegg, 1973; Crowe and Clegg, 1978). These organisms solve the problems that are caused by desiccation by an adaptive mechanism involving the replacement of lost water by a “compatible solvent” (Clegg et al; 1982) such as large concentrations of polyhydroxy alcohols, as well as certain sugars.
Trehalose and sucrose have been shown to preserve both structure and function of isolated proteins during drying (Carpenter et al., 1987; Crowe et al., 1987). Blakeley et al., (1990) reported the use of trehalose in preventing the denaturation of monoclonal antibiotics during dessication and that the dehydrated antibodies had a high level of accuracy in reaction with their antigens. Colaco et al., (1992) showed that extremely fragile biomolecules such as DNA restriction and modifying enzymes could be dried “in vitro” in the presence of trehalose with no loss of activity even after prolonged storage. Uritani et al., (1995) reported that disaccharides such as trehalose, maltose, or sucrose protected restriction enzymes during vacuum desiccation and further storage. Likewise Rossi et al. (1997) showed the ability of trehalose and sucrose in protecting the restriction enzyme ECORI most effectively during storage at 37°C and 45^{o}C.

2.3.2 Protective Mechanisms of Sugars on Biological Materials during Drying

2.3.2.1 Membranes

Liposomes are artificial vesicles composed of concentric lipid bilayers separated by water compartments. The typical characteristic of bilayer-forming lipids is their amphiphilic nature: a polar headgroup covalently attached to one or two hydrophobic hydrocarbon tails. When these lipids are exposed to an aqueous environment, interactions between themselves (hydrophilic interactions between polar headgroups and van der Waal’s interactions between hydrocarbon chains) and with water (hydrophilic interactions, hydrophobic effect) lead to spontaneous formation of closed bilayers. Liposomes can differ in size, they can range from the smallest vesicle (SUV) obtainable on theoretical grounds (diameter-20mm) to liposomes which are visible under the light microscope, with a diameter of 1mm or greater (LUV), equal to the dimensions of living cells. They can also differ in terms of lipid composition and structural organization, corresponding to uni-, oligo – or multi-lamellar vesicles (mvv or MVV). Liposomes are built in such a way that the solute can be encapsulated in the aqeous compartment (polar solutes) or embedded in the lipid bilayers (lipophilic or amphiphilic solutes). The properties of liposomes and their subsequent applicability depend on the physical and

physico-chemical characteristics of the liposome membrane. Usually, a zwitterionic or non-ionic lipid is used as the basic lipid for the penetration of liposomes. The net surface charge of liposomes can be modified by the incorporation of positively charged lipids, such as stearylamine, or negatively charged lipids, such as diacetylphosphate, phosphatidyl glycerol or phosphatidyl serine (Frezard, 1999; Ananta, 2005).

Liposomes are a commonly used model system for studying lipid bilayers and have helped to elucidate the protective effects of carbohydrates on biological membranes during dehydration. It appears that two requirements must be met to achieve this stabilization: inhibition of fusion between liposomes during drying and rehydration and depression of the gel to liquid crystalline phase transition temperature (T_m) in the dry liposomes, at least for unsaturated lipids (Crowe and Crowe, 1982; Crowe and Crowe 1995). One important effect of the presence of disaccharides is the reduction of the thermotropic phase transition in the dry membranes. If a lipid bilayer is dried in the absence of any added sugar, then as the water molecules are removed, the phospholipid headgroups are forced closer together. This close approach of lipid molecules leads to increased van der Waal's interactions between the fatty acyl chains, and the membrane is forced into gel phase (Crowe et al., 1984a; 1997a).

Fourier transform infrared spectroscopy (FTIR) (Crowe et al; 1989c), differential scanning calorimetry (DSC), or X-ray diffraction can be used to measure this effect, which appears as a shift in the gel-to-liquid crystalline phase transition (T_m) toward higher temperature. When such a membrane is rehydrated, it must go through another phase transition, (T_m) which returns it to the liquid crystalline state (Crowe and Crowe, 1988; Crowe et. al., 1989c).

In contrast, if a lipid bilayer is dehydrated in the presence of disaccharides, such as sucrose or trehalose, then as the water is removed, sugar molecules are thought to insert into the membrane and interact directly with the lipid headgroups by hydrogen bonding. This is known as "water replacement hypothesis" because the sugar molecules are thought to substitute for water molecules between the lipid headgroups during dehydration. Thus, adequate spacing is maintained that often preserves the membrane in a liquid crystalline state, even when dry. When a membrane dried in this way is rehydrated, there is no concomitant phase transition therefore the barrier properties of the membrane are retained (Crowe et al., 1984a, b; Caffrey et al., 1988; Leslie et al., 1995; Crowe and Crowe, 1992, 1995). Interaction of the carbohydrates with the polar headgroups depresses T_m to such an extent that a phase transition during drying and rehydration is largely prevented.

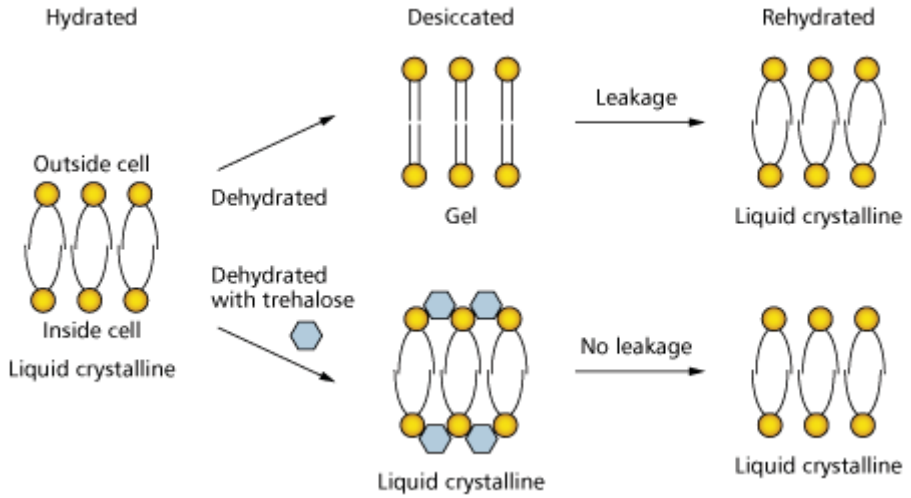


Figure 2.4: A diagrammatic representation of the effect of sugar on leakage of trapped solute when phospholipid bilayers are dried and rehydrated at room temperature.
(Adapted from www.chemsoc.org/chembytes/ezine/2002/wharton_oct 02.htm)

In addition to a transition from the liquid crystal to the gel phase, certain phospholipids can undergo a transition from liquid crystal to hexagonal II phase, as water is removed, as a result of interaction between adjacent phospholipids headgroups. This type of transition is especially common in membranes high in phosphatidylethanolamine, such as the inner membrane of *E.coli*. While a transition from the liquid crystal phase to gel phase differs from a transition to hexagonal II phase, the overall result for cells' loss of membrane integrity is the same (Crowe and Crowe, 1982; Gordon-Kann et al.,1984).

Dried sugar matrices, due to their ability to form vitrified (glassy) matrices, are supposed to protect the contained labile biomaterial during storage. A glass is a kinetically metastable, time dependent physical state presented in amorphous or partially crystalline materials characterized by near absence of molecular movement. The concept of "glassy" and "rubbery" states has been introduced to interpret the stability of low-moisture foods and biomaterials (Slade and Levine, 1991a, b; Slade and Levine, 1994). Sugar and other carbohydrates form glasses by adequate dehydration from a solution. The most important parameter describing the glassy state of amorphous material is the glass transition temperature (T_g) below which the materials exhibit extremely high viscosities which give them solid like properties. Above the T_g, viscosity drops sharply in the "rubbery" state and the mobility of the system increases accordingly. The T_g of low molecular weight carbohydrates is specific to each anhydrous material but is extremely sensitive to water, which plasticizes the amorphous structure (Roos and Karel, 1990; 1991).

The formation of glassy solid results in a reduction of translational molecular motion and rates of chemical reactions and relaxation rates for various processes in glassy matrices may be very low, especially at temperatures well below the glass transition temperature (Franks,

1993). The glass forming properties (vitrification) of certain carbohydrates, as one of the factors, in the stabilization of biomaterials at low moisture have been extensively analysed.
It has been shown that vitrification is required for stabilization of liposomes in the dry state (Crowe et al., 1994). If the liposomes are permitted to warm above the glass transition (T_g), during freeze drying or during storage in the dry state they undergo massive fusion, leading to production of large, multilamella vesicles and leakage of the contents of the original liposomes. Many solutes are capable of forming glasses and inhibiting this fusion, but only a few seem able to prevent leakage during freeze-drying and storage, suggesting that while vitrification (and the consequent prevention of fusion) is required, it is not in itself sufficient. Mixtures of hydroxyl ethyl starch (HES) and glucose used in drying liposomes showed that HES, having an elevated T_g only inhibited fusion between liposomes during drying but it did not depress T_m thus leakage was not prevented. HES are a large polymer sterically hindered from penetrating the bilayer in the dry state. However, glucose, with a relatively low T_g , was not vitrified at room temperature during drying and did not inhibit fusion or leakage but only depressed T_m thus, as a result the combination preserved the dry liposomes (Crowe et al., 1997b).

2.3.2.2 Proteins

The biological function of proteins depends on their three – dimensional structure, which is determined largely by water. Loss of water, which forms the hydration shell of the protein, can lead to loss of native structure, resulting in loss of biological function upon rehydration (Prestrelski et al., 1993).
Studies have been made on soluble proteins such as trypsin (Lee and Timasheff, 1981; Lopez-Diez and Bone, 2004), restriction enzymes (Colaco et al., 1992; Buera et al., 1999), and amylase (Terebiznik et al., 1997). Enzyme inactivation as a result of drying may involve a conformational change in the active site due to protein denaturation (unfolding and/or inter – or intraprotein hydrophobic aggregation) or to blockage of specific active groups by the formation of covalent links (i.e. condensation of amino groups of proteins with carbonyl compounds) that modify the active site of the enzyme or make it inaccessible to its substrate (Burin et al., 2004). The water replacement theory and the glass transition theory have also been advanced to explain the protective role of sugars in the desiccation tolerance of proteins.
The protective effect of sugar on stabilization of native protein state during dehydration can be explained by water replacement hypothesis. Using infrared spectroscopy method it was shown that in the presence of sugar the amide bands of the dried proteins were similar to those of hydrated proteins and the OH vibrations of sugar are altered by the protein so that

they closely resemble the OH groups of the hydrated sugars (Leslie et al., 1995; Ananta 2005). Disaccharides form hydrogen bonds with the proteins when water is removed, thus preventing protein denaturation by replacing water molecules needed to maintain the protein structure (Carpenter and Crowe, 1988, 1989). However, the failure of glucose to prevent lysozyme unfolding during freeze-drying showed that hydrogen bonding between carbohydrate and protein alone is not sufficient to confer protection to a protein during lyophilization (Allison et al., 1999). Thus, the difference in the efficacy for protein preservation by different sugar despite the ability to hydrogen bond to protein (at sites which normally binds water) could be explained by the extent of interaction between protein and sugar (Lopez-Diez and Bone, 2004).

Levine and Slade (1992) made a hypothesis that the glassy state is the sole important factor in long-term stabilization of stored restriction enzymes dried in trehalose matrix because translational diffusion-relaxation processes (such as protein denaturation) are strongly inhibited. Studies conducted by Schebor et al. (1996) and Cardona et al. (1997) showed that enzyme inactivation was observed in heated glassy matrices of maltodextrin and PVP well below their glass transition temperatures. This indicated that the formation of a glassy matrix was not a fundamental requisite in protein stabilization. Uritani et al. (1995) and Rossi et al. (1997) experimentally found that enzymes stabilization was observed during desiccation and storage of rubbery dried trehalose and sucrose systems, thus suggesting that the ability of sugars to protect the enzyme may be attributed to the stabilization of the biological structure through a preferential interaction rather than to a modification of the properties of water.

2.3.2.3 Intact Cells

Compared to freezing and freeze-drying, convective drying is an economical alternative for the preservation of starter cultures of bacteria. A prerequisite is that inactivation during drying is kept at a minimum. At low drying temperatures thermal inactivation is negligible, but dehydration inactivation may impose serious problem. A potential disadvantage of drying in the preservation of cell cultures is the damage caused to bacterial cells during the process. One of the most susceptible sites in bacterial cells is the cytoplasmic membrane, which is indicated by the increased sensitivity of the sublethally injured bacteria to NaCl (Teixeira et al., 1997). Other possible sites in the cell where damage may occur include the cell wall (Brennan et al., 1986) and DNA (Teixeira et al., 1997). The loss of antagonistic activity in *Carnobacterium divergens* has been reported after drying (Silva et al., 2002). This phenomenon was explained by a possible loss of plasmid responsible for the inhibitory activity. There is an evidence of lipid oxidation in *Lactobacillus bulgaricus* cells during drying (Teixeira et al., 1996) indicating the induction of lesions in the cellular lipid - containing structures.

Endogenous trehalose is known as an effective protectant for bacteria and yeast cells in the absence of water (Eleutherio et al, 1993; Newman et al., 1993; Louis et al., 1994). Also the accumulation of trehalose upon drying of fungal spores, nematodes, and cysts of the brine shrimp Artemia (Crowe et al., 1984a; Chapman, 1994) or of sucrose in pollen and seeds of higher plants (Crowe et al., 1988; Hoekstra et al., 1992) improves the tolerance to desiccation. Added carbohydrates decrease dehydration inactivation of dried *L. plantarum* cells (Linders et al., 1997b). The residual activity of these fluidized bed-dried cells improves when the bacteria are dried in the presence of maltose, sucrose and sorbitol. It has previously been described for anhydrobiotic organisms that the protective effect of carbohydrates particularly concerns cell membranes (Crowe et al., 1984a).

Using Fourier transform infrared spectroscopy (FTIR) it is possible to measure phase transitions in intact cells (Crowe et al., 1989 b, c). The T_m of freeze- dried *Escherichia coli* and *Bacillus thuringiensis* was depressed as a result of the penetration of trehalose and sucrose into the cells thus improving the survival of the bacteria after drying. In order to allow the internal accumulation of sugars, cells were incubated in 100mM sugar solution at T_m (10^oC) when membranes were having a higher level of permeability and sugar flew down its concentration gradient and into the cells (Leslie et al., 1995).

The importance of membrane phase transitions was also studied in relation to the desiccation tolerance of *L. plantarum* cells after the addition of maltose, trehalose and sorbitol (Linders et al., 1997c). The authors found out that only maltose and sorbitol improved the residual activity significantly irrespective of the inability of the sugars to depress the T_m of the dry cells while trehalose, a super glass former, was not protective but rather lowered the residual activity of the cells. It was explained that sugars might not get access to the cytoplasm based on the fact that the cells were grown on glucose and fructose and thus not adapted to the uptake of other carbohydrates. The protective effect of sorbitol was likely due to its antioxidant properties.

Sugar has to be present in both sides of the membrane, to allow sufficient protection of cellular components critical for viability including membranes and proteins (Crowe et al., 1985; Leslie et al., 1995; Diniz-Mendes, 1999); however the threshold level of internal sugar concentration sufficient to confer protection varies greatly depending on the cell type, drying conditions and residual moisture content. A minimum amount of sugar is needed intracellularly to hydrogen-bind to all of the lipid molecules in the plasma membrane or to form intracellular glassy state (Chen et al., 2001). In general, anhydrobiotic organisms have concentrations of internal sugar (especially trehalose) that range from 20 to 50% of the dry weight of the organisms (Crowe et al., 1998; Ananta, 2005).

2.3.3 Drying of Probiotic Bacteria

2.3.3.1 Introduction

Bacteria can be dried either by freeze drying, vacuum drying, spray-drying, or fluidized bed drying (Champagne et al., 1991; Teixeira et al., 1995b; King and Lin, 1995; Bayrok and Ingledew, 1997). Most of dried bacterial preparations are currently produced by freeze-drying due to the possibility to operate at mild conditions, so that the degree of injury could be minimized. However, some drawbacks of freeze- drying process, such as long processing time and high energy consumption led to efforts in evaluating alternative drying processes. Spray drying is one of the promising processes for production of dry probiotic preparations (Gardiner et al., 2000; Desmond et al., 2002; Corcoran et al., 2004; Ananta et al., 2004) since under optimized conditions it allows high processing rates and low operating costs and also maintenance of a high degree of survivability.

2.3.3.1.1 Spray drying

As earlier stated a lot of investigations (Teixeira et al., 1995a,b,c; Teixeira et al., 1997; Gardiner et al., 1998; Gardiner et al., 2000; Desmond et al., 2001, 2002; Corcoran et al., 2004; Ferreira et al., 2005; Ananta et al., 2005) have been made in order to evaluate the possibility of producing lactic acid bacteria preparations by means of spray drying process.
In the spray drying process the feed solution is transformed from a fluid state into a dried form by spraying the feed into a hot drying medium. The process itself involves the atomization of a liquid feedstock into a spray of droplets, which come in direct contact with electrically heated air in a drying chamber. There are three modes of contact between hot air and liquid feed: co-current, counter-current and mixed flow (Masters, 1991). Figure 2.5 shows a schematic drawing of a lab-scale spray dryer working with in a co-current flow of drying air and feed solution.

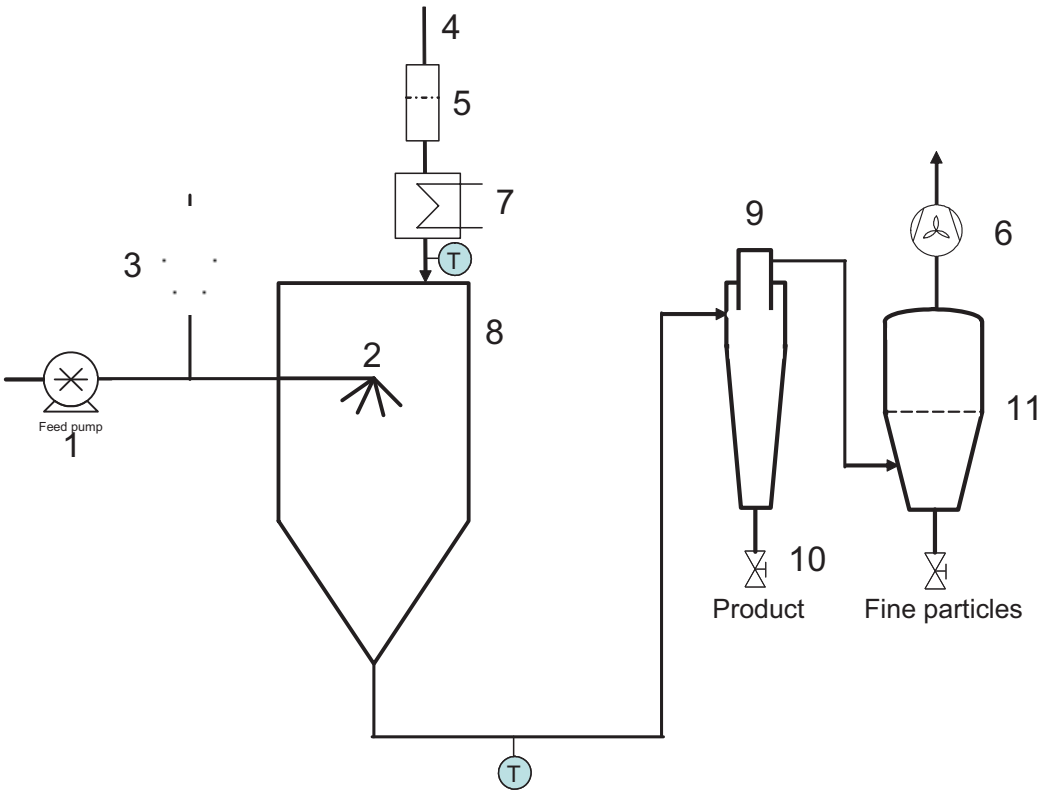


Figure 2.5: Schematic view of a spray dryer installation with its essential components. The role of each component is as described below. (Ananta, 2005).

The feed (1) is pumped from the product feed tank to the atomizing device which is normally located in the air disperser in the top of the drying chamber. The sprays are produced by a rotary (wheel) or nozzle atomizer (2), resulting in fine droplets of the range 2 – 150 μm diameter. When two-fluid nozzle is used to generate spray, pressurized air (3) is required. The drying air (4) is drawn from the atmosphere via a filter (5) by an aspirator (6) and is passed through the air heater (7) to the air disperser. In the drying chamber (8), the atomized droplets meet the hot air and the evaporation takes place cooling the air at the same time. Evaporation of moisture from the droplets and formation of dry particles proceed under controlled temperature and airflow conditions. The construction of the drying chamber is made under consideration of the adequate residence time and droplet trajectory distance for achieving the heat and mass transfer. After the drying of the droplets in the chamber, the majority of the dried product falls to the bottom of the chamber. The dried product can either be discharged continuously from the bottom of the drying chamber or passed into a solid-gas separator (9) where the solids from the gas stream are recovered and the powder is collected at its bottom (10). The fine particles are usually collected in an outlet filter (11).

Operating conditions and dryer design are selected according to the drying characteristics of the product and powder specification, most importantly the residual moisture content of the powder and the particle size. The first variable is affected by the evaporation rate and the dryer ΔT (inlet air temperature minus the outlet air temperature), which in turn dictates the

amount of drying air needed and ultimately the sizing and cost of almost all of the system components. The particle size requirement affects the choice of atomization method and can also affect the size of the dryer.

The dehydration of the atomised liquid particles proceeds from the particle surface to the inner core, resulting in the formation of protective vapour film, which surrounded the droplet and keeps the particle at the vapour saturation temperature. As long as the particle does not become completely dry, evaporation still takes place and the temperature of the solids may decrease (due to evaporative cooling) or does not approach the dryer outlet temperature (Mermelstein,2001; Roos, 2002). This is why many heat sensitive products (enzymes, micro-organisms, volatile aroma compounds, etc.) can be spray dried at relatively high temperatures to produce powders with low moisture load without the danger that the product may be harmed (Ananta, 2005). The earlier mentioned investigations made on spray drying of microbial cells demonstrated the production of low moisture content skim-milk based preparations containing bacterial cultures coupled with stability of dried cultures under different storage temperatures and relative humidity (where applicable); amongst others.

2.3.3.1.2 Vacuum drying

Drying under vacuum traces its origin to the early 1920s in Sweden but commercial applications began in Europe, notably in Germany, Italy and Russia in 1965. The various vacuum drying processes are divided into two categories: the continuous vacuum drying process, in which the vacuum is maintained continuously throughout the drying process; and the discontinuous type, in which phases of convection heating at atmospheric pressure alternate with phases under vacuum.

Vacuum drying is ideal for materials that would be damaged or changed if exposed to high temperatures. It prevents oxidation of materials and it is also ideal where a solvent must be recovered or where materials must be dried to very low moisture levels.

Unlike atmospheric drying, drying under reduced pressure lowers the boiling point and provides a greater temperature difference between the heating medium and the product. This results in faster drying and more efficient heat recovery. Drying at lower temperatures reduces energy consumption. The dryers' closed-system designs minimize product loss caused by atmospheric contaminants, dusting, oxidation, and discoloration and chemical changes. The potential for explosions is reduced, therefore workers are protected.

Laboratory ovens employed in vacuum drying applications, curing and moisture content testing are called vacuum ovens. Common applications include drying heat sensitive samples, moisture determination, and drying samples under controlled atmosphere. The heat-treating process takes place inside a vessel that is air-tight. This allows a vacuum to be drawn inside

the vessel. Since there is no air in the vacuum chamber, heat is transferred from the heating elements to the interior chamber wall, then to the shelves, and finally to the samples.

Some authors employed vacuum ovens in the drying of biological materials such as enzymes and yeasts in sugar matrices (Uritani et al., 1995; Rossi et al., 1997; Cerrutti et al., 2000). Cerrutti et al. (2000) reported that there was lower loss of viability when S*. cerevisiae* cells were dried under vacuum compared to when freeze dried. The result was explained by the fact that during freezing/freeze-drying, the cells did not have the opportunity to compensate for the osmotic stress produced by this procedure, but this reaccomodation only was made possible during vacuum drying.

The reported spray-drying experiments on probiotics were conducted in skim milk powder, which protective ability was associated with proteins though in an unknown way. The protective abilities of sugars especially trehalose should be investigated during spray-drying of probiotics. Vacuum drying in a laboratory oven, considering the available technology and financial situations in some developing countries, also warrants investigation. Some authors investigated the effectiveness of anti-oxidants such as monosodium glutamate (MSG) when included in carrier media during drying processes. Survival of cell concentrates of *Lactobacillus delbrueckii ssp. bulgaricus* following spray-drying in skim milk with monosodium glutamate was evaluated during storage at different conditions of temperature and water activity (Teixeira et al., 1995b). Ferreira et al. (2005) also evaluated the survival of *Lactobacillus sakei* during heating, drying and storage in the presence of monosodium glutamate. MSG is widely advertised and used in cooking at homes and in large occasions in many Asian and African countries, therefore its feasibility in producing spray dried probiotics in conjunction with trehalose warrants investigation.

Monosodium glutamate

A compound such as monosodium glutamate (MSG) was reported to have good storage properties (Carvalho et al., 2003; McLaughlin, 2007). MSG commonly known as Ajinomoto, Vetsin or accent is the sodium salt of glutamic acid. It is also known as 2-aminoglutaric acid. In the past, MSG was extracted from natural protein rich foods such as seaweed which was a time-consuming practice. Recently, it is made from an industrial process involving fermentation of cornglucose and other raw materials. MSG contains about one-third of the sodium of table salt and it is used in smaller amounts. When added to foods, it produces a flavouring function similar to the glutamate that occurs naturally in foods. Glutamate is a naturally occurring amino acid that is found in nearly all foods, especially high protein foods such as dairy products, meat and fish, and in many vegetables. Foods often used for their flavouring properties, such as mushrooms and tomatoes, have high levels of naturally occurring gluta-

mate. The human body also produces glutamate and it plays an essential role in normal body functioning.

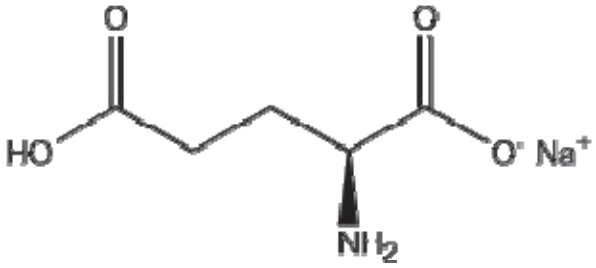


Figure 2.6: The chemical structure of monosodium glutamate (MSG).

Although MSG is readily available, inexpensive, and easily soluble in water and neither absorb humidity nor solidify, it has been given a bad reputation as a suspicious additive that many consumers believe gives allergies but many studies have found that it does not cause ill effects (McLaughlin, 2007).

3 Materials and Methods

3.1 Bacterial strains and growth conditions

Bacterial strains, *Lactobacillus rhamnosus* E-97800(*E800)* and *Lactobacillus rhamnosus GG (LGG)* used were obtained from VTT culture collection (Espoo, Finland). The cultures which were sent in freeze-dried form in glass ampoule were later stored as glass beads cultures (Roti$^{(R)}$-Store, Carl-Roth, Karlsruhe, D) in a –80^{o}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^{o}C over a period of 24h and monitored spectrophotometrically at 600nm (Graphicord uV-240, Schimadzu, JPN).

3.2 Osmotic agents

Laboratory grade sucrose (Merck KgaA, Darmstadt, Germany) and trehalose (Carl Roth GmbH, Karlsruhe, D) and lactose (Merck KgaA, Darmstadt, Germany) were used.

3.3 Media

3.3.1 Methods of preparation of media

Unless otherwise stated, all media were prepared using deionised water and sterilized by autoclaving for 15 min at 121^{o}C.

The media used for osmotic treatment were prepared by incorporating the sugars into growth media. High osmolarity media were obtained by adding trehalose or sucrose (0.1M, 0.3M, 0.4M, 0.6M, 0.9M, 1.2M and 1.5M) to the MRS broth (5ml) [Oxoid, Basingstoke, UK] at concentrations indicated between the brackets. MRS broth without trehalose or sucrose served as controls. The osmolarities of the solutions were measured (51308 Vapour Pressure Osmometer Wescor Inc.).

The medium used for plate count enumeration was MRS agar (Oxoid, Basingstoke, UK). This was also prepared according to the manufacturers instructions. 62g of the powder was sterilized in 1 liter of water and then cooled to 50^{o}C before being distributed into petri-dishes.

3.4 Plate enumeration method

Treated and non-treated samples were serially diluted in Ringer's solution (No. 15525, Merck, Darmstadt, D) and appropriate dilutions were drop plated in duplicate on MRS agar. The viable cell numbers were determined after 48h of incubation at 37°C under anaerobic conditions produced by anaerobic kits (Anaerocult® A, Merck, Darmstadt, D).

The impact of physical treatments 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 physical exposure, while N_o refers to the initial count before physical exposure.

3.5 Physical treatments

3.5.1 Screening for the sub-lethal and lethal Conditions

3.5.1.1 Osmotic Treatments

High osmolarity media obtained by adding trehalose and sucrose (0.1M, 0.3M, 0.4M, 0.6M, 0.9M, 1.2M and 1.5M) to the MRS broth (5ml) at concentrations indicated between the brackets and MRS broth without these osmotic agents (control samples) were inoculated with the bacterial strains at $OD_{0.1}$. Samples were incubated at 37°C for 24h, during which the growth of cells were recorded by taking optical density measurements.

3.5.2 High Pressure Treatments

The high pressure unit (U111, Unipress, Warsaw, PL) was developed to meet the conducting kinetic studies up to pressures of 700MPa and a wide temperature range between –40 and 100°C (Arabas, Szeczepek ,Dmowski, Hein and Fonberg, 1999). This unit consists of five pressure chambers which are seperated from each other via high-pressure valves. The chambers are immersed in a water bath with a thermostat. This design allows a simultaneous treatment of five different samples in one pressure build-up step to isothermal conditions. Each chamber is equipped with a K-type thermocouple and a pressure sensor to monitor the temperature history of each sample during the treatment cycle.

The exponentially grown cultures ($OD_{0.5}$) were filled into sample containers (Nunc Cryo Tubes Nr.375229, Nunc A/S, and Roskilde. DK), which were subjected to 100MPa for 10 min.

3.6 Characterization of stress responses

After the screening for sub-lethal and lethal stress conditions of the two bacterial strains as stated above, bacterial adaptive response and cross resistance to other stresses were investigated. Cultures were grown as earlier described and cells in the exponential growth phase were harvested at O.D $_{600}$ 0.5. To measure tolerance to challenges, non-treated and pre-treated cells were removed by centrifugation, washed and subjected to selected lethal stress conditions (Laplace et al., 1999; Saarela et al., 2004).

3.6.1 Response of Log-phase cultures to osmotic treatments

Growth and survival of exponentially grown cultures at 0M, 0.1M, 0.3M, 0.4M, 0.6M, 0.9M, 1.2M and 1.5M of trehalose were screened. Exponentially- grown cells were incubated at 37^{o}C in MRS broth with or without the osmotic agents for 60 min. Bacterial counts were determined after incubation under anaerobic conditions (Anaerocult® A, Merck, Darmstadt, D). Osmotic treatment by sucrose was also conducted.

Measurements of kinetics of growth and survival were done by withdrawing samples from 0.6M MRS-sucrose/trehalose media at time intervals of 0, 15, 30, 45, 60 min. 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.

3.6.2 Assessment of osmotic pre-treated cells' response to lethal heat stress

Exponentially-grown cultures were prepared. They were pre-treated osmotically in MRS broth containing 0.6M osmotic agents at 37^{o}C for 45 min after which a time-zero sample was collected and bacterial counts were determined. Non-treated cells served as controls. Cultures were then incubated at 60^{o}C for 1h. Samples were withdrawn at intervals of 1.5, 3, 5, 7 and 10 min after which bacterial counts were determined. The impact of heat 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 lethal heat exposure, while N_o refers to the initial count before lethal heat exposure.

3.6.3 Assessment of osmotic pre-treated cells' response to lethal osmotic stress

Exponentially-grown cultures were prepared, the cells were harvested and the supernatant removed. Samples were re-suspended in MRS without osmotic agent (control) and also in MRS containing 0.6M osmotic agents, followed by incubation at 37^{o}C for 45 min. A time-zero sample was collected and bacterial counts were determined. The cells were harvested and re-suspended in MRS containing 1.5M sucrose for 60min, during which bacterial counts were determined at intervals of 10, 20, 30, 40, 50, and 60 min. The impact of lethal 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 lethal osmotic exposure, while N_o refers to the initial count before lethal osmotic exposure.

3.6.4 Assessment of osmotic pre-treated cells' response to bile stress

Exponentially-grown cultures were prepared. They were pre-treated in MRS broth containing 0.6M stress agents at 37°C for 45 min after which a time-zero sample was collected and bacterial counts were determined. Non-treated cells served as controls. Cultures were then incubated in 1% bile (Sigma) for 1h. Samples were withdrawn at intervals of 5, 10, 15, 20, 25, 30 and 60 min after which bacterial counts were determined. The impact of bile 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 bile exposure, while N_o refers to the initial count before bile exposure.

3.6.5 Assessment of pressure pre-treated cells' response to lethal heat stress

Exponentially-grown cultures were prepared. Pre-treatment at 100MPa and a temperature of 20^{o}C and 30^{o}C for 10 min was conducted. Non-treated samples served as controls. Cultures were then incubated at 60^{o}C for 1h. Samples were withdrawn at intervals of 0, 1.5, 3, 5, 7 and 10 min after which bacterial counts were determined. The impact of heat 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 heat exposure, while N_o refers to the initial count before heat exposure.

3.6.6 Electron Microscopy

Thin layers of control, trehalose or sucrose treated stationary-phase cells were mounted on stubs, dried with acetone by the CO_2 method using the CPD 030 Critical Point Dryer (BAL-TEC) followed by the coating of dried cells with gold (20nm). Cells were examined and photographed with S-2700 Scanning Electron Microscope (HITACHI) operating at 20kV. Osmotic pretreated and non-treated cells subjected to bile stress were also examined (Leverrier et al., 2003).

3.7 Flowcytometry.

3.7.1 Stress Treatments.

Stationary growth phase cultures were harvested, washed in Ringer's solution and concentrated to a theoretical value of OD_{600} value of 10 using Ringer's solution. This corresponded to a cell concentration of 3.0 - 3.4x10^9 cfu mL^{-1}. Equal volume of the cell concentrate was treated with equal volume of the MRS-sugar solution at 37^{o}C for 30-45 min.

3.7.2 Esterase activity and membrane integrity.

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

3.7.3 cF extrusion activity and kinetics of extrusion.

Equal volumes of cF stained cells were further incubated together with 20mM glucose for 20 and 40min at 37^{o}C in order to measure the performance of treated or control cells in extruding intracellular accumulated cF (Bunthof et al., 1999). For kinetic measurements, culture samples from *Lactobacillus rhamnosus E800* were withdrawn every 10 min for 40 min and from *Lactobacillus rhamnosus GG,* every 5 min for 20 min to monitor release of cF from glucose energized cells.

3.7.4 Flowcytometric Measurements

Analysis was performed on a Coulter® EPICS® XL_MCL flow cytometer (Beckman Coulter Inc., Miami, FL, USA) equipped with a 15mW 488nm 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 530nm following excitation with laser light at 488nm, whereas red fluorescence at 635nm is emitted by PI- stained cells.
A set of band pass filters of 525nm (505-545nm) and 620nm (605-635nm) 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 Flow-Check^{TM} Flourospheres (Beckman-Coulter Inc., Miami, FL, USA).

3.7.4.1 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 Eq. (1).

$$EA\ (\%) = (\#4p/\ \#4ctrl) \times 100 \qquad (1)$$

Where EA is the residual enzymatic activity in response to a particular osmotic treatment, #4p is the percentage of population in #4 following osmotic treatment; #4ctrl is the percentage of population in #4 prior to osmotic treatment.

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

$$cFA\ (\%) = (1- \#4_{Glu} / \#4) \times 100 \qquad (2)$$

Where cFA is the measure of performance in extruding cF, $\#4_{Glu}$ is the percentage of population in #4 following glucose addition and 20-40min incubation; #4 is the percentage of population in #4 prior to glucose addition.

The kinetics of relative number of population extruding the intracellular accumulated dye is calculated in Eq. (3) thus:

$$RcF\ (\%) = (\#4_{t\text{-}Glu} / \#4_{t=0}) \times 100 \quad (3)$$

Where RcF is the relative number of cells still stained with cF in #4 following glucose addition, $\#4_{t\text{-}Glu}$ is the percentage of cells still stained with cF in #4 following glucose addition and incubation t min, $\#4_{t=0}$ is the percentage of cells still stained with cF in #4 prior to glucose addition.

3.7.4.2 Statistical Analysis

The correlation between the cell viability and osmotic induced changes on the physiology of *Lactobacillus rhamnosus* strains 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).

3.8 *Drying experiments*

3.8.1 Vacuum 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. Cells from the exponential phase were mixed with equal volume of 20% (w/w) trehalose, sucrose or lactose. However cells from the stationary phase were concentrated to OD_{10} before mixing with equal volumes of 20%, 25% or 30% (w/w) trehalose. Control cells were mixed with equal volume of Ringer's solution. 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 or 48h. The final pressure achieved in the vacuum oven was 26.7kPa (200Torr).

3.8.1.1 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 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 dilu-

tion was in duplicate on MRS agar. Plates were placed in an anaerobic jar (Anaerocult®, A, Merck, Darmstadt Germany) and incubated at 37°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.

3.8.1.2 Flow cytometric measurements of samples dried in vacuum-oven

Staining procedure with LIVE/DEAD BacLight

LIVE/DEAD BacLight bacterial viability kit 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 dried 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 flow cytometric 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. 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 10 min were entirely encountered in the area surrounded by gate DEAD (Ananta, 2005).

3.8.1.3 Storage tests

Dried samples were stored at 4°C, 25°C and 37°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 ($\log N/N_o$). N refers to the bacterial count at a particular storage period while N_o refers to the bacterial count at the beginning of storage.

3.8.1.4 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.

3.8.1.5 Screening of bacterial toletrances to acid, bile, lysozyme and pepsin

Equal volume of fresh and 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 1mg mL^{-1} or 5mg mL^{-1} Lysozyme (Sigma Aldrich, Steinheim, Germany) (Kamaya, 1970) and 1mg mL^{-1} or 3mg mL^{-1} Pepsin (Sigma- Aldrich, Steinheim, Germany). Samples were incubated at 37°C in waterbath for 3h and for 2h by acid treatment. Appropriate dilutions of samples were drop plated on MRS agar and incubation was conducted for 48h at 37°C.

3.8.2 Spray drying

3.8.2.1 Preparation of samples

One bead from deep-frozen cultures was transferred into 10mL MRS broth and incubated overnight at 37°C. An overnight culture was inoculated, at an initial $O.D_{600}$ of 0.1, into a fresh MRS broth in which 12.5g L^{-1} trehalose was incorporated. Growth was allowed to continue in the presence of trehalose until cells reach the stationary phase. Thereafter, cells were harvested by centrifugation (4000g/10min), washed in Ringer's solution (pH 7.0) and re-

suspended in an equal volume of 20% (w/w) sterilized trehalose. This was followed by the heat challenge experiments and the drying process.
Sterilized trehalose supplemented with 12.5g L^{-1} monosodium glutamate (MSG) (Sigma-Aldrich) was also used as a carrier medium.

3.8.2.2 Heat challenge experiments

The thermal tolerance of *L. rhamnosus GG* and *L. rhamnosus E800* were compared in 20% (w/w) trehalose solutions. 50µl of samples were put into sterile A-R glass capillaries (Kleinfeld Labortechnik GmbH, Gehrden, Germany) (Mathys et al., 2007) and placed in a water bath at the test temperatures of 60^{o}C, 65^{o}C and 70^{o}C. At intervals, capillaries were removed and cooled in ice bath after which a sample volume of 30µL was serially diluted in Ringer's solution on micro plates (Carl Roth GmbH, Karlsruhe, Germany) and plated on MRS agar. Survivors were enumerated after 3 days of anaerobic incubation at 37°C in an anaerobic jar.

3.8.2.3 Drying process

The spray-drying process of *LGG* and *L. rhamnosus E800* in the various media, non-supplemented trehalose and trehalose supplemented with monosodium glutamate (MSG), was undertaken in a laboratory scale spray dryer (Büchi B-191, Flawil, Switzerland), which is schematically shown in Figure 3. The feed solution was pneumatically atomized into a vertical, co-current drying chamber using a two-fluid nozzle at a constant flow rate (5 mL min^{-1}). The outlet temperature was adjusted from 60^{o}C to 75^{o}C by varying the air inlet temperature. The dried powder was collected in a product container connected in bottom part of the single cyclone separator. Once the outlet temperature stabilized, the heated glass container was disconnected and replaced with another container in order to minimize uncontrolled thermal stress on the dried bacteria.

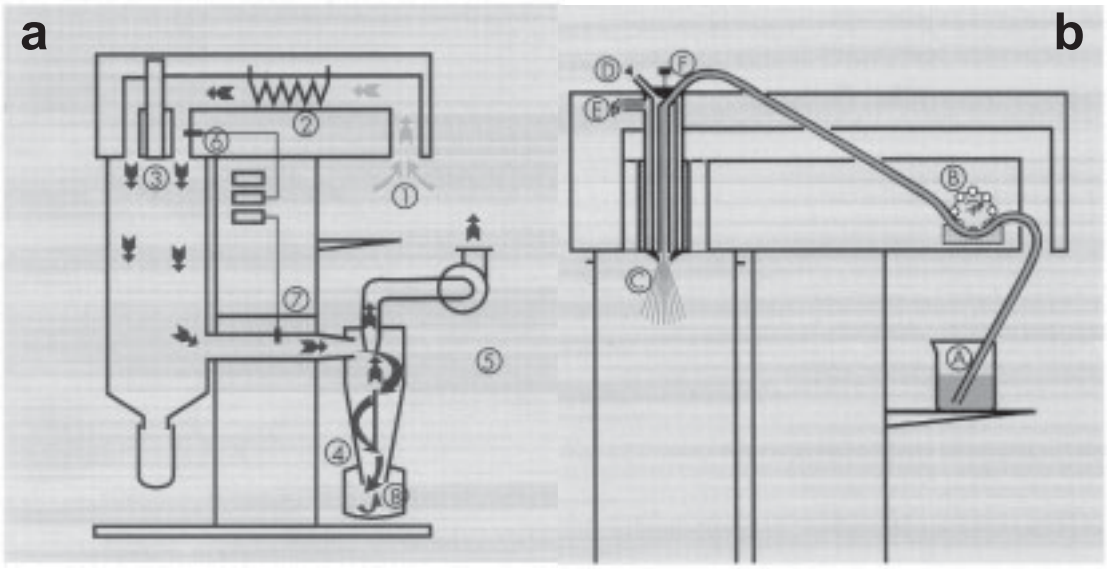


Figure 3:
(a) Pathway of drying air in the spray dryer Büchi B191
Cold air is aspirated through the air inlet tunnel (1) and then electrically heated (2) prior to entrance in the spray cylinder, in which drying of the droplets into solid particles takes place (3). Dried powder is separated from fine particles in the cyclone (4) and collected in the glass container (8). Outlet filter is placed to remove fine particles and to prevent them from entering aspirator (5) that generates the air-flow. Temperatures are measured in the entrance of spray cylinder (6, termed as air inlet temperature) and in the intermediate piece between spray cylinder and cyclone (7, termed air outlet temperature)
(b) Pathway of feed solution and pressurized air in the spray dryer Büchi B191
Feed solution (A) conveyed by peristaltic feed pump (B) and atomizing air (D, inlet) is passed separately to the nozzle head (C), where the atomization of the feed solution into fine droplets takes place. The co-current two-fluid nozzle is located at the centre of the upper part of the spray cylinder. Atomization is created by compressed air at a pressure of 0.5 to 2 bars. Nozzle diameter is 0.7 mm. Powders produced with this adjustment have particle size ranged from 5 to 15 µm on average (Desmond et al., 2002).

Generally, the level of outlet temperature which is determined by the drying rate could be adjusted by two different settings of the spray dryer. In this study, the adjustment of outlet temperature was performed by holding flow rate of the feed suspension at a constant value (25% pump capacity ~ 5 mL min^{-1}) for all outlet temperatures, whereas the inlet temperature was varied, as shown in Table 3. A rise in the outlet temperatures due to the fouling of the inner wall of the spray chamber can be compensated by slightly increasing the feed flow rate.

Table 3: Applied parameters for spray drying of L. rhamnosus GG and L. rhamnosus E800

Pump capacity :	25 ± 2 % ~ flow rate of feed suspension 5 mL min^{-1}
Flow rate of drying air :	100% ~ 60 m^3 h^{-1}
Atomization pressure :	6 bar
Flow rate of pressurization air :	700 L h^{-1}

Aimed outlet temperature (°C)	**Adjusted inlet temperature (°C)**
60-65	111
65-70	118
70-75	125

On the other hand, it is also possible to vary the flow rate of the feed solution (thereby varying the amount of water per time unit which needs to be evaporated) by holding the inlet temperature at typically high level to obtain the aimed outlet temperature. This latter operational procedure is thought to be more flexible when different drying rates are frequently applied since changing the feed flow rate is faster and easier than changing inlet temperature (Ananta, 2005).

3.8.2.4 Determination of moisture content in dried samples

The residual moisture content of spray-dried samples was determined by oven drying the powders at 102^0C, determining the difference in weight, and expressing the weight loss as a percentage of the powder weight (International Dairy Federation, 1993).

3.8.2.5 Enumeration of probiotics after spray drying

To determine the survival rate of the probiotic bacteria, spray dried powders were rehydrated with sterile Ringer's solution to obtain the same solids concentration as the initial feed solution. Afterwards, they were serially diluted and drop plated in duplicate on MRS agar. Plates were placed in an anaerobic jar and incubated at 37°C for 48 h. Survival rates were calcu-

lated as follows: %survival = N/N_o x 100, where N_o represented the number of bacteria before drying and N was the number of bacteria after drying.

3.8.2.6 Storage tests

Spray dried samples were kept at 25°C and 37°C under a relative humidity of 11%. The relative humidity was kept constant by storing the powders in hermetically closed jar. Only samples which were spray-dried at 65-70°C were subjected to storage tests. Storage experiments were performed in three replicate trials for 6-8 weeks. Storage inactivation data were expressed as logarithmic value of relative survival fraction ($\log N/N_o$). N refers to the bacterial count at a particular storage period while N_o refers to the bacterial count at the beginning of storage.

3.8.2.7 Sensitivity tests

In order to study the potential cellular damage arising from drying methods, the sensitivity of cultures to NaCl and Lysozyme before and after drying processes was determined (Teixeira et al., 1995b; Gardiner et al., 2000). The tolerances of cells to simulated gastric juices and bile were also investigated. Fresh cultures and dried ones were plated on MRS agar plates supplemented separately with 5% NaCl (Merck), 5mg mL^{-1} Lysozyme (Sigma), 1%Bile (Sigma). All chemical additives except NaCl were added to MRS molten agar after filter sterilization. Simulated gastric juice was made from 2g NaCl, 3.2g pepsin and distilled water to make 1,000mL, pH 1.6) (Porubcan, 2006). Cultures (0.1g) were incubated with 9.9mL simulated gastric juice at 37°C for 90min. Serial dilutions were conducted and appropriate dilutions were drop plated. The plates were examined after 2-6 days of anaerobic incubation at 37°C and viable numbers were compared with numbers obtained on MRS plates without these selective agents.

3.8.2.8 Flow cytometric measurements of spray- dried samples

Double staining with carboxyfluorescein diacetate (cFDA) and propidium iodide (PI)

A 100 µL aliquot of rehydrated powder was mixed with 900 µL Ringer solution and centrifuged for 10 min at 4000 g. The pellet was resuspended in 100 µL PBS buffer 0.05 M pH 7.0 and mixed together with 100 µL of 100 µM cFDA stock solution (Molecular Probes, Inc. Leiden, The Netherlands), so that the concentration of cFDA in the reconstituted pellet suspension was 50 µM. The suspension was incubated at 37°C for 10 min to allow intracellular en-

zymatic conversion of cFDA into cF (Carboxyfluorescein). After excessive cFDA was removed by centrifugation, 30 µM PI (Molecular Probes, Inc. Leiden, NL) was added. The cell suspension was kept in an ice bath for 10 min to allow labelling of the membrane-compromised cells prior to flow cytometric measurement.

Flow cytometric measurement

All measurements were made with a Coulter®EPICS®XL-MCL flow cytometer (BeckmanCoulter Inc., Miami, USA) with 488 nm excitation from an argon-ion laser at 15 mW. The green fluorescence from carboxyfluorescein was collected through a 525 nm band-pass filter; and a 620 nm band-pass filter was used to collect red fluorescence from propidium iodide. Data were analysed with the software package Expo32 ADC (BeckmanCoulter Inc., Miami-FL, USA). Acquisition of fluorescence data was performed by pre-setting a gate in the forward-angle light scatter (FS) *versus* sideward scatter (SS) plot, which enabled bacterial cells of interest and artefacts to be discriminated. The flow rate was set at typical values of 300-600 bacterial cells per s (Ananta et al., 2005).

3.8.3 Recovery rates

The stability of the spray-dried cells was monitored in powdered chocolate product stored at 25°C and apple juice stored at 4°C over a period of 10 weeks and 14 days respectively. Samples were mixed separately with spray-dried trehalose powders containing probiotic cells *to* a final concentration of 10^9 CFU g^{-1} or mL^{-1}. The resultant samples were transferred into sterile plastic jars under aseptic conditions and stored at the above-stated temperatures. Samples were collected at intervals over a period of 10 weeks and 14 days, serially diluted in Ringer's solution and plated on MRS agar plates. Survivors were enumerated after 3-5 days of anaerobic incubation at 37°C in an anaerobic jar.

4 Results and Discussion

4.1 Effects of osmotic conditions of MRS- media on growth of bacterial strains

4.1.1 Osmotic strength of growth media

High osmolarity media prepared by additions of trehalose and sucrose to MRS broth at same molarity had similar osmotic strength (Figure 4. 1).

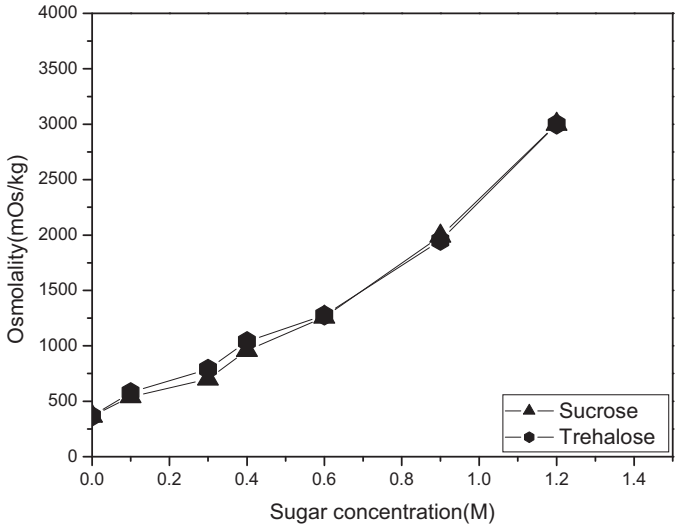


Figure 4.1: The osmotic strength of MRS-sucrose and MRS-trehalose growth media used in stress treatments of Lactobacillus rhamnosus strains.
Plots are representatives of the means of duplicate experiments.

In order to obtain equiosmolar conditions by an addition of trehalose and sucrose to MRS broth, the measurement of osmolarity was necessary rather than calculation from the number of particles in the solution. The amount of solutes added to a solvent does not usually produce a proportional increase in osmotic pressure due to interaction of the solutes with the solvent (Glaasker et al., 1998), which in the case of MRS is likely to be complex because several other solutes are also present. The osmotic strength of MRS broth before trehalose addition was 365mOs.$Kg^{-1}H_20$. The incorporation of trehalose and sucrose subsequently generated higher osmotic conditions (Figure 4. 1). The osmolarity of MRS medium containing 1.5M of these osmotic agents was greater than 3000 mOs.$Kg^{-1}H_20$. The influence of these conditions on the viability of the *Lactobacillus rhamnosus* strains were tested, since one of the alternative methods of improving the viability of probiotic cultures is the utilization of their stress adaptation.

Trehalose and sucrose are both non-reducing disaccharides having almost same mass and formula; they also serve as compatible solutes during osmotic stress. However, trehalose has mostly been reported to be involved in several physiological stress responses including osmotolerance and anhydrobiosis (Mardin and Crowe, 1975; Gadd et al., 1987; Larsen et al., 1987; Hounsa et al., 1998; Garcia de Castro and Tunnacliffe, 2000). Moreover on the ability to protect the structure and function of enzymes against thermal inactivation, trehalose is described to be more effective than other sugars because it has larger hydrated volume, for example, than sucrose and therefore can substitute more water molecules in the solution (Sola-Penna and Meyer-Fernandes, 1998).

Preliminary investigations were made to determine the growth kinetics of *L. rhamnosus* strains. The *Lactobacillus rhamnosus* strains, (*LGG)* and *L. rhamnosus E97800 (E800)* were cultivated in batch culture to determine the times taken to reach the exponential and stationary phases of growth. These were the required stages needed to determine the stress responses of an organism. Both strains reached the exponential phase with viable numbers of $1.8x10^7$ - $5.1x10^7$ at approximately 4h and the stationary phase, with viable numbers of $3.0x10^{9}$ - $3.4x10^9$ at about 12h.

4.1.2 Sub- lethal and lethal concentrations of osmotic agents

To determine the sublethal and lethal concentrations of osmotic agents on the growth of *L. rhamnosus GG* and *Lactobacillus rhamnosus E-97800 (E800),* measurement of cells growth was taken over a period of time. Figure 4.2 is a graphical representation of the inhibition of growth of these strains, which followed a similar pattern at increasing concentrations of trehalose and sucrose. It could be observed that at 12h of incubation, growth could hardly take place above 0.9M osmotic agents. Trehalose or sucrose at 0.6M was chosen as the sublethal concentration since at this level cell growth was still recorded and 1.5M was chosen as the lethal concentration because at this level no growth was recorded. Linders et al. (1997a) reported the inhibition of growth of *L. plantarum* by NaCl at increasing concentrations and a loss of growth above 1.4 mol l^{-1}. Uguen et al. (1999) also reported decreases in the growth rate and the growth yield of *L. lactis ADRIA 85L030* with the increasing osmolarity of the medium with NaCl, KCl and sucrose.

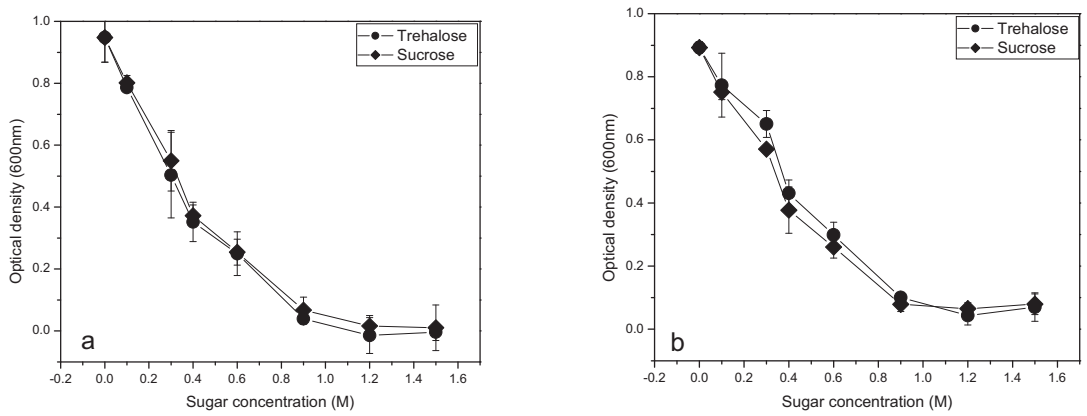


Figure 4.2: Growth of (a) L. rhamnosus GG and (b) L. rhamnosus E800 cultivated in high osmolarity media as monitored by optical density measurements.
Initial OD_{600} was 0.1 and growth was measured at 37°C within a period of 12h.

Growth and survival mechanisms could be the accumulation of these osmotic agents into bacterial cytoplasm in order to restore cells turgor pressure. Glaasker et al. (1998) reported that the cytoplasmic concentrations of lactose and sucrose in growing *Lactobacillus plantarum* cells are always similar to the concentrations in the medium. High concentrations of these sugars only impose transient osmotic stress because external and internal sugars equilibrate after some time by facilitated diffusion. Low levels of *Lactobacillus rhamnosus GG* growth in MRS medium containing different sugars was also reported by Corcoran et al. (2005).

4.2 Responses of exponential-phase cultures to osmotic stress induced by trehalose and sucrose

In order to reduce the acquired tolerances related to growth phases of these strains, cells at the exponential phase of growth were used further for stress treatments. The bacterial counts, represented as logarithmic value of relative survival fraction (log N/N_o), observed to decrease during osmotic treatments, are as shown in Figure 4.3 a, b.

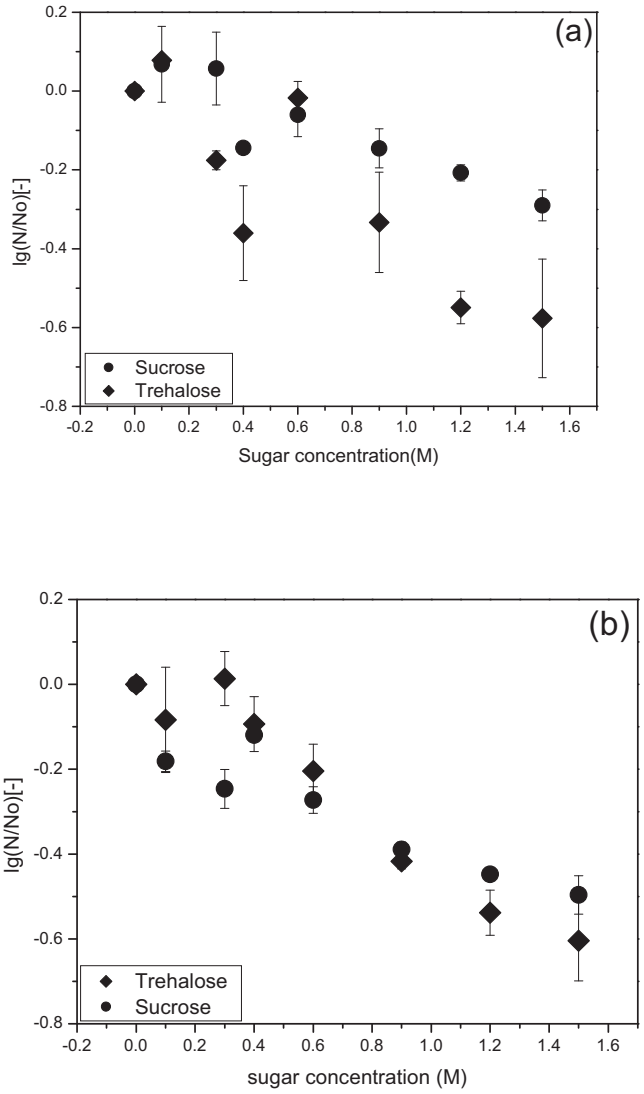


Figure 4.3: Effect of increasing sucrose and trehalose concentrations in MRS growth medium on the viability of (a) Lactobacillus rhamnosus GG and (b) Lactobacillus rhamnosus E800.
Cells were incubated at 37°C for 60 min. Data represent means of 3 independent experiments and standard deviations are indicated by error bars.

There was no significant difference ($p>0.05$) in the viability of non-treated and treated cells. In a similar way, as discussed under section 4.1, cells were able to adapt to the new environment and survive through an uptake coupled with accumulation of trehalose and sucrose. Similar results were obtained when stationary-phase cells were exposed to same treatments (Sunny-Roberts et al., 2007; Sunny-Roberts and Knorr, 2007; Sunny-Roberts and Knorr, 2008) but in some published works (Kim et al., 2001; Ananta, 2005) the viability of stationary-phase cultures following exposures to lethal levels of other stresses was significantly higher than that of cells in the exponential phase. However, there was no significant difference between the two phases for NaCl stress (Kim et al., 2000).

Subjection of exponential phase cells to 0.6M osmotic agents over a period of 60 min revealed a take-off in growth rate of cells, though insignificant, at 15-30 min of osmotic treatments (Figure 4.4 a, b).

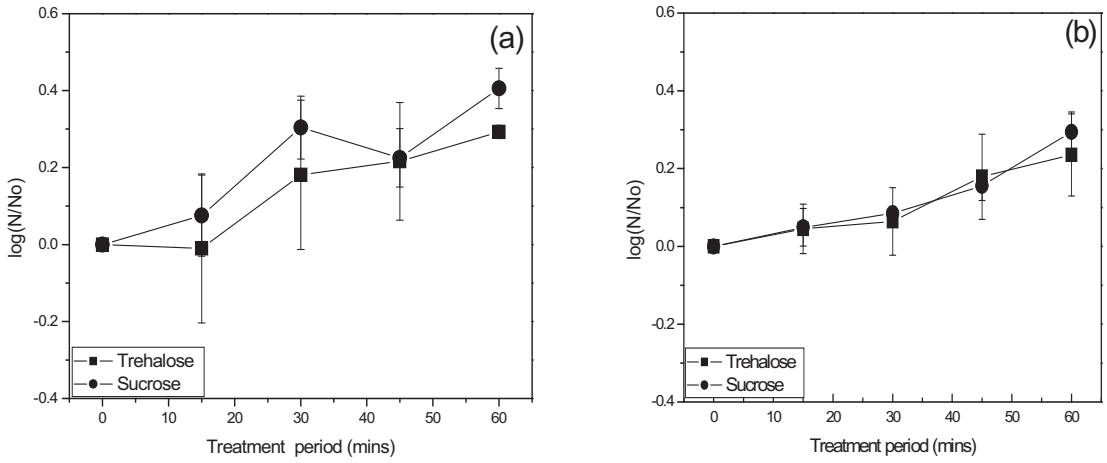


Figure 4.4: The growth kinetics of (a) L. rhamnosus GG and (b) L. rhamnosus 800 during osmotic pretreatment.
Equal volume of cells was inoculated into equal volume of 0.6M osmotic medium. Cells were incubated at 37°C for 60 min. Plots are means of triplicate experiments and standard deviations are indicated by error bars.

Thus, 30 min was taken as the minimum period of sub-lethal treatment at which cells must have established an adaptation mechanism to their environment. This was in agreement with the report of Glaasker et al. (1998). These authors reported that an uptake of glycine betaine (a component of MRS medium used in this study) was inhibited after ca. 12 mins by high concentrations of sugars in the bacterial cytoplasm.

4.2.1 Impact of non-ionic pretreatments on the viability of strains subjected to selected environmental stresses

Having established the duration required for pre-treatments, bacterial cells were further subjected to some environmental stresses, which they may encounter either during food processing or in human intestinal tracts.

Sucrose

There was no significant difference ($p>0.05$) in the response of 0.6M osmotic agents pretreated cells and non- treated cells in terms of culturability on MRS agar when exposed to a lethal concentration of sucrose (Figure 4. 5a, b).

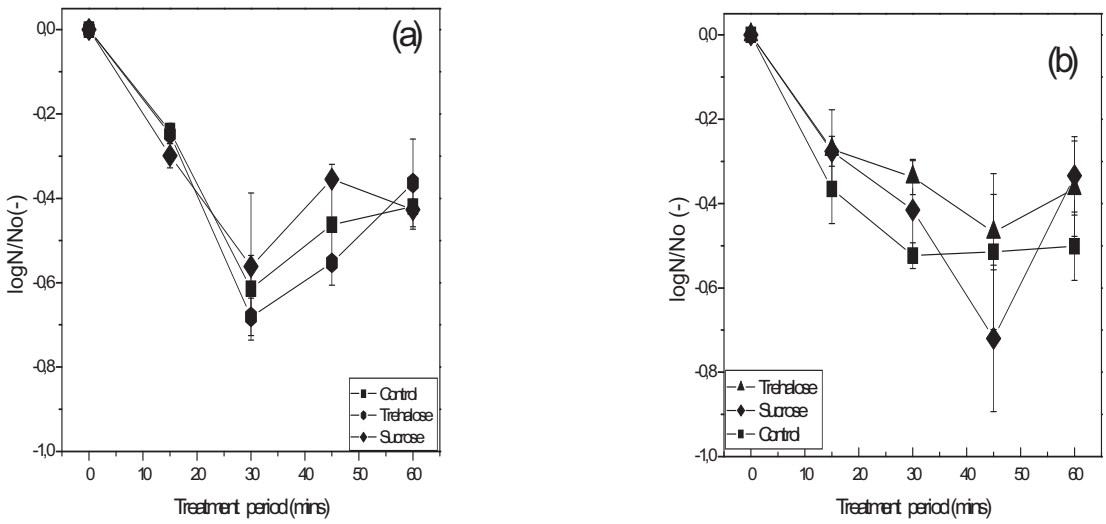


Figure 4.5: Survival of sub-lethal osmotic pre-treated and control (a) Lactobacillus rhamnosus GG and (b) Lactobacillus rhamnosus E800 cells during lethal osmotic (1.5M sucrose) treatment.
Pre-treatment was made separately at 0.6M sucrose and trehalose concentrations. Data represent means of at least two independent experiments.

It was also noted that for both treated and non-treated cells, it took almost 30 mins to adapt to the new environmental condition before they could resume growth. It could be possible that within the initial period, just as control cells, sucrose pre-treated cells and trehalose- pre-treated cells might try to take up sucrose from their new environment during the course of adaptation in order to maintain an osmotic balance; but *Lactobacillus plantarum* cells grown on glucose and fructose were not adapted to the uptake of other carbohydrates employed in drying processes (Poolman, 1993; Linders et al., 1997c). Therefore, non-ionic osmotic pre-treatment may not be necessary when these bacteria are to be employed in processing foods containing such amount of sugars. A market survey revealed the composition of food products such as jams, honey, cookies and candies to include at least 30% sucrose equivalent of 0.9M sucrose.

Heat

The effect of sub-lethal level of osmotic pre-treatment on the thermotolerance of the study strains was tested (Figure 4.6a, b) The lethal temperature of 60°C for heat challenge was adapted from studies made with other probiotic lactobacilli (Kim et al., 2001; Ananta and Knorr, 2003; Desmond et al., 2004) .

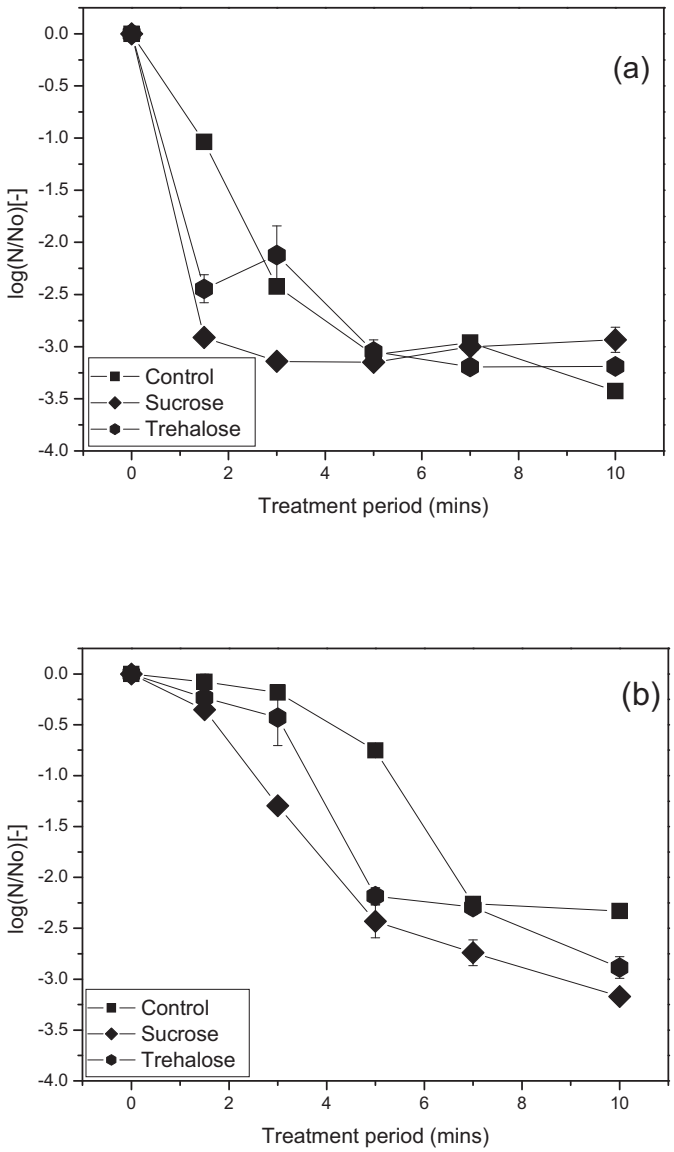


Figure 4.6: The viability of control cells and osmotic pre-treated cells of (a) Lactobacillus rhamnosus GG and (b) Lactobacillus rhamnosus E800 after exposure to heat treatment (60°C, 10 min).
Pre-treatment was made separately at 0.6M sucrose or trehalose concentrations for 45 min. Data represent means of at least two independent experiments.

Control cells of *L. rhamnosus E800* were more tolerant ($p<0.05$) to heat treatment than *LGG*. Drastic reductions in logarithmic value of bacterial counts within the first 4 min of heat treatment, especially in sucrose-treated *LGG* cells, were observed (Figure 4.6 a). Contrary results were observed in *L. rhamnosus E800;* its inherent thermotolerance must have played an important role. Generally, osmotic pre-treatment could not improve the heat tolerance of both strains. These results signify difficulties in improving/ maintaining these probiotics' stability and viability in the processing of sugar-based foods involving heat treatment, unless if they are separately preserved and added into the heat-processed final product. Gouesbet et al.

(2001) reported an increase in the thermotolerance of *L. delbrueckii ssp. bulgaricus* strains when subjected to a heat challenge of 65°C for 10 min in trehalose and sucrose solutions. Fay (1934) reported no increase in the thermal resistance of bacteria when heated in lactose solutions. Pre-treatment with NaCl has mostly been reported to give a good protection to bacterial cells against lethal heat treatment (Gouesbet et al., 2001; Kim et al., 2001; Desmond et al., 2001). There was an increase in the thermal resistance of *Lactobacillus bulgaricus* when heated in skim milk (Teixeira et al., 1994). Heat resistance experiments of bacteria were also performed in Ringers solution (Franz and von Holy, 1996). Based on the fact that different experimental parameters including study strains were used by different authors, comparative analysis of data was made difficult.

Bile salts

Results of experiments on bile salt stress are as shown in Figure 4.7a, b. An exposure of the exponential phase cultures to 1% bile salt reduced the viability of non-treated *L. rhamnosus GG* drastically. Contrarily non-treated *L. rhamnosus E800* exhibited a high tolerance to bile but there was a significant loss of tolerance, N/N_o~2log reduction, upon osmotic pre-treatment. The bile tolerance of LGG was significantly improved as a result of osmotic pre-treatment with trehalose. It could be assumed that pre-treatment with sucrose might produce a similar effect, but this may be verified.

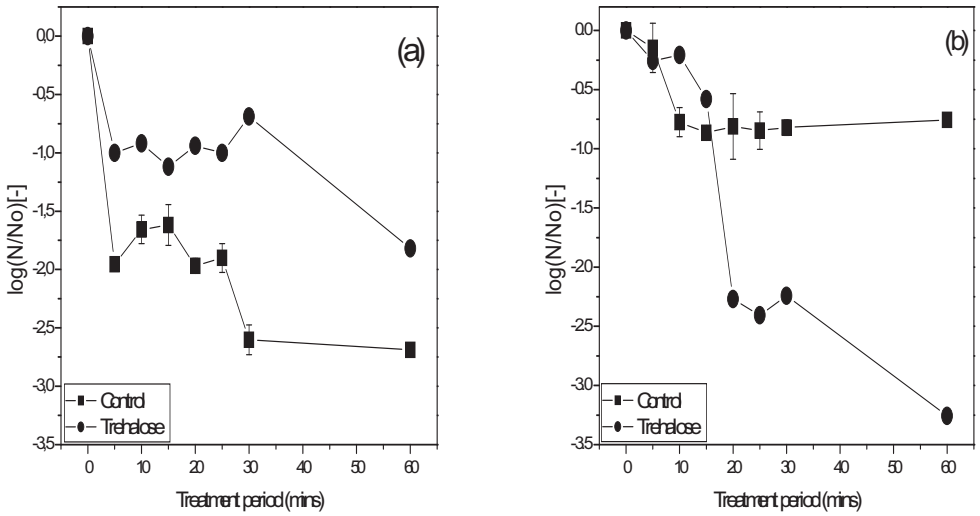


Figure 4.7: The viability of control cells and osmotic pre-treated cells of (a) Lactobacillus rhamnosus GG and (b) Lactobacillus rhamnosus E800 after exposure to bile salts treatment.
Data represent means of two independent experiments.

Lankaputhra and Shah (1995) reported that different strains of one species can have different levels of tolerance to bile. Pre-treatment of *L. acidophilus* cells with NaCl gave a protec-

tion to lethal bile treatment (Kim et al., 2001). Saarela et al. (2005) reported the tolerance of fresh cells of B. animalis ssp. lactis E-012010 to bile acids. Bile salts are highly toxic for micro-organisms. They act on membranes, exposing the bacterial periplasm and cytoplasm (Thanassi et al., 1997). Resistance to bile is recognized as an important feature of lactic acid bacteria as probiotics and in the human intestine, bile concentration is variable with maximum of 2%.

Bacterial Morphology

Bacterial cells grown under standard conditions exhibited one of the characteristic morphology (rod-shaped) of lactic acid bacteria {Fig 4.8a (i); 4.8b (i)}. Under electron microscopy, *LGG* cells exposed to 0.6M trehalose had same morphology as control cells but at 1.5M trehalose, there was a change in the morphology of the strain. Sucrose treated cells at 0.6M and 1.5M also exhibited changes in morphology. Moreover it was observed that structures similar to capsules, or slime layer, which could serve as an additional form of protection in such a high osmotic environment, were formed around the *LGG* cells (Figure 4.8a). Tolerance to harsh conditions can be due to an adaptive response which may include morphological changes.

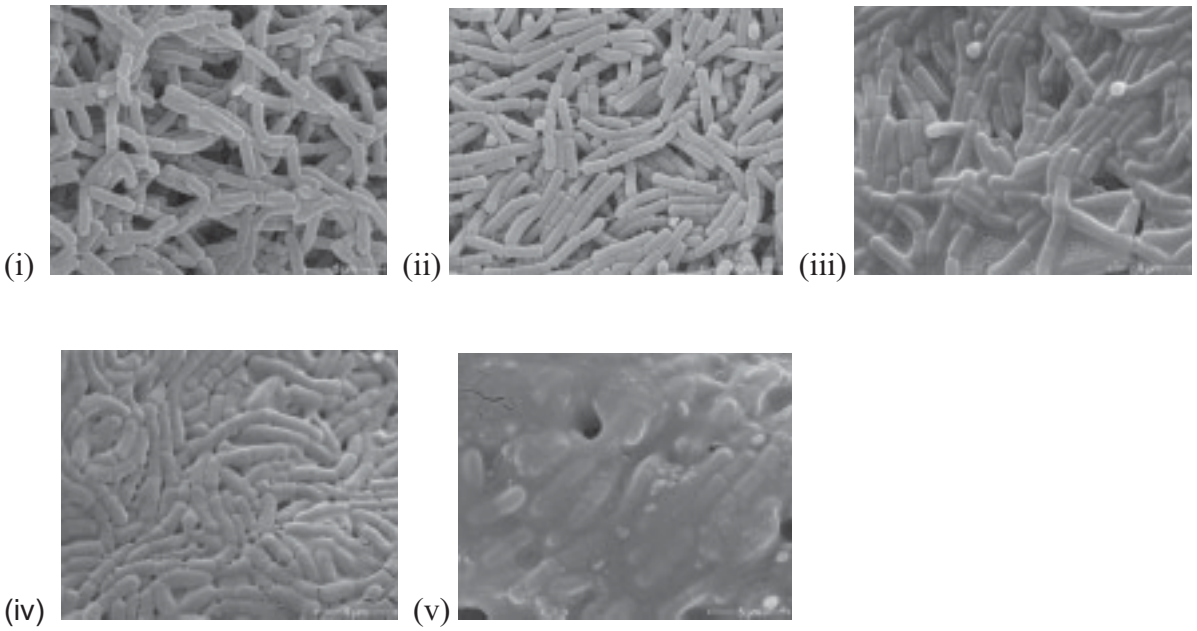


Figure 4.8 (a): Morphology of control cells of Lactobacillus rhamnosus GG (i) and during exposure to (ii) 0.6M trehalose (iii) 1.5 M trehalose (iv) 0.6M sucrose or (v) 1.5M sucrose.
Cells were incubated at 37°C for 60 min and examined by scanning electron microscopy. Bar = 5µm

These morphological changes were not exhibited by *L. rhamnosus E800* cells (Figure 4.8b). Irrespective of these, the viability of cells was not significantly reduced.

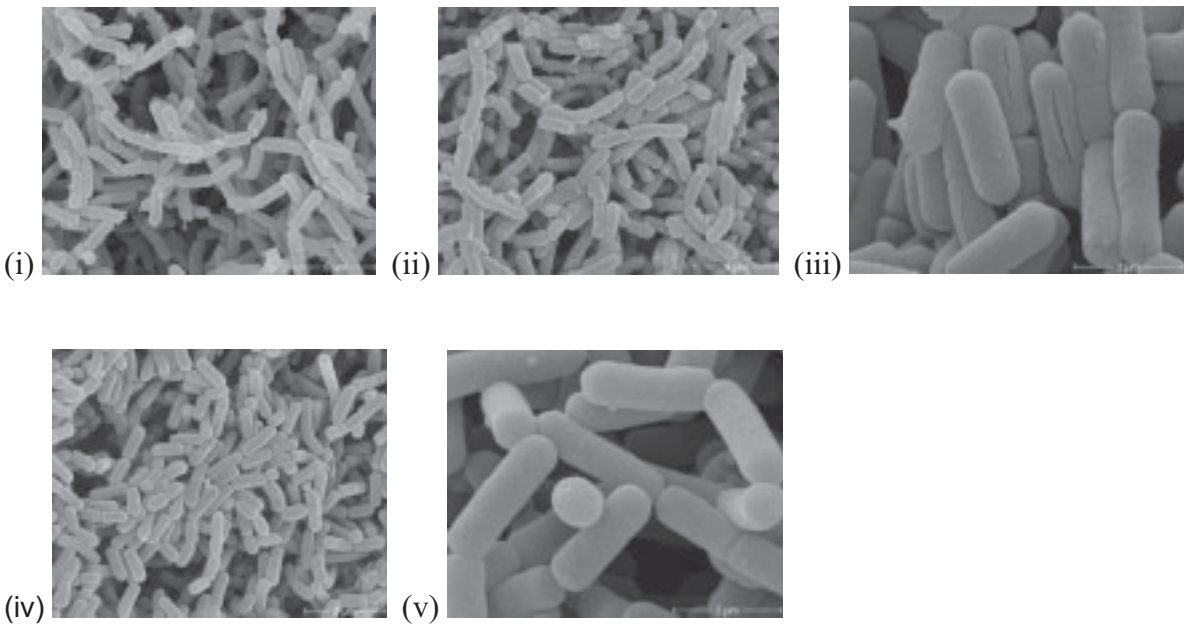


Figure 4.8 (b): Morphology of control cells (i) of Lactobacillus rhamnosus E800 and during exposure to (ii) 0.6M trehalose (iii) 1.5 M trehalose (iv) 0.6M sucrose or (v) 1.5M sucrose.
Cells were incubated at 37°C for 60 min and examined by scanning electron microscopy. Bar = 5μm (samples i ii and iv). Bar = 2μm- (samples iii and v).

Cells exposed to 1.5M trehalose exhibited another feature. This was shown in Figure 4.8b (iii). The crackings were injuries, which the cells were able to repair when cultured on fresh media; this was indicated by an insignificant reduction in cells' culturability on MRS medium (Figure 4.3b).

The morphology of osmotic pre-treated and control cells exposed to bile treatments (Figure 4.8 c, d) revealed that some control *LGG* cells experienced chains breakage after bile salts treatments. In contrast, adaptation to osmotic treatment before bile salts treatment did not cause notable changes in the morphology of LGG (Figure 4.8c). However, there were changes in the cell sizes. Control cells had an average length of 2.48 ± 0.40μm while trehalose pre-treated and sucrose pre-treated cells, exposed to bile treatments, were 1.92 ± 0.45μm and 1.95 ± 0.45μm in length respectively. On the other hand, trehalose pre-treated *L. rhamnosus E800* cells showed some cracked surfaces (Figure 4.8d, iii), which signified membrane damages. This could be a contributory factor to the significant loss in viability reported in Figure 4.7b. In this strain, cell sizes did not seem to decrease except in sucrose pre-treated cells. Control cells had an average length of 1.83 ± 0.45μm while trehalose or sucrose pre-treated cells exposed to bile salts challenge had 1.86 ± 0.36μm and 1.70 ± 0.39μm average length respectively. Decrease in cell length may be attributed to the effect of additional stress from bile salts which resulted into shrinkage or breaking of formed chains.

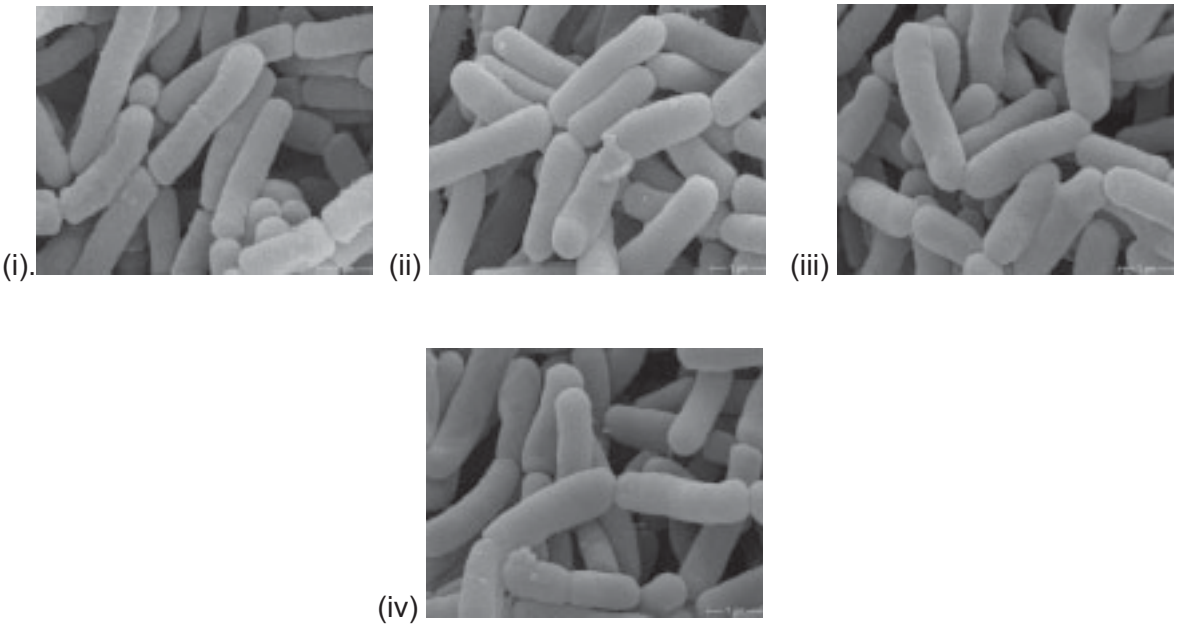


Figure 4.8 (c): Morphology of control cells of Lactobacillus rhamnosus GG (i) and during exposure to (ii) bile salts challenge [1%, 1 h] (iii) trehalose pre-treated cells[0.6M, 45 min] exposed to 1% bile salts challenge or (iv) sucrose pre-treated cells [0.6M, 45 min] exposed to 1% bile salts challenge.
Cells were incubated at 37°C and examined by scanning electron microscopy. Bar = 1µm.

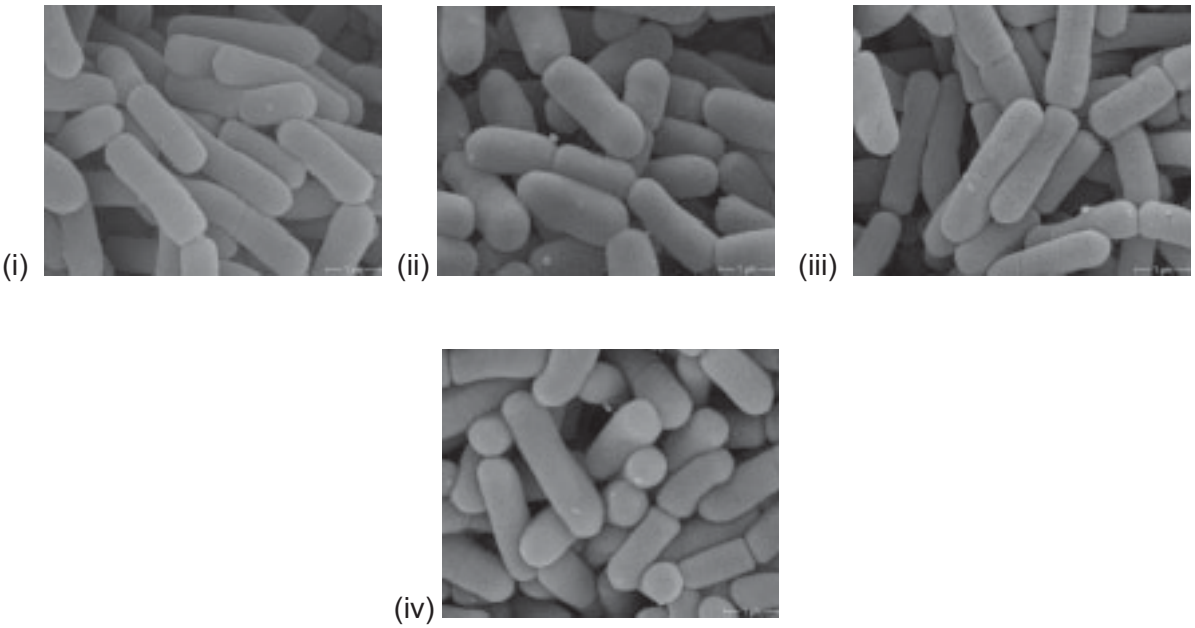


Figure 4.8 (d): Morphology of control cells of Lactobacillus rhamnosus E800 (i) and during exposure to (ii) bile salts challenge [1%, 1 h] (iii) trehalose pre-treated cells[0.6M, 45 min] exposed to 1% bile salts challenge or (iv) sucrose pre-treated cells [0.6M, 45 min] exposed to 1% bile salts challenge.
Cells were incubated at 37°C and examined by scanning electron microscopy. Bar = 1µm.

Shrinkage of bile stressed Escherichia coli was shown to result from the induction of the bolA morphogene belonging to the rpoS regulon (Hartke et al., 1998). But gram positive bacteria

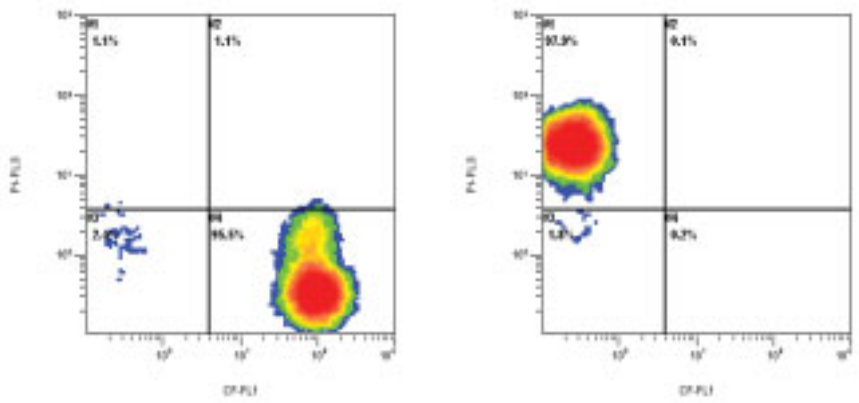
cannot plasmolyse because of the strong adhesion between the cytoplasmic membrane and peptidoglycan or very high internal osmotic pressure (turgor pressure of 15 to 25 atm) (Glaasker et al., 1998).

Leverrier et al. (2003) reported drastic changes in control cells' morphology after bile salts stress. This suggested leakage of intracellular material, which was detected by SDS-polyacrylamide gel electrophoresis analysis of the extracellular fraction. This phenomenon must have occurred greatly in *LGG* control cells considering its ~2.5 log reduction reported in Figure 4.7 a. Leakage of proteins and permeabilization of *L. acidophilus* were also observed (Noh and Gilliland, 1993; Zarate et al., 2000). These results illustrate, at the morphological level, that the observed notable changes might not neccesarily signify the protective effect of osmotic adaptation to bile salts treatments.

4.3 Fluorescence properties of control and osmotic-treated L. rhamnosus strains as indicated by cFDA/PI staining

4.3.1 Analysis of flow cytometric data

To resolve the fluorescence properties of the bacterial population measured by flow cytometer, the density plot analysis of green fluorescence (FL1) versus red fluorescence (FL3) were applied (Figure 4.9a, b). With this, the population was differentiated according to their fluorescence behaviours. Populations in #1 have their membranes compromised and they possess no esterase activity; those in #2 have their membranes minimally damaged and possess esterase activity; #3 possess no esterase activity and membranes are intact; #4 possess esterase activity and their membranes are intact.



(a) (b)

Figure 4.9: Basic fluorescence density plots (cF, green fluorescence vs PI, red fluorescence) of (a) intact and (b) heat treated cells of L. rhamnosus strain following staining with cFDA and PI.
Heat treatment at 95°C for 15 min was performed to yield dead, compromised cells. The population in each quadrant is shown in percentages.

The quadrants were set so that only viable cells with intact membranes were found in #4 (Figure 4.9a). This population actively accumulated cF and excluded PI, thereby exhibiting high green fluorescence and low red fluorescence. All bacterial strains were found in #4 before heat treatment (Figure 4.9a) but after heat treatment, there was membrane rupture and loss of cF accumulation capacity thus cells were not able to exclude PI. This population with membrane damage was encountered in #1 (Figure 4.9b).

4.3.2 Membrane integrity and esterase activity of trehalose treated and control cells

Bacterial populations were already differentiated into four quadrants (Figure 4.9).

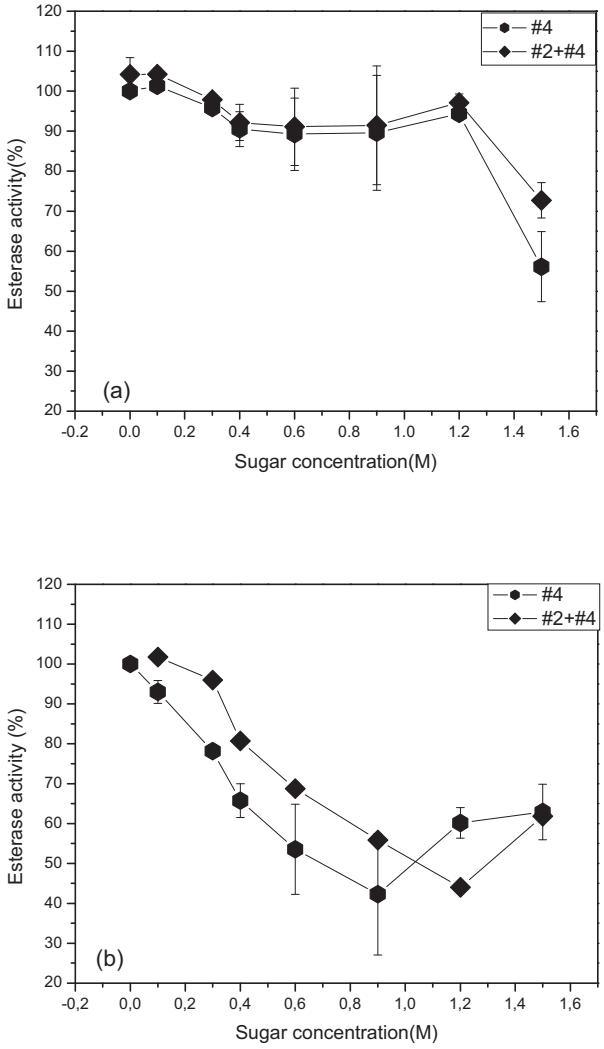


Figure 4.10: Graphical representation of the impact of trehalose treatment at 37°C for 30 min on esterase activity of (a) Lactobacillus rhamnosus GG and (b) Lactobacillus rhamnosus E800 at different molarities as derived from fluorescent density plots in Figure 4.11a, b.
Results were means of at least 3 independent trials.

Cells populations with high cF fluorescence and thus high green fluorescence are normally gated in #2 and #4. The presence of greater part of trehalose-treated population with such characteristics, similar to non-treated cells, was encountered in #4. Solely labelled cF cells were gated in #4 while some population double stained by cF and PI were gated in #2. Double stained cells indicated cell membrane damage which allowed the penetration of PI and the retention of cF. This unique population in #2 was observed following trehalose treatment beyond 1.2M. Thus residual esterase activity reduced significantly after 1.2M osmotic treatment in *LGG (Figure 4.10a).* In contrast, with increase in trehalose molarity, drastic and gradual reductions of esterase activity were recorded in *L. rhamnosus E800* (Figure 4.10b). This occurence was made explicit by FL1 and FL3 density plots.

FL1 -FL3 density plots showed the existence of greater percentage of non-treated cells in #4. These cells had intact membranes and possessed esterase activity but some of these cells moved to #3 (Figure 4.11a, b) when treated with trehalose. The presence of some populations in #3 could be taken to indicate either a loss of esterase activity or cF extrusion. Further study, conducted by the fluorescence measurement of bacterial supernatant, indicated that membranes were permeabilized thus allowing intracellular accumulated cF, molecular weight 376, to leak (unpublished). 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 suggested that the degree of permeabilization was low, thus signifying the ability of the cells to maintain their membrane integrity. It is proposed that trehalose lipid incorporates into phosphatidylcholine membranes and segregates within lateral domains, which may constitute membrane defects or pores through which the leakage of small solutes might take place (Zaragoza et al., 2009). The subjection of LGG to heat treatment at 60^{o}C for 300s also revealed the absence of PI labelling accompanied by increasing loss of cF accumulating activity. In this case, the occurrence of esterase inactivation was reported (Ananta, 2005). Bunthof (1999) observed a gradual decrease of intracellular cF in cF-labelled non-energized *Lactobacillus lactis* cells.

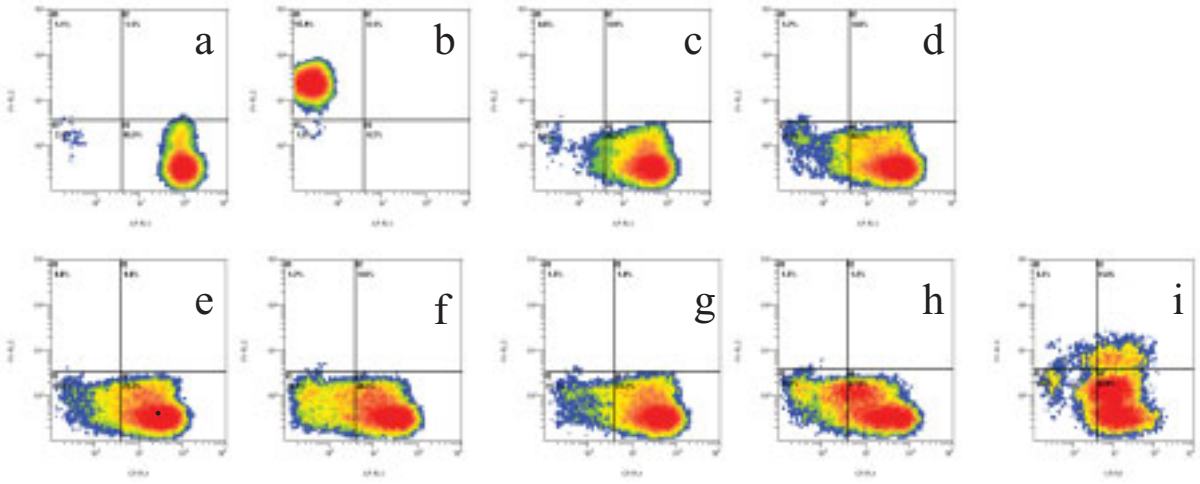


Figure 4.11(a): Flow cytometry density plots of FL1 vs FL3 of LGG for evaluating the impact of incubation in trehalose solution at different molarities on their membrane integrity and cF accumulation capacity. The bacteria were (a) non-treated, (b) treated at 95^{o}C for 15 min; incubated in trehalose 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 37^{o}C for 30 min.
The density plots shown are representatives of four or more replicative trials.

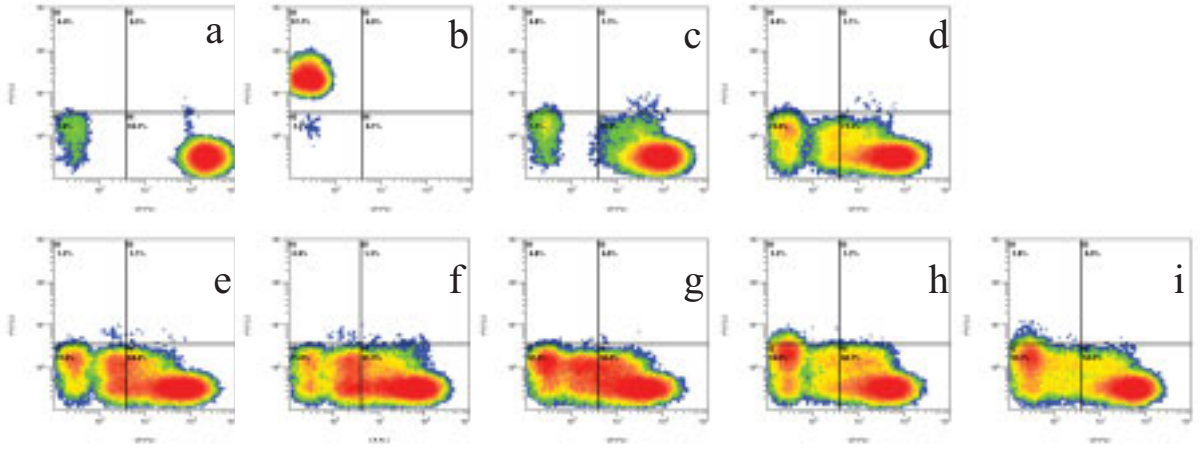


Figure 4.11(b): Flow cytometry density plots of FL1 vs FL3 of *Lactobacillus rhamnosus E800* for evaluating the impact of incubation in trehalose solution at different molarities on their membrane integrity and cF accumulation capacity. The bacteria were (a) non-treated, (b) treated at 95^{o}C for 15 min; 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 37^{o}C for 30 min.
The density plots shown are representatives of four or more replicative trials.

4.3.3 Membrane Integrity and esterase activity of sucrose treated and control cells

The percentage of cF-stained cells was also used to estimate the viability of sucrose stressed cells. Sucrose (0.1-1.2M) treated LGG cells possessed residual esterase activity as the control cells. This is graphically represented in Figure 4.12a and also shown in Figure 4.13a by the presence of a high percentage of the population in #2+#4 with high cF fluorescence and thus high green fluorescence. The cF accumulated cells were further separated

into two sub-populations: solely labelled cF cells in #4 and another sub-population in #2 where as a result of severe osmotic stress, part of the population was double stained by cF and PI. Insignificant percentages of cells were found in #2 at 0.1-1.2M whereas about 25% was encountered in #2 at 1.5M treatment.

As earlier mentioned the presence of such double-stained population, at 1.5M, indicated that the cell membranes were irreversibly damaged and penetration of PI into the cells was allowed but intracellular accumulated cF could still be retained. This could be regarded as an intermediate state of membrane damage. This physiological status was considered as a transient phase in the progressive change towards cell death. However, inactivation is not irreversible and double stained cells may recover (Bunthof, 2002). There was no leakage of cF out of the cells despite the perturbation of the cell membranes.

The calculation of the residual esterase activity based on the total percentage of population in #2+#4 showed that despite the membrane damage, cells were still able to accumulate cF thus signifying the preservation of membrane integrity and enzymatic activity. The cell membrane damages seemed not to be low since relatively big molecule PI, molecular weight 668g/mol, could get into the cells; however cF (molecular weight: 376g/mol) did not passively diffuse out of the cells. Compared to the original non-fluorogenic substrate cFDA (molecular weight: 460g/mol), which is moderately permeant to cell membrane, the presence of additional negative charges on cF at physiological pH may possibly inhibit its leakage out of the cells (Haughland, 2002). Major part of the population did not accumulate PI and were mostly encountered in #4 irrespective of the presence of a double stained population at this molarity (Figure 12a). The occurrence of a sub-population with this characteristic was also recorded in bile salt stressed bifidobacterium cells, ethanol stressed malolactic starter cultures, and high pressure inactivated LGG (Ben Amor et al., 2002; da Silveira et al., 2002; Ananta et al., 2004). Shapiro (2001) speculated that the occurrence of double stained population resulted from clumps containing bacteria with opposing physiological status.

Comparatively to sucrose-treated LGG cells' responses, all sucrose-treated *L. rhamnosus E800* cells had intact membranes and also possessed esterase activity (Figure 4.12b & 4.13b). This is well represented by the presence of a greater percentage of sucrose-treated population in #2+#4, in which cells with high cF fluorescence and thus high FL1-value were encountered (Figure 4.12b).

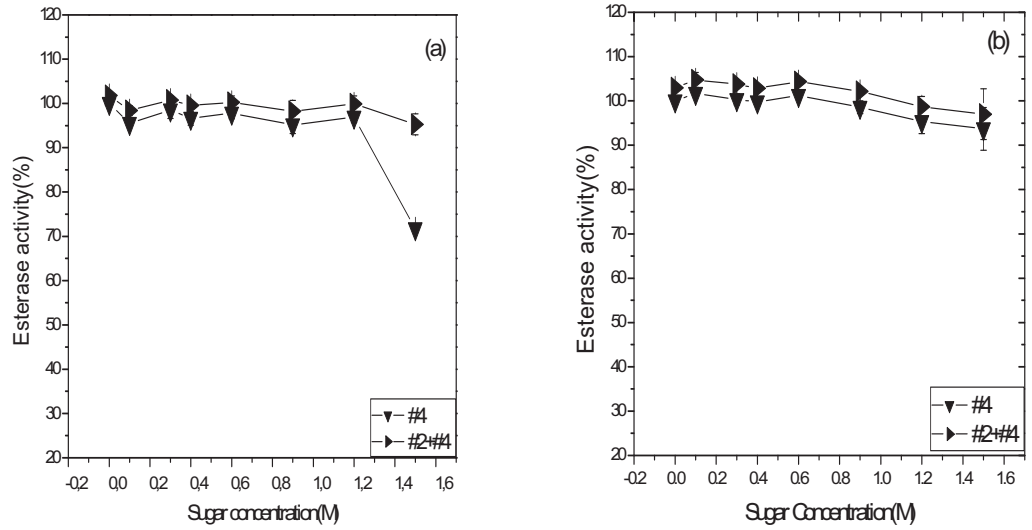


Figure 4.12: Impact of sucrose treatment at 37°C for 30 min on esterase activity of (a) Lactobacillus rhamnosus GG and (b) Lactobacillus rhamnosus E800 at different molarities as derived from fluorescent density plots in Figure 4.13a, b.
Results were means of at least 3 independent trials.

However, none of *L. rhamnosus E800* cells treated at 1.5M sucrose was encountered in #2. Absence of double-stained cells in #2 and some other populations in #3 revealed that none of the membranes of these cells were permeabilized (Figure 4.13b). This was in contrast to the transient membrane permeabilization observed in FL1-FL3 density plots when this strain was treated with trehalose (Sunny-Roberts and Knorr, 2007), having same osmotic strength as sucrose.

It could be inferred from these results that at high molarity trehalose solutions (1.5M) the membranes of LGG were well protected ,as shown by low degree of damages, than when treated in same molarity of sucrose solutions in which high degree of damages allowing the penetration of PI were recorded. On the contrary, cells of *L. rhamnosus E800* had their membranes permeabilized only in trehalose solutions.

The fact that intracellular fluorescein accumulation owing to enzyme-substrate reactions in cells exposed to cFDA is not energy-dependent is a major draw back in using esterase substrate to identify bacterial viability status (Davey et al., 1999). It was reported that cells killed by H_2O_2, heat and y-irradiation still possessed esterase activity and accumulated cF (Breeuwer et al., 1994; Bunthof et al, 1999; Vives-Rego et al., 2000). Pump activity was found to be a more sensitive indicator of cell stress since this activity ceases prior to electrical depolarisation (Nebe- von Caron et al., 2000).

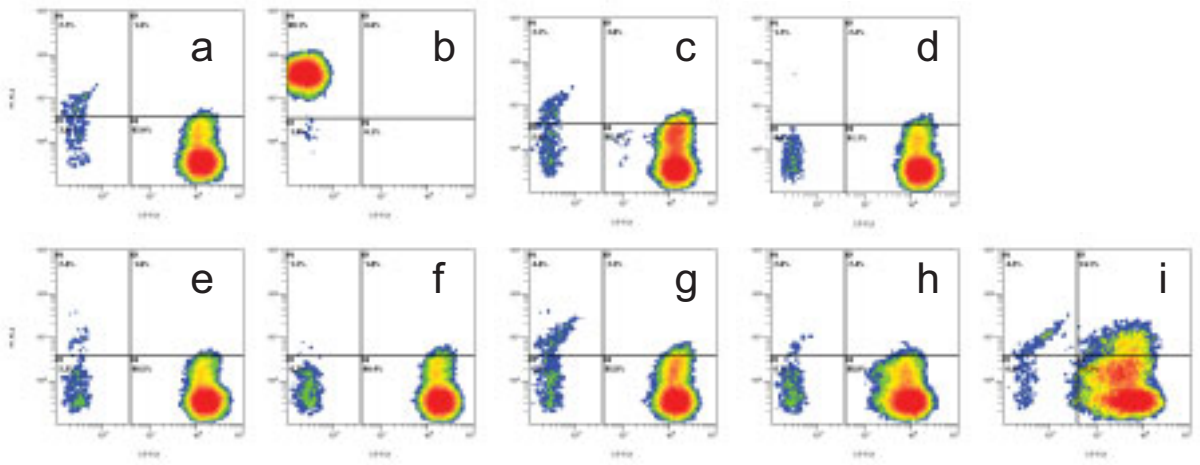


Figure 4.13(a): Flow cytometry density plots of FL1 vs FL3 of Lactobacillus rhamnosus GG for evaluating the impact of incubation in sucrose solution at different molarities on their membrane integrity & cF-accumulation capacity. The bacteria were (a) untreated, (b) heat treated at 95°C for 15 min, incubated in sucrose solution of (c) 0.1M, (d) 0.3 M, (e) 0.4 M, (f) 0.6 M, (g) 0.9 M, (h) 1.2 M or (i) 1.5 M at 37°C for 30 min.
The density plots shown are representative graphs for four or more replicative trials.

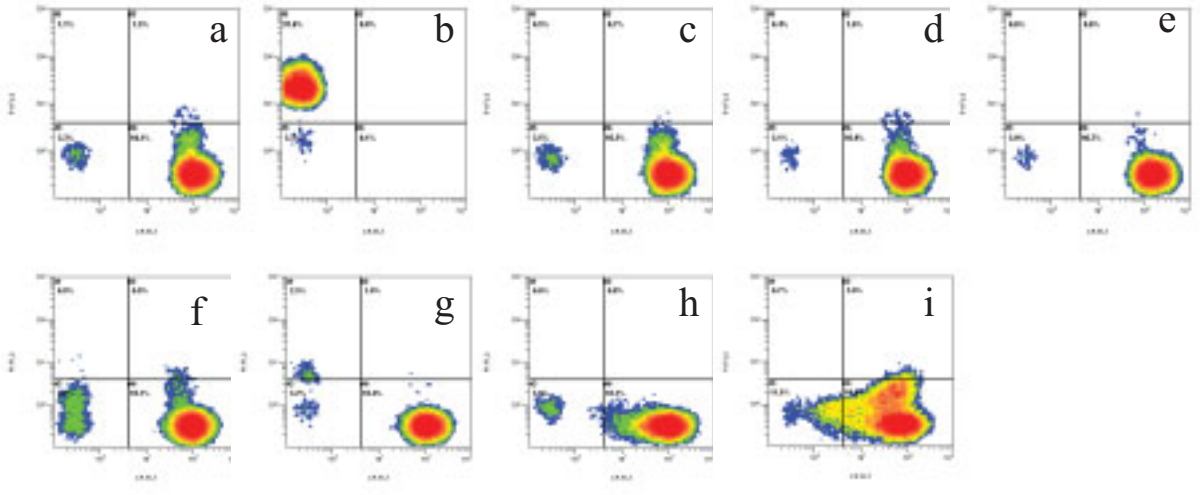


Figure 4.13(b): Flow cytometry density plots of FL1 vs FL3 of Lactobacillus rhamnosus E800 for evaluating the impact of incubation in sucrose solution at different molarities on their membrane integrity & cF-accumulation capacity. The bacteria were (a) untreated, (b) heat treated at 95°C for 15 min, incubated in sucrose solution of (c) 0.1M, (d) 0.3 M, (e) 0.4 M, (f) 0.6 M, (g) 0.9 M, (h) 1.2 M or (i) 1.5 M at 37°C for 30 min.
The density plots shown are representative graphs for four or more replicative trials.

4.4 Comparison of the viability status of cells' response to cFDA/PI labelling and standard culturability assay

The effect of different levels of osmotic treatments can be further explained and quantified by comparison of cF/PI uptake (Figure 4.10a,b &4.12a,b) with culture method on MRS agar (Figure 4.14a,b).

4.4.1 Osmotic stress induction by sucrose

Sucrose treated LGG cells maintained same level of culturability on MRS agar as the control cells at all molarities (Figure 4.14a) but when the cells were treated at 1.5M sucrose solution, the capacity of cells in accumulating cF (Figure 4.12a) was not found to be in correlation with the culturability on plates. This could be due to a repair of the permeabilized membranes during the cultivation of these cells on fresh media.

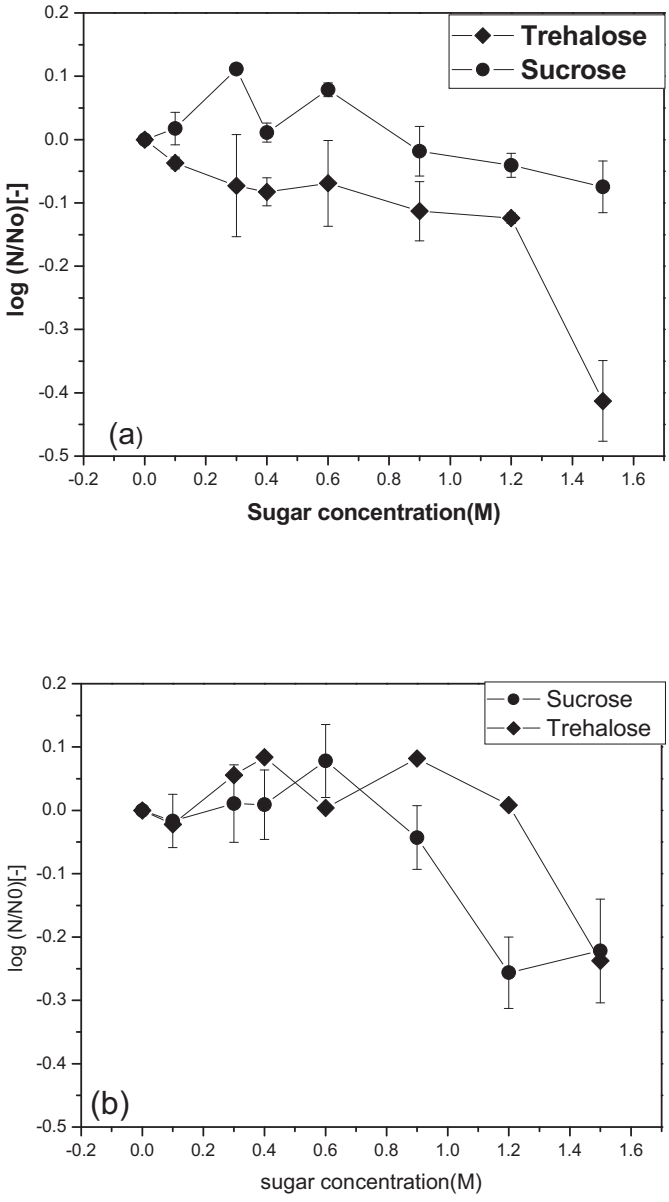


Figure 4.14: The impact of non-ionic treatment on the viability of (a) LGG and (b) Lactobacillus rhamnosus E800 as assessed by plate count method and exhibited as the logarithmic value of surviving cells (N/N_o).
Incubation was at 37°C for 48 h. Results are means of at least 3 independent experiments.

Although all sucrose-treated *L. rhamnosus E800* cells possessed similar cF accumulating capacity as the control cells (Figure 4.12b) , only the cells cultivated in the solutions having concentrations up to 0.9M exhibited same viability as the non-treated cells (Figure 4.14b) on MRS medium; although at higher osmotic conditions, 1.2M and 1.5M, culturability was slightly affected (log N/N_o ~ -0.3). These indicated that the retention of intracellular esterase might not be crucial in the maintenance of culturability. Bunthof et al. (1999) classified treatments that did not affect plate counts significantly as mild treatments. Therefore in this context, stress treatments by non-ionic agents may be considered to be mild. However, it should be noted that the growth phase of the micro-organisms subjected to stress treatments determines the type of exhibited response.

4.4.2 Osmotic stress induction by trehalose

Similar results, as in sucrose treatments, were obtained when the cells were subjected to stress by trehalose treatments. As reported earlier, the bacterial cells had their membranes permeabilised causing a leakage of cF out of the cells while PI was also excluded. Although this feature was most pronounced on *L. rhamnosus E800* (Figure 4.10b), culturability on MRS agar was not significantly affected until after 1.2M (Figure 4.14b). This showed a non-correlation between cF accumulating capacity and the reproduction capacity. Porter et al. (1995) reported such occurrence when *E.coli* cells were incubated at low temperatures; cells were viable but not culturable. There was a good correlation between the cF accumulating capacity and the reproduction capacity in trehalose-treated LGG cells (Figure 4.10a & Figure 4.14 a).

Since it was also revealed by the plate counts method that osmotic stress induced as a result of trehalose and sucrose treatments produced different effects on the viability of the test strains, these effects are, therefore, classified as being strain-specific.

4.5 Extrusion of intracellular accumulated dye in response to glucose addition

Bacteria have a very efficient efflux pump, which results in a rapid efflux of dye, thus hampering interaction with target molecule. Dye efflux serves as an additional measure of cell viability (Bunthof et al., 2000). Apart from the ability of cells to accumulate cF and exclude PI, the ability to extrude accumulated cF upon energizing using fermentable sugar could also be ascertained as an additional marker of vitality in order to study the mode of osmotic agents'

actions on bacteria. These combined methods could give more information about the physiological condition than cF labelling alone does (Bunthof et al., 1999).

This pump action is most likely mediated by an ATP-driven transport system because *L. lactis* cells treated with valinomycin and nigericin produced ATP upon addition of lactose despite the dissipation of proton motive force (Bunthof et al., 1999). Ananta (2005) reported that the dye extrusion was substrate specific since extrusion did not take place in *LGG* upon lactose addition.

4.5.1 Extrusion activity in L. rhamnosus GG

In FL1-FL3 density plot analysis, the extrusion ability of sugar-stressed cells upon glucose addition was determined as a result of the shift of initially stained population from #4 to #3 by intracellular fluorescence loss (Figure 4.15a,b). Graphical representation of this events showed that cells treated with sucrose up to 0.6M had a similar extrusion activity as the control samples (Figure 4.15c). Lower percentages of extrusion activity were recorded at 1.2M and 1.5M sucrose treatments. Cells treated with trehalose up to 0.9M had comparable extrusion ability as the control. Results of the plate counts (Figure 4.14a) correlated well with the extrusion activity only by trehalose treatments.

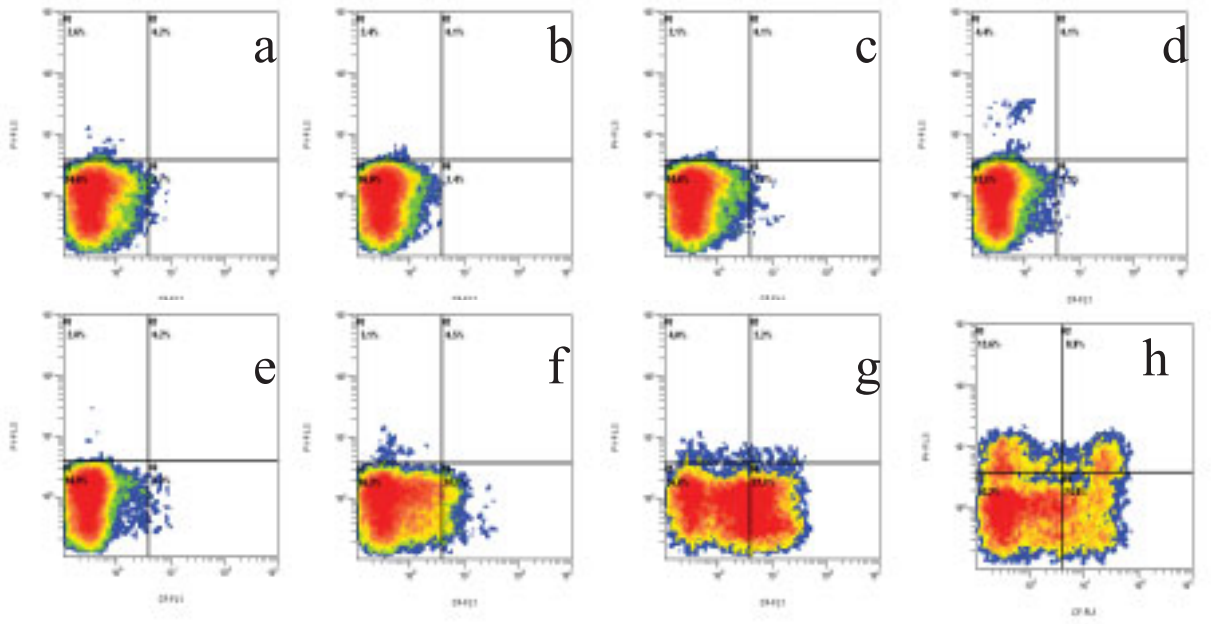


Figure 4.15(a): Flow cytometry density plots of FL1 vs. FL3 of Lactobacillus rhamnosus GG to assess the impact of incubation in sucrose at different molarities on their cF-extrusion activity. The bacteria were (a)untreated; incubated in sucrose solution of (b) 0.1M (c) 0.3M (d) 0.4M (e) 0.6M (f) 0.9M (g) 1.2M or (h) 1.5M at 37^{o}C for 30 min.
The density plots shown are representatives of four or more replicative trials.

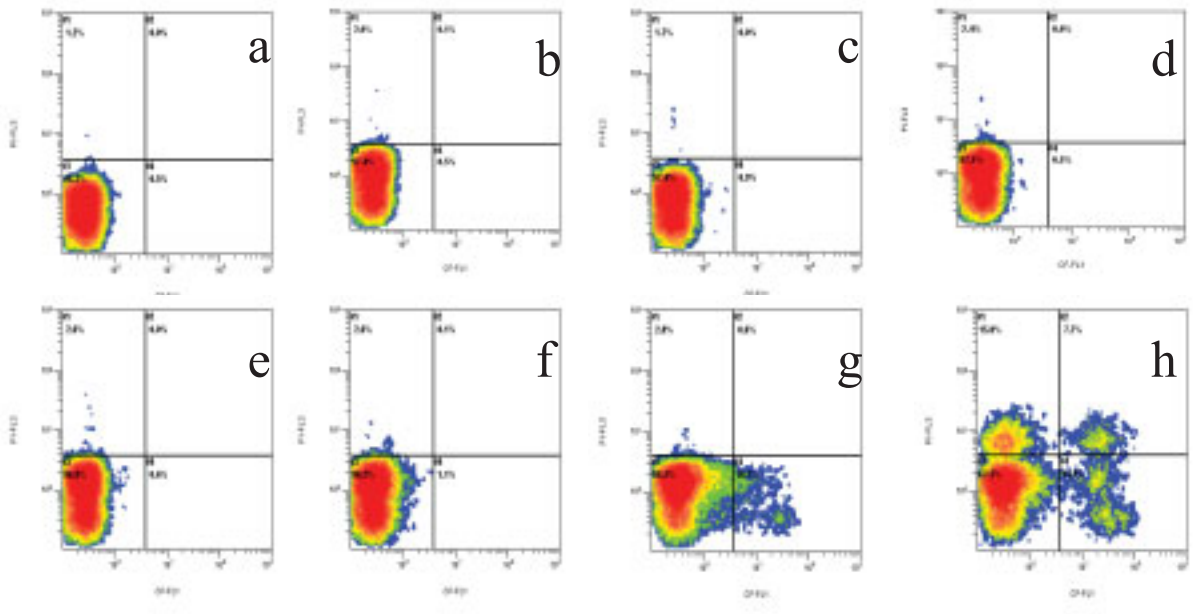


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Figure 4.15(b): Flow cytometry density plots of FL3 vs. FL3 of Lactobacillus rhamnosus GG. to assess the impact of incubation in trehalose at different molarities on their cF-extrusion activity. The bacteria were (a)untreated; incubated in trehalose 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^{o}C for 30 min.
The density plots shown are representatives of four or more replicative trials.

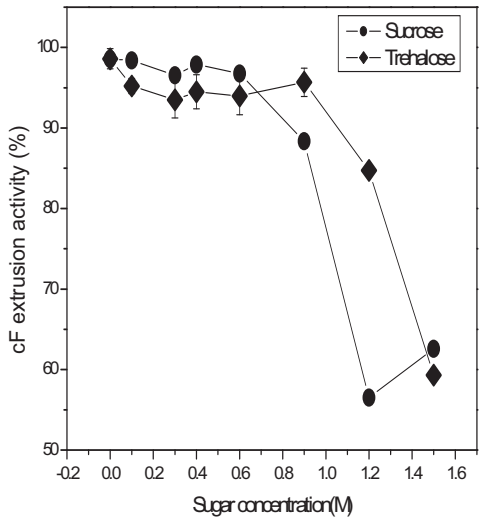


Figure 4.15(c): Graphical representation of cF-extrusion activity of LGG, as affected by osmotic treatments.
These results were determined by the fluorescence properties of population in #4 as shown in Fig. 4.15a&b. Results were means of at least 3 independent experiments.

4.5.2 Extrusion activity in L. rhamnosus E800

FL1-FL3 density plot analysis showed the extrusion of intracellular cF upon energizing with 20mM glucose. This is also represented by the shift of population in #4 to #3 (Figure 4.16a,b) due to fluorescence loss. Some bacteria were reported to complete extrusion of accumulated

dye in 20 min (Bunthof, 1999; Ananta et al., 2004; Sunny-Roberts et al., 2007)), but it took the non-treated strain 40 min to complete cF extrusion. Although some of these authors prepared cells suspensions in buffer while Ringers solution was used in this study, nevertheless, the duration of extrusion could be described as being strain-specific. Therefore the extent of population shift after 40 min incubation in the presence of glucose determined the degree of injury on this cellular pump. The extrusion of intracellular cF upon energizing with 20mM glucose for 40 min was as shown by FL1-FL3 density plot (Figure 4.16a, b). Some percentages of the initially cF- stained populations in #4 became unstained upon glucose addition and thus differentiated in #3. Cells treated in 1.2M and 1.5M sucrose solutions could not completely extrude accumulated cF in 40 mins (Figure 4.16a). An extension of the incubation period up to 90 min showed that there was no decrease in the percentage population in #4 (data not shown), suggesting that there was an occurrence of irreversible injury on this dye extruding system which affected the reproduction capacity of the bacterial strain (Figure 4.14b). CF-efflux activity and culturability seemed to be strongly correlated at these sugar molarities. Graphical representation of this density plot showed that only cells treated with 1.2M and 1.5M sucrose had a strong perturbation of extrusion activity (Figure 4.16c). There was no significant record of the perturbation of the extrusion activity in trehalose-treated cells (Fig 4.16b,c) but culturability was affected at 1.5M (Figure 4.14b). Trehalose treatment produced an improved cells' pump activity.

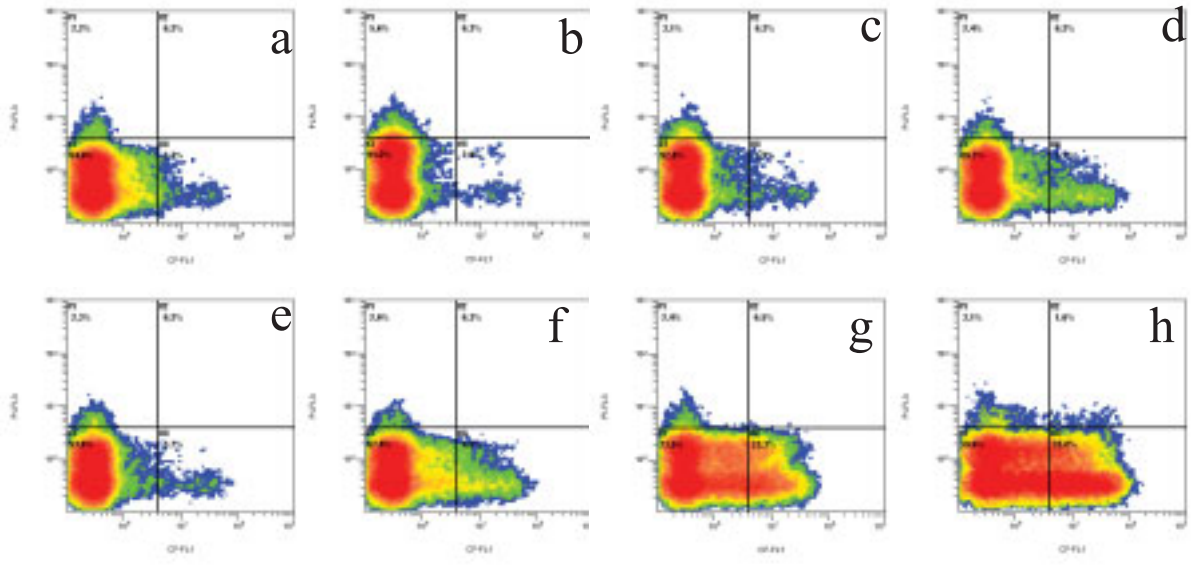


Figure 4.16(a): Flow cytometry density plots of FL1 vs. FL3 of Lactobacillus rhamnosus E800 in assessing the impact of incubation in sucrose at different molarities on their cF-extrusion activity. The bacteria were (a)untreated; incubated in sucrose solution of (b) 0.1M (c) 0.3M (d) 0.4M (e) 0.6M (f) 0.9M (g) 1.2M or (h) 1.5M at 37^{o}C for 30 min.
The density plots shown are representatives of four or more replicative trials.

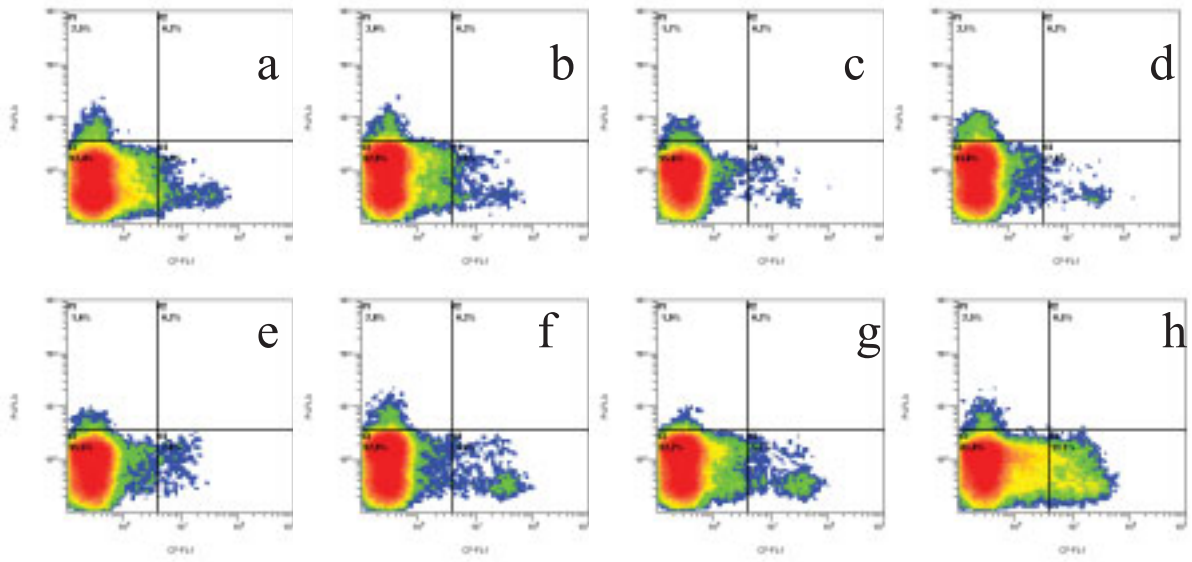


Figure 4.16(b): Flow cytometry density plots of FL1 vs. FL3 of Lactobacillus rhamnosus E800 to assess the impact of incubation in trehalose at different molarities on their cF-extrusion activity. The bacteria were (a)untreated; incubated in trehalose solution of (b) 0.1M (c) 0.3M (d) 0.4M (e) 0.6M (f) 0.9M (g) 1.2M or (h) 1.5M at 37^{o}C for 30 min.
The density plots shown are representatives of four or more replicative trials.

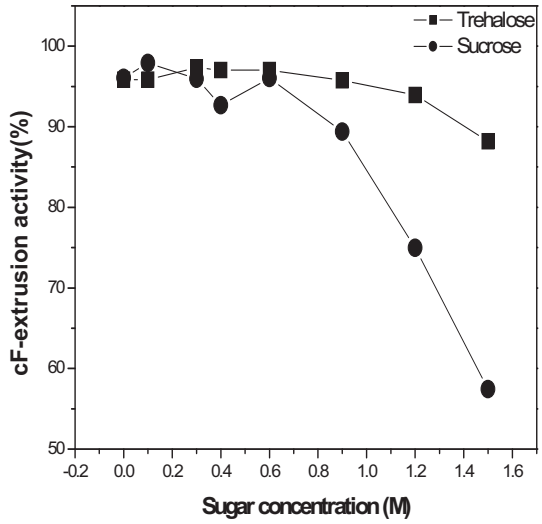


Figure 4.16(c): Graphical representation of cF-extrusion activity of Lactobacillus rhamnosus E800 as affected by osmotic treatments.
These results were determined by the fluorescence properties of population in # 4 as shown in Fig. 4.16a&b. Results were means of 2 independent experiments.

4.5.3 Kinetic measurements of cF-extrusion rates

As indicated earlier, LGG cells subjected to 0.6M of the osmotic agents, sucrose and trehalose, exhibited no significant difference ($p>0.05$) with the control samples in respect to the reproductive capacity, esterase activity and cF extrusion performance. Therefore, osmotically treated cells at 0.6M were further used in kinetic measurements. The kinetics of the movement of cF-stained cells from #4 to #3 showed that both the control cells and sucrose-treated cells had similar extrusion rate (Figure 4.17a) although in the first 10 min, 20% sucrose-

treated cells were seen to have extruded intracellular accumulated dye. Both sets of cells completed extrusion in 20min.This contradict the report of Ananta et al. (2004) on extrusion rate of non-treated LGG cells. This variability could be due to loss or gain of plasmid leading to inconsistency in the metabolic traits or activity. Cells treated in 0.6M trehalose solution had a higher extrusion rate than both the control cells and sucrose treated cells. In the first 10 min, 80% cells population had moved from #4 to #3, this eventually resulted in extrusion activity being completed in 15 min. The improved pump activity given by trehalose treatment must have accounted for the ability of pre-treated LGG cells, over the control cells, in tolerating bile salts as seen in Figure 4.7a. Thanassi et al. (1997) reported that the tolerance of enteric bacteria, such as *Escherichia coli,* to bile salts was as a result of an active efflux mechanism that is responsible for the efflux of bile salts.

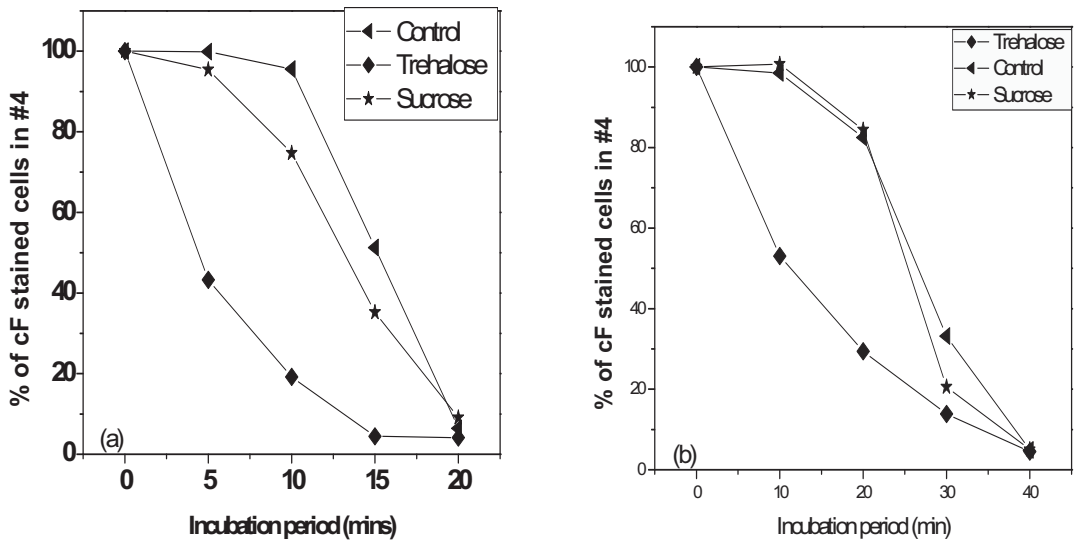


Figure 4.17: Kinetics of cF extrusion from (a) L. rhamnosus GG cells and (b) L. rhamnosus E800 cells represented by the decrease in the percentage of cF-stained population in #4.

Kinetic measurements of the rate of cF extrusion by *L. rhamnosus E800* cells showed that 0.6M sucrose-treated cells exhibited same extrusion rate as the non-treated cells (Figure 4.17b). Though trehalose improved the pump activity more than sucrose, but completion of cF extrusion was made at same time as other cells (Figure 4.17b). The longer period taken in completing pump activity besides the impact of osmotic stress may account for the low tolerance level of trehalose pre-treated cells to bile salts (Figure 4.7b).

The fact that efflux of fluorescence compound can give information about the crucial transport mechanism located in membranes of cells of lactic acid bacteria has been reported in literature (Bunthof et al., 2000; Ulmer et al., 2000; Ananta and Knorr, 2004; Sunny-Roberts and Knorr, 2007). Although sucrose was not as effective as trehalose in improving the pump

activity of these strains, nevertheless, sucrose treatment up to 0.9M may not subject the probiotics to the actions of toxic compounds or drugs e.g. antibiotics, bile etc.

For comparative purposes, probiotics were dried using two different drying equipments- vacuum oven and spray dryer. Although spray dryer is the most widely used, drying was also conducted in vacuum oven considering the economic situations in developing countries: based on its inexpensive costs, its ease of application (not sophisticated) and moreover, it could be fabricated locally (if not affordable) in the course of probiotics' research interests.

4.6 Survival rates of probiotic cells after drying in vacuum oven

4.6.1 Cells in the Exponential phase

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 62±5.9% of the cells survived the drying process in trehalose while < 1% survivor was found in sucrose and lactose. *L. rhamnosus E800* vacuum-dried in trehalose medium recorded 63±9.3% survivors in 24h. Extending the drying period to 48h showed a decrease in the survival rates of *LGG* and *L. rhamnosus E800* cells to 10±0.1% and 13±2.1% respectively. This could be due to a prolonged exposure to osmotic stress due to slow drying process. 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., 1997c). 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.
Meng et al. (2008) reported that sugars are likely to form highly viscous glasses at room temperature when they are dehydrated, and the improved storage of anhydrobiotes and liposomes has been associated with the presence of a glassy state. In this study, trehalose and sucrose samples were found as viscous syrups irrespective of the period of drying. Vacuum drying of enzyme solution stored at 37°C 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 suggested that the dried systems, in this study, may not be in the glassy state. Therefore the protective ability of tre-

halose could be explained as a result of the hydroxyl groups of the sugar making a direct interaction with the polar residues of the membrane 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), which could have accounted for the minimal survival rates of the *L. rhamnosus* strains. 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).

Some sucrose and lactose samples were also found in crystalline forms. These forms would not protect the bacterial cells against dehydration because upon crystallization these sugars would not be associated with the bacterial membranes thus the bacterial cells would be exposed to dehydration-related damages. 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 (Daemen and van der Stege, 1982; Abadias et al., 2001; Ananta, 2005). Skim milk protein can prevent cellular injury by stabilizing cell membrane constituents (Castro et al., 1995) and form a protective coating on the cell wall proteins, while calcium in milk increases survival after dehydration (King and Su, 1993).

4.6.2 Cells in the Stationary phase

Cells in the stationary phase of growth were 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 during drying (Table 4. 1). The improved tolerance to drying signified that cells of *L. rhamnosus GG and L. rhamnosus E800* must have been able to take up trehalose during the first few hours of drying period when they remained as solutions, in order to protect the functional proteins embedded in the bacterial membranes besides the membranes. The presence of sugars delayed the drying of cells, thus cells were subjected to a longer period of drying time than control cells, and in which no solutes were incorporated hence drying occurred at a faster rate.

Although there was no significant difference between the survivals rates of *Lactobacillus* strains dried in 20% trehalose but due to obtained lower values, samples dried in 30% treha-

lose were chosen for further analysis. The survival rates of control LGG was 2.70 x 10^6 cfu mL $^{-1}$ while that of *L. rhamnosus E800* was 2.20 x 10^6 cfu mL $^{-1}$. The improved survival rates of both organisms in trehalose matrices are as shown in Table 4.1.

Table 4.1: The impact of trehalose concentration during drying of LGG and L. rhamnosus E800 under vacuum on the % surviving cells (N/N_o) on MRS agar and % viable cells encountered in 'LIVE' gates.

Parameters	%Trehalose (w/w)					
	20		25		30	
	LGG	L. rhm E800	LGG	L. rhm E800	LGG	L. rhm E800
% surviving cells (N/N_o)	1.03±0.2^g	0.80±0.02^g	7.46±0.6^f	1.40±0.2^g	16.58±3.5^e	13.20±1.6^e
Survival rates (cfu mL^{-1})	2.80x 10^7	2.85x10^7	1.87x10^8	4.80x10^7	4.20x10^8	2.95x10^8
*% SYTO9 stained cells indicated by 'LIVE' gate	59.55±8.7^d	86.10±1.3^b	57.00±5.0^d	91.50±0.7^a	73.70±5.1^c	92.25±0.8^a

An initial cell count of bacterial solution was ~10^9 cfu mL^{-1}. Data are means of 3 drying experiments. Values with same letters on the superscripts are not significantly different from one another, but different letters show significant difference ($p \leq 0.05$).
*The FL1-FL3 density plots are as shown in Fig 4.18a, b.

Increased sensitivity of sublethally injured bacteria to NaCl has been associated with cell membrane damage (Gardiner et al., 2000). As observed in this study, there were no significant differences prior to drying between the colony counts of these strains on MRS agar containing 5% NaCl and MRS alone, but after drying cells' numbers decreased in the presence of NaCl. Almost 60% of *LGG* cells exhibited sensitivity to NaCl while 20% *L. rhamnosus E800* were sensitive. This indicated that cell membrane damages must have occurred and made more extensive in *LGG* 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., 1997c). Colony sizes were also reduced (data not shown). Low sensitivity of

L. rhamnosus E800 to NaCl but low survival rate indicated the possibilities of damages to other cell wall components which are vital for reproductive growth.

4.6.3 Flow cytometric analysis of bacteria dried under vacuum

Flow cytometric analysis was also applied to evaluate the cellular injury sites affected by the drying method and also to relate data on the survival of bacteria obtained by this analysis with plate count data. The bacterial cells were stained with SYTO9 and PI and the onset of cellular damage was identified by characteristic population shift from gate 'LIVE', where cells with intact membranes were predominantly located, towards gate 'DEAD', in which cells with completely disintegrated membranes were encountered.

The flow cytometric measurements of osmotically treated cells showed the presence of majority of fresh cells in 'LIVE' gates (Figure 4.18 A, B). After drying, some of these cells moved towards the 'DEAD' gates. This was an indication that protection provided by compatible solute, such as trehalose, to cells during osmotic stress differs from the one given when cells are subjected to drying processes; which bring much reduction in available cell water.

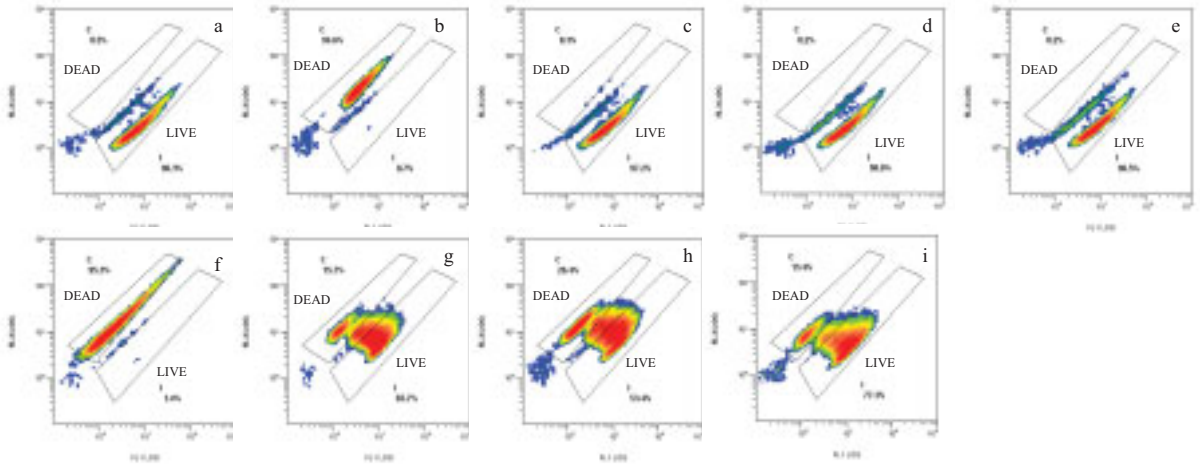


Figure 4.18 (A): Flow cytometry density plots of FL1 (fluorescence collected at 525nm) vs FL3 (fluoresecence collected at 620nm) of Lactobacillus rhamnosus GG to assess the impact of drying on the integrity of cellular membranes of stationary phase cells.
Two gates were fixed for discrimination of the 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^{o}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.

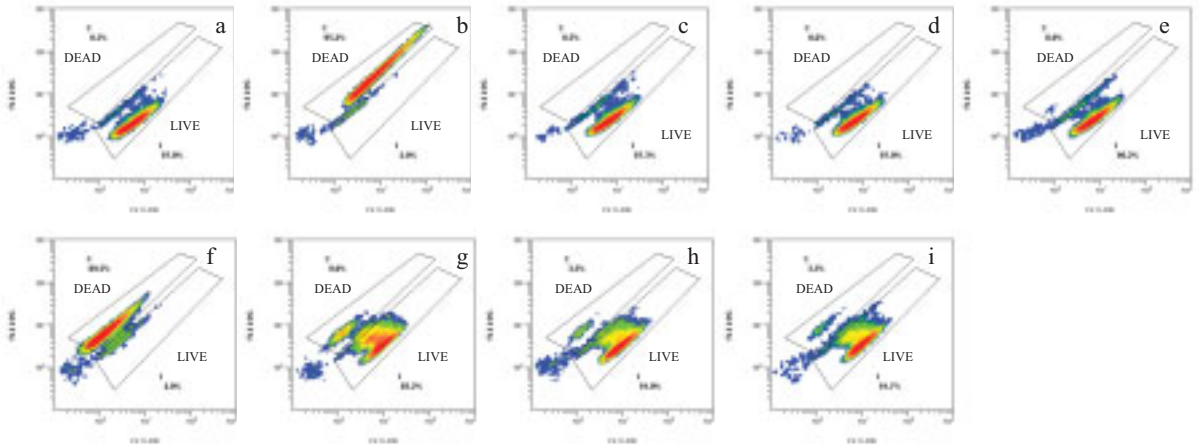


Figure 4.18 (B): Flow cytometry density plots of FL1 (fluorescence collected at 525nm) vs FL3 (fluoresecence collected at 620nm) of L. rhamnosus E800 to assess the impact of drying on the integrity of cellular membranes of stationary phase cells.
Two gates were fixed for discrimination of the two extreme 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^{o}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.

The results of flow cytometric measurements differ significantly ($p<0.05$) from the values obtained by plate counts as revealed in Table 4.1& Figure 4.18 A,B. 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. 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). The presence of such non-culturable bacteria with a high degree of membrane intactness in food is relevant since the subsequent storage conditions determine the recovery or complete inactivation of this fraction. From another point of view, 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.
In contrast, the flow cytometric density plots of cells at the exponential phase revealed a lower percentage of viable *LGG* cells after drying in trehalose medium to be 25%, compared to 65% culturable cells earlier reported (Section 4.6.1) on MRS agar plates. This difference could be as a result of the ability of some cells found in 'DEAD' gates, which might have been sublethally injured, 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. On the contrary, 80% *L. rhamnosus E800* cells were found at the 'LIVE' gate but not all were culturable. Similar observations were made on cells in sucrose matrices but in lactose matrices, >90% cells were gated 'DEAD' and correspondingly <1% cells were culturable. 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.
The weight of vacuum dried samples was estimated to be about 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.

4.6.4 Bacterial tolerances to lysozyme, pepsin, acid and bile

Bacterial cells were treated with some selective agents in order to evaluate the sites of cellular injury as a result of drying process. There was no significant difference in the tolerance of individual strain to different concentrations of lysozyme (1mg/ml or 5mg/ml) and pepsin (1mg/ml or 5mg/ml). *LGG cells* showed log reductions of ~ 0.4 to lysozyme after drying. This sensitivity, though minimal, could be a manifestation of damage to the cell wall components. Log reduction of ~ 0.85 was observed with pepsin treatment. *L. rhamnosus E800 cells,* on the other hand, exhibited ~ 1log reduction to lysozyme treatment. This is an indication of a severe damage to cell wall components besides cell membrane; which must have contributed to a low survival rate during drying process. The components of gram positive bacterial cell wall include peptidoglycan, teichoic acid and teichuronic acid found in considerable quantities. Polysaccharides are also found in considerable amounts in certain species of gram positive bacteria. Lysozyme is an important component in the prevention of growth in foods of animal origin (Hughey and Johnson, 1987). It can have applications as a preservative in foods that do not naturally possess it. This enzyme has bacteriostatic and bactericidal actions and it is present in body fluids. It acts by dissolving the mucocomplex substances essential for maintenance of cells' morphological structure. *L. rhamnosus E800 cells* could not tolerate pepsin action (~ 2log reduction). Trehalose could not protect samples against the actions of acid and bile since no colony growth was observed. Resistance to bile and acid are recognized as an important feature of LAB used as probiotics but the inability of cells to undergo reproductive growth due to the actions of bile did not indicate that they were not viable. Many possible probiotic effects of bacteria depend on viability and not culturability. Marteau et al. (1997) explained that the damaging effects of bile salts on yoghurt bacteria seemed to have positive consequences. Bile could liberate the lactase activity from yoghurt bacteria in the small bowel, which could partially explain the better lactose digestion after digestion of yoghurt by lactase-deficient subjects. The bacterial lysis in the small intestine depends on bile salt and could thus be considered as a way to deliver specific biologically active compounds to the duodenum using ingested microorganisms.
Reducing the period of drying under vacuum may help to reduce these damages since irrespective of the drying period; wet mass or syrups were obtained. An earlier study revealed

the ability of *LGG* to tolerate bile action after its subjection to osmotic stress by trehalose treatment (unpublished) without drying process. Therefore, 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).

4.6.5 Probiotic survival rates in vacuum-dried trehalose during storage

The storage stability of vacuum dried *L. rhamnosus GG and L.rhamnosus E800* in 30% (w/w) trehalose during prolonged storage at 4°C, 25°C and 37°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 rates of both strains was higher ($p<0.05$) than at other storage temperatures (Figure 4.19a, b); but rate of *L. rhamnosus GG* survival was higher and significantly different from that of *L.rhamnosus E800*. For a shelf life of 5 weeks of storage, there was a reduction of 2 log units at 25°C (Figure 4.19a) which was equated to be a level ~ 10^6 cfu mL^{-1} in *LGG* while great losses of survival occurred in *L.rhamnosus E800* (Figure 4.19b).

In general a level of ~10^6 cfu/g, at the time of consuming probiotic, 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°C, strains of study exhibited increased fluidity and within a week, viability reduced drastically by more than 4 log cycles (data not shown). There were significant differences between the storage stability of these strains ($p<0.05$) under the selected storage temperatures. 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).

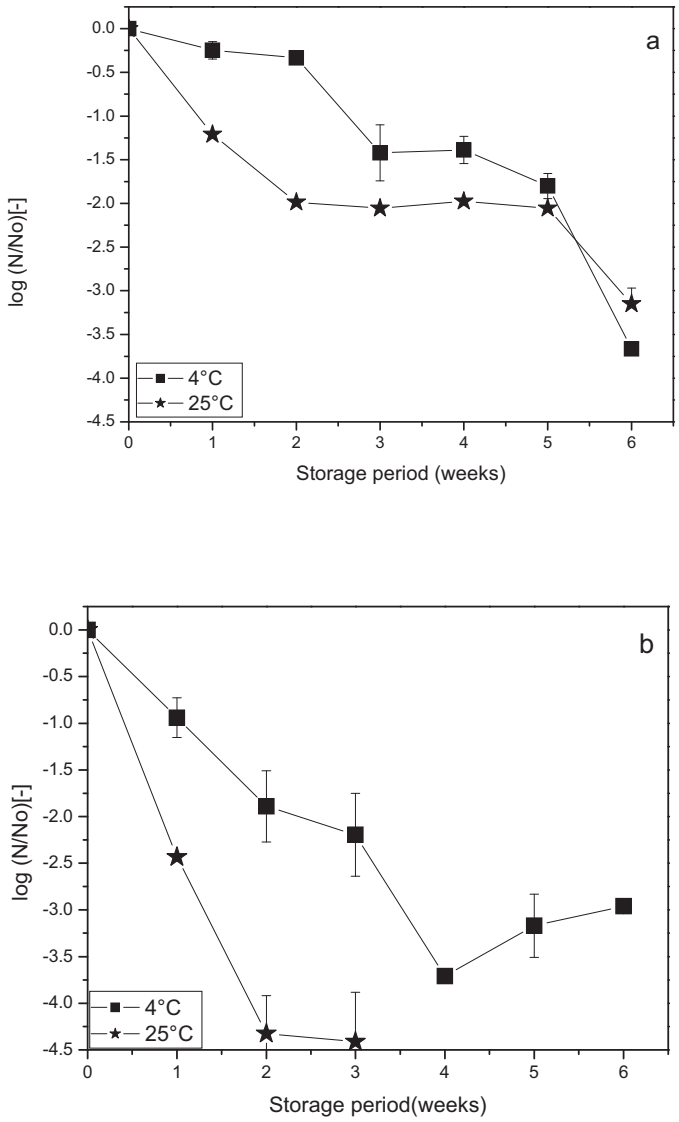


Figure 4.19: Viability loss of dried (a) Lactobacillus rhamnosus GG and (b) Lactobacillus rhamnosus E800 expressed as the logarithmic values of relative survival fraction (log N/No) as described in Materials and Methods during storage at 4°C and 25°C.
Initial concentrations were 4.20 x10^8 cfu mL^{-1} and 2.95 x10^8 cfu mL^{-1} for Lactobacillus rhamnosus GG and Lactobacillus rhamnosus E800 respectively.

Storage at refrigeration temperature, though impractical from a commercial point of view, is optimal for culture viability in these 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 probiotic lactobacilli (~10^8 cfu mL^{-1}) were obtained.

4.7 Spray drying of probiotic bacteria

4.7.1 Thermal tolerance of probiotic strains

Probiotic cultures must be capable of withstanding the harsh conditions often encountered during food processing in order to be successfully used in functional food application. Heat is one of the environmental conditions to which probiotic cultures are exposed in the course of food processing. Therefore the bacterial strains under study were subjected to heat treatment in the carrier medium, which would be subsequently used for spray drying experiments. The results of heat resistance tests showed that *L. rhamnosus GG* and L. *rhamnosus E800* differ in their ability to survive at all treatment temperatures (Figure 4.20a, b). This is in agreement with the observations of Teixeira et al. (1997) on *L. bulgaricus* cells heat treated in skim milk at different temperatures.

When *L. rhamnosus strains* were heat-treated at 65^{o} C, there was a reduction in the cell numbers from 1.80x10^{9} cfu mL^{-1} to 3.80x10^{3} cfu mL^{-1} following incubation in 30s while *L. rhamnosus E800* exhibited a reduction in cell numbers from 1.80x10^{9} cfu mL^{-1} to 1.90 x10^{6} cfu mL^{-1}. Heat treatment at other temperatures (60°C and 70°C) also showed that the latter organism possessed a greater heat resistance than the former; there was a rapid decline in survival rate at 70°C (Figure 4.20a, b). Teixeira et al. (1997) reported a significant decline in survival rate of *L. bulgaricus* cells at 65°C and above. This lower heat resistance was attributed to damages to cytoplasmic membrane, cell wall and proteins.

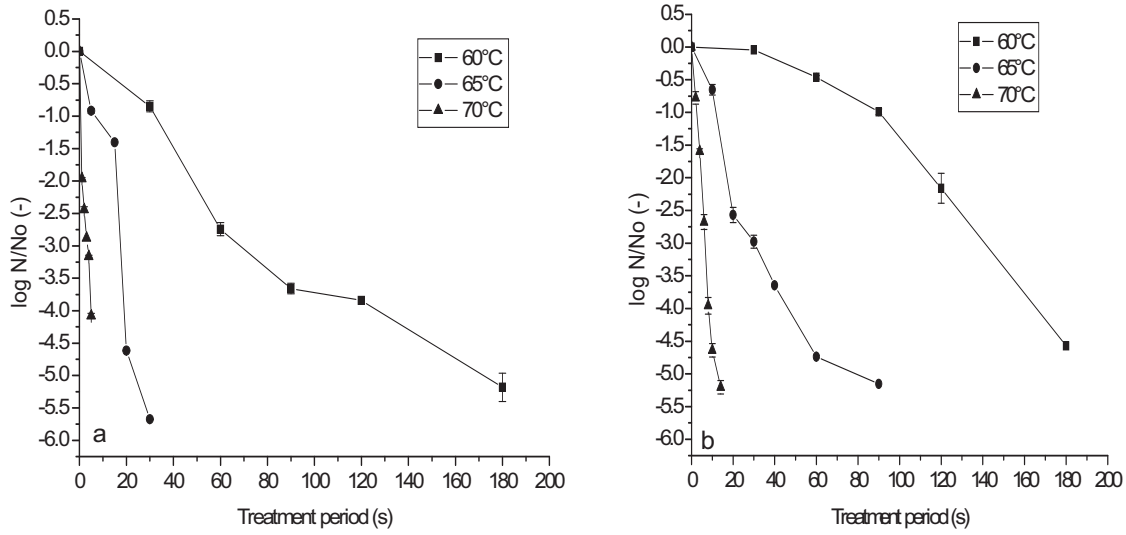


Figure 4.20: Survival of (a) L. rhamnosus GG and (b) L. rhamnosus E800 heat-treated at 60°C, 65°C and 70°C in 20% (w/w) trehalose.
Results are based on data from duplicate heat challenge experiments; error bars represent the standard deviations of the mean.

These results were further explained by the thermotolerance parameter, D-value (i.e. time required to kill 90% of the initial population) calculated by determining the absolute value of the inverse of the slope of the lines at the treatment temperatures applied (Stumbo, 1965). The D- values obtained for *L. rhamnosus GG were* 31 s, 5.1 s and 1.4 s at 60°C, 65°C and 70°C respectively. The corresponding values for *L. rhamnosus E800* were 55 s, 14 s and 2.4 s respectively.

Both cultures were in the stationary phase of growth, which might have contributed to increased heat resistance. It has been demonstrated previously that stationary-phase cultures are more resistant to heat stress than cells in the exponential phase of growth (Teixeira et al., 1994; Gardiner et al. 2000). However, it was noted in a preliminary investigation that D-values of *LGG* when heat-treated in Ringer's solution at same temperatures were 97 s, 10.3 s and 1.6 s while that of *E800* were 187 s, 23 s and 2.2 s. These D-values were higher than the reported values in trehalose medium. This could be as a result of differences in osmotic strength. The osmotic strength of Ringer's solution was 122 mOs kg^{-1} while that of trehalose was 853 mOs kg^{-1}. Therefore, the latter must have imposed severe osmotic stress on the bacterial cells which probably might have reduced the ability of the cells to tolerate heat.

The reported D-values were lower than the D-values reported for some *Lactobacillus* in skim milk (Teixeira et al., 1994; Gardiner et al., 2000; Corcoran et al., 2004). Since most determinations of the heat resistance of the probiotic cultures prior to spray drying were made in skim milk, coupled with the application of different temperature ranges, there was a difficulty in comparison of data.

4.7.2 Survival rates during spray drying

Most liquid or frozen probiotic cultures require refrigeration for storage and distribution, thereby adding expense and inconvenience to their widespread use. However these cold chain requirements may be greatly reduced or eliminated through the use of dry powders, which are potentially superior to liquid or frozen state in their sterility and stability (Meng et al., 2008).

Preliminary spray-drying experiments of probiotics in 20% (w/w) trehalose were conducted to determine the optimum outlet temperature for probiotic viability and production of powders with moisture contents not exceeding 4% (Masters, 1985). This solids content was used because increasing the solids content of trehalose decreased the percentage of surviving bacteria although preservation of bacteria in increased amounts of trehalose improved their stability in storage (Conrad et al., 2000). These authors further explained that dissolution of higher amounts of trehalose becomes difficult on a production scale whereas 20% wt trehalose is slightly above its room temperature solubility and therefore less to be problematic.

Increase in solids content increases suspension viscosity and more concentrated feed suspensions produce larger particles (Re, 1998) which require longer drying times to achieve a given level of residual moisture. This can reduce cell viability due to the longer contact time of the particles with hot air (Ananta, 2005). The temperature ranges of 60-75°C were chosen because at higher temperatures, there will be higher microbial inactivation and possibly pronounced browning reaction (Bielecka and Majkowska, 2000), and at low drying temperatures below 60°C, there could also be formation of lumps in powder due to high moisture content.

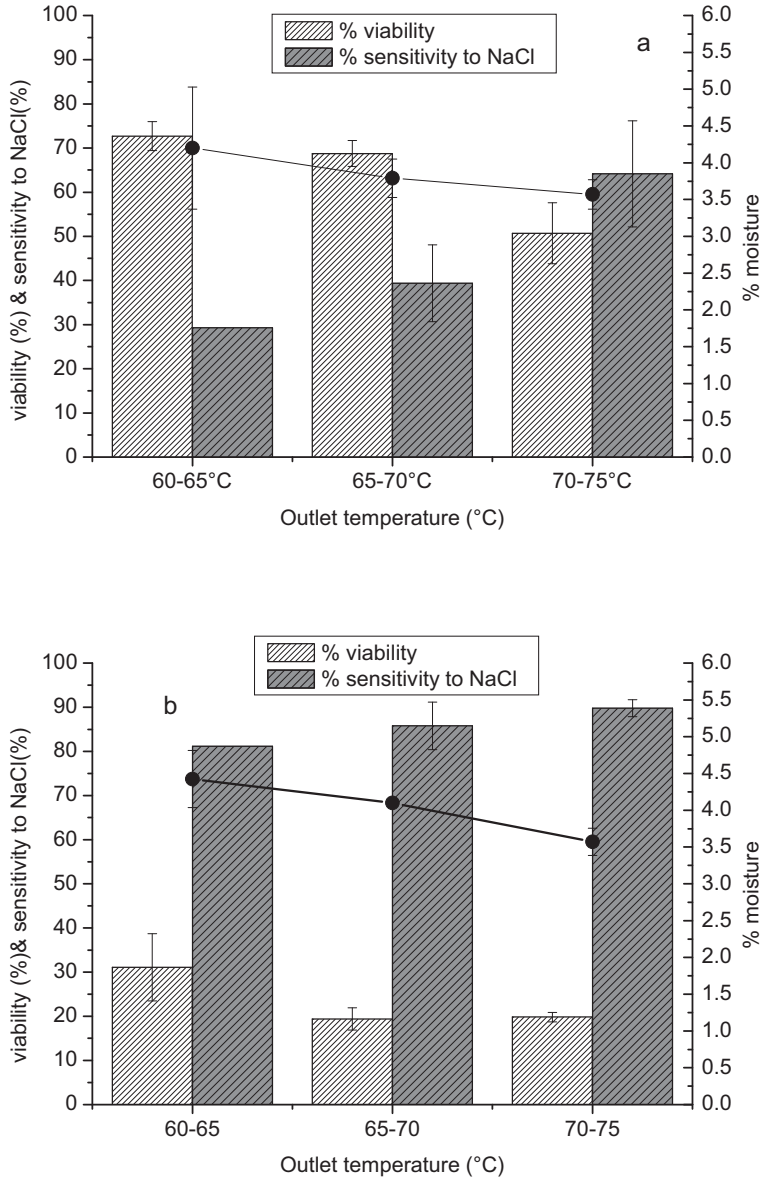


Figure 4.21: Survival of (a) L. rhamnosus GG and (b) L. rhamnosus E800 and corresponding sensitivity to NaCl during spray-drying in 20% w/w trehalose at different outlet air temperatures.
The line indicates the moisture contents of resulting powders. The results are means based on data from three spray-drying trials. Standard deviations are indicated by vertical bars.

Moreover, spray drying at lower temperatures saves more energy than at higher outlet temperatures. Also considering a potential carrier medium, trehalose is a non –reducing disaccharide and can only undergo browning reaction under extreme hydrolytic conditions and trehalase activity.

When outlet temperatures between 60-75^{0}C were investigated, the survival rates were found to be inversely proportional to outlet temperatures. The residual moisture content, from 3.57% (w/w) to 4.2% (w/w) in *LGG* and 3.57% (w/w) to 4.43% (w/w) in *L. rhamnosus E800*, was seen to decrease as the outlet air temperature increased. The residual moisture content of 3.79% and 4.1% was achieved upon spray drying at an outlet temperature of 65-70^{0}C (Fig 4.21a, b).

Since this moisture level was regarded as a good quality parameter of dried dairy products for prolonged storage and stability (Masters, 1985), and coupled with less stickiness of the powder on the walls of the drying chamber and aerocyclone (as indicated in the % powder recovery) (Figure 4.21c), an outlet temperature of 65-70^{0}C was used for further assessments. This powder yield could be improved by supplementing trehalose with agents such as skim milk, starch etc. but the protective effects of trehalose during the drying processes could be masked by the resulting effects of any suggested components.

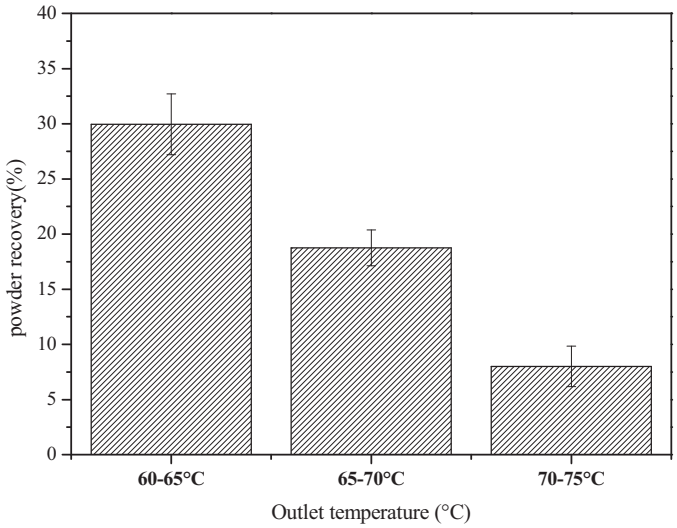


Figure 4.21(c): The effect of the applied outlet temperatures on the powder recovery (relative to the total solids from feed suspension) of the probiotic strains.
Data are means of six replicate spray drying trials and error bars represent the standard deviations of the mean.

The spray drying of the two *L. rhamnosus* strains resulted in different survival rates. There was a significant difference ($p<0.05$) in the survival rates of *LGG* and *L. rhamnosus* E800,

which were about 69% and 23% respectively (Figure 4.21a,b), corresponding to viable counts of $1.8x10^9$ cfu mL^{-1} and $3.65x10^8$ cfu mL^{-1} (Table 4.2).

Trehalose was reported to be non-effective in protecting *L. plantarum* against desiccation during drying (Linders et al., 1997c) because of its inability to gain access into the cytoplasm. In this study, this hurdle was overcome by growing the strains in MRS-trehalose medium unitl they reach the stationary phase at which they were harvested for drying. As micro-organisms are unlikely to be able to accumulate compatible solutes during the short drying period, these solutes should be accumulated before drying i.e. during the growth phase (Ferreira et al., 2005). Irrespective of this, the survival of L. rhamnosus *E800* after drying process was low and significantly different ($p< 0.05$) from the survival of LGG. Tymczyszyn et al. (2007) demonstrated the preservation of dehydrated bacteria after growing them in a low water activity medium such as MRS medium supplemented with trehalose or sucrose. Ferreira et al. (2005) reported ca. 60% survival in spray-dried states when cells of *L. sakei* were grown in the presence of sucrose or MSG.

The survivability of these strains was also investigated in skim milk powder at same drying temperatures, 65-70°C. It was discovered that the survival rates of *LGG* and L. rhamnosus *E800* were 75% and 55% respectively. Although skim milk seems to give a higher survival rates of *L. rhamnosus E800*, but still the application of trehalose during spray-drying could be effective for incorporating these probiotic microorganisms into foods especially sugar-based foods and dairy beverages.

Table 4.2: The survival rates of L. rhamnosus strains (LGG and L rh.E800) and the effects of selective agents on their viability after spray drying experiments at an outlet temperature of 65-70°C.

[b] Carrier media	Survival rate (cfu mL^{-1})		Survival rate (%)					
			Bile		Lysozyme		Simulated gastric juice	
	LGG	L.rh.E800	LGG	L.rh.E800	LGG	L.rh.E800	LGG	L.rh.E800
Trehalose	$1.80x10^9$ [a](68.8±2.9)	$3.65x10^8$ [a](23.4±2.5)	97.5 ±0.6	80.6 ±11.4	78.5 ±1.6	3.1 ±0.2	n.d.	n.d.
Trehalose and MSG	$3.05x10^9$ (80.8±15.1)	$1.30x10^9$ (89.3±15.2)	68.5 ±2.6	69.2 ±3.7	94.4 ±13.1	62.6 ±16.9	0.60 ±0.01	0.01 ±0.00

[a] Values in brackets are survival rates in percentage.

[b]Initial bacterial counts before spray drying in non-supplemented trehalose and trehalose supplemented with MSG were $2.55x10^9$ cfu mL^{-1} and $3.35x10^9$ cfu mL^{-1} (LGG) ; $1.90x10^9$ cfu mL^{-1} and $1.50x10^9$ cfu mL^{-1} (L.rh.E800) respectively.

Values are means of three drying experiments± standard deviations; n.d means not detected.

The application of sucrose may be cost effective as a carrier medium in spray drying but it may not be worthwhile because when dried samples are exposed to moist air (which depresses T_g), sucrose samples may not be maintained in the vitrified state, although in biological systems the glassy state was necessary to increase stability but not sufficient to ensure absolute stability (Higl et al., 2007). Trehalose has a very high glass transition temperature compared to other disaccharides. This makes trehalose to remain stable even at greater range of temperature extremes; thus it provides additional stability to glass systems into which it is incorporated. Trehalose glasses are also more resistant to moisture gain than other disaccharide systems. Sucrose can absorb up to 1% moisture by weight; this may increase the moisture content of the dried samples thus they are prone to microbial attacks. Moisture content of ~ 3.5% is being preferred for shelf stable products (Zayed and Roos, 2004). Leslie et al. (1994) reported high survival of bacteria freeze-dried in the presence of trehalose immediately after freeze-drying and upon long exposure to moist air. In contrast, bacteria freeze-dried in sucrose showed lower initial survival and rapid decrease in viability upon exposure to moist air. This occurrence could be as a result of lowering of T_g or the glycosidic bond in sucrose being more susceptible to hydrolysis; these expose the embedded probiotics thus reducing their stability and/or viability. Crowe et al. (2001) stated that trehalose is not special under ideal conditions for drying and storage but under sub-optimal conditions, it provides stability when other sugars do not.

The experimental results on the survival of the *Lactobacillus rhamnosus* strains in the presence of monosodium glutamate (MSG) are as shown in Table 4.2. It was observed that the survival rates of these cells increased significantly ($p<0.05$), especially in *L. rhamnosus E800*, when spray-dried in the presence of 12.5g L^{-1} MSG. The survival rate of *L. rhamnosus E800* was 89.3%, which was not significantly different ($p>0.05$) from that of *LGG,* 80.8% (Table 4.2). No significant differences were observed in survival during freeze-drying of some *Lactobacillus* species after the addition monosodium glutamate (Carvalho et al., 2003).

Besides the carrier media, the final pH of the growth media of the cells might have influenced survival during spray drying (Meng et al., 2008). In this study, the final pH of the growth medium at the time of harvest was ~ 3.8. This could be explained in relation to effect of acid shock, which might have influenced the synthesis of heat shock proteins; thus improving bacterial resistances to drying. Similar reports were made (Palmfelds and Hahn-Hagerdel, 2000; Silva et al., 2005) in experiments with uncontrolled pH. However, Linders et al. (1997a) indicated that pH control during the growth of *L. plantarum* cells resulted in higher residual activity during drying. Kessler (1993) found no significant influence of pH control during growth on the residual activity of *Lactococcus diacetylactis* after drying.

Several authors also reported variations in survival rates amongst different probiotic species (Gardiner et al., 2000; Cocoran et al., 2004). In the present study, *L.rhamnosus GG* exhibited

a weak thermal tolerance yet it was the best survivor during spray drying. This indicated that thermal tolerance alone is not an accurate predictor of performance during spray drying and that other phenomena, such as dehydration-related stresses (osmotic, accumulation of toxic compounds) affect cell viability during drying (Fu & Etzel, 1995; Corcoran et al., 2004). However, the issue of thermal inactivation cannot be neglected (Figure 4.21a, b).

Ananta (2005) demonstrated that thermal inactivation in aqueous solution was higher than inactivation during spray drying of *LGG* and that the real temperature experienced by bacteria was much lower than the adjusted outlet temperature. During spray drying, dehydration of the atomised liquid particles proceeds from the particle surface to the inner core, resulting in the formation of protective vapour film, which surrounds the droplet and keeps the particle surface wet and maintain the temperature at the vapour saturation temperature. This keeps the drying rate constant. Due to evaporation, the temperature of the solids may decrease or does not approach the dryer outlet temperature. At subsequent drying stage, the particle surface becomes dry and the temperature may increase maximally to the dryer temperature since evaporative cooling is no longer possible. Temperature effect will be higher but due to lower moisture content, cells will show higher thermal resistance (Härnulv et al., 1977; Mermelstein, 2001).

4.7.3 Tolerance of cells to NaCl, lysozyme and simulated gastric juice

L. rhamnosus GG and *L. rhamnosus E800* cells developed sensitivity to 5% NaCl and lysozyme before and after spray drying. About 16% of LGG cells were sensitive to NaCl before spray drying experiments while less than 5% of L*. rhamnosus E800* cells were sensitive. This sensitivity probably occurred as a result of sublethal injury on the cells due to osmotic stress imposed by the carrier medium. A transient permeabilization of membranes of these cells when subjected to osmotic stress was earlier reported (Sunny-Roberts et al., 2007; Sunny-Roberts & Knorr, 2007). Following spray drying at an air outlet temperature of 65-70^{o}C, the probiotic strains also became sensitive to NaCl as revealed by reductions in cell numbers. Despite the fact that *L. rhamnosus E800* cells had a minimal sensitivity to NaCl prior to spray- drying, it was discovered that 86% of these cells were sensitive to NaCl but only 40% of *LGG cells* were sensitive after spray drying (Figure 4.21a,b). This suggested that *L. rhamnosus* E800 cell membranes suffered more damages during the drying process than those of LGG, thus resulting in lower survival rate of *L. rhamnosus E800*. These results are similar to the results of Gardiner et al. (2000) on spray-dried *L. paracasei* and *L. salivarus* strains.

These damages also occured at other air outlet temperatures, though, at varying intensities. Increased sensitivity to NaCl of sublethally injured bacteria has been associated with cell

membrane damage (Teixeira et al, 1995b; Teixeira et al; 1997). Moreover, average colony sizes were found to be reduced (0.96±0.58mm) compared to the colony sizes prior to spray drying (3.51±0.71mm). These morphological changes were additional indications that spray drying stressed the cells. Sensitivity of cells to lysozyme could be a manifestation of damage to the cell wall components (Brennan et al., 1986). Spray drying of the study strains in non-supplemented trehalose affected the cell walls at different intensities (Table 4.2). This was shown by 3.1±0.2% of *L. rhamnosus E800* cells showing tolerance to lysozyme while 78.5±1.6% of *LGG* cells was tolerant. In the presence of MSG (Table 4.2), tolerance of *L. rhamnosus E800* cells to lysozyme was seen to be greatly improved, indicating a pronounced protective mechanism of MSG on membranes and cell wall. *Lactobacillus rhamnosus GG* dried in trehalose supplemented with MSG had a survival rate of 0.6%, which corresponded to $1.70x10^{7}$ cfu $mL^{-1,}$ in simulated gastric juice. The tolerances of spray-dried cells in supplemented trehalose or non-supplemented trehalose to bile were significantly different from one another. The ability of high percentage of cells in tolerating bile could be an indication of the possibility of such dried probiotic to withstand the environmental stresses in the human gastrointestinal tract.

4.7.4 Flow cytometric analysis of spray-dried bacterial cells

In order to further clarify the cellular sites that were affected by spray drying, flow cytometric analysis was applied on a spray-dried *L. rhamnosus* strain to evaluate cellular injury sites after drying. An attempt was made to relate the data on the bacterial survival as obtained by flow cytometric analysis with data from plate count method. *L. rhamnosus* strain was stained with both cFDA and PI. Basically this staining technique reveals the capability of viable cells in converting non-fluorescent cFDA into a membrane –impermeant fluorescent product cF, which can be accumulated in cells' cytoplasm. Cells require a high degree of membrane integrity and functional cytoplasmic enzymes in order to retain this dye. Besides intracellular retention of cF, cells with intact membranes exclude nucleic acid dyes (PI). PI only enters cells with compromised membrane and binds to RNA or DNA thus forming a complex which emits red fluorescence when excited.

Bacterial populations were differentiated based on their fluorescence properties as shown in Figure 4.22. The quadrants on the density plot were set in such a way that viable cells with intact membranes were in #4 (Figure 4.22a), where active cells showed high green fluorescence and low red fluorescence which signified accumulation of cF and exclusion of PI.

Control cells (in Ringer's solution) of *Lactobacillus rhamnosus* strain were mainly encountered in #4 but as a result of thermal treatment (95°C, 15 min), the cells' membranes were compromised and the cF retention ability was lost and cells were not capable of excluding PI.

Therefore, cells showed low green fluorescence and high red fluorescence and were gated in #1 (Figure 4.22 b).

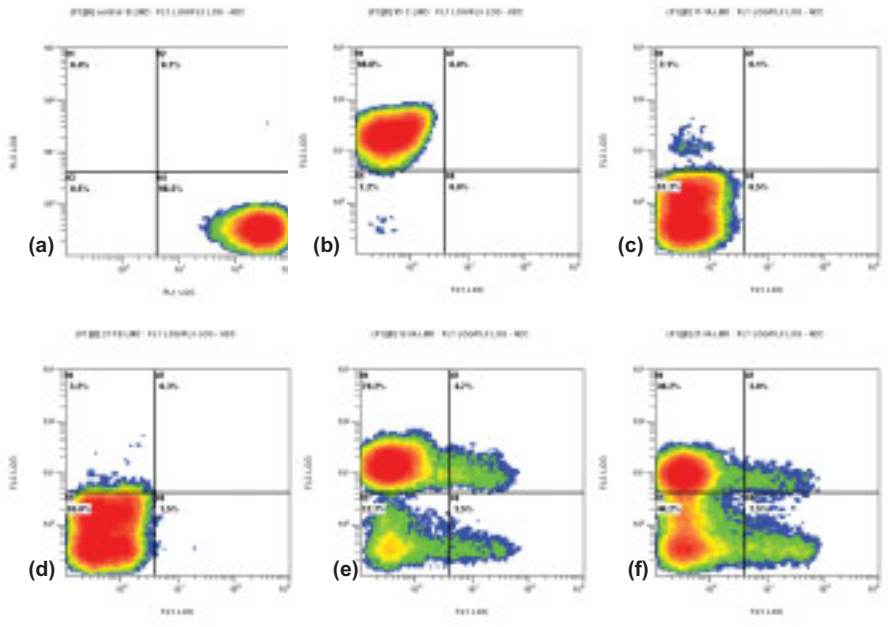


Figure 4.22: Representative flow cytometric density plot analysis of Lactobacillus rhamnosus strain spray dried in 20% (w/w) trehalose at 65-70°C air outlet temperature. Control cells (in Ringer's solution) (a) ; dead, membrane compromised cells following heat treatment at 95°C,15 min (b); cells in non-supplemented 20% w/w trehalose prior to drying (c) ; cells in trehalose supplemented with MSG prior to drying (d) ; cells in non-supplemented 20% w/w trehalose after drying (e) ; and cells in trehalose supplemented with MSG after drying (f).

Prior to spray drying the control cells were added to the carrier media (non-supplemented trehalose or trehalose supplemented with MSG) to form the feed suspensions. Analysis of the feed suspensions by FCM density plots revealed that more than 90% control population gated in #4 moved to #3 as a result of the osmotic stress imposed upon the cells by the carrier media (Figure 4.22 c, d). The presence of such population in #3 could be taken to indicate either a loss of esterase activity or cF extrusion (Ananta et al., 2004). In previous studies on these strains, populations gated in #3 were reported to exhibit membrane permeabilization (Sunny-Roberts et al., 2007; Sunny-Roberts and Knorr, 2007), therefore they could not retain cF but the penetration of PI into the cells was not allowed. This signified that membrane integrity was still maintained. For example, the survival rate of *LGG* on MRS plates was ~ 98% which was significantly different from the percentage of cF stained cells (~1.4%) gated in #4 (Figure 4.23). The high survival rate could be as a result of the ability of cells gated in #3 to resume growth on MRS agar plates after a repair of transient permeabilized membrane. Reproductive growth requires both membrane integrity and metabolic activity.

Upon drying in non-supplemented 20% (w/w) trehalose, more than half of the populations in #3 moved to #1(Figure 4.22e). Populations in #1 had their membranes ruptured thus allowing the penetration of PI into the cells therefore they are referred to as 'dead'. Death was due to additional stress (oxidative, dehydration, osmotic) encountered in the course of spray drying. Similar situations were observed in FCM density plot analysis of cells dried in trehalose supplemented with MSG but higher percentage of cells were still gated in #3 (Figure 4.22f). This difference was reflected in survival rates on MRS agar plates. The survival rates of *LGG* in supplemented and non-supplemented trehalose were 80.8% and 68.6% (Figure 4.23) while that of *E800* were 89% and 23% respectively (Table 4.2). The percentage of cF-stained population in #4 (<10%) was significantly different from the survival rates on MRS agar plates (Figure 4.23). Bunthof et al. (1999) reported that the retention of intracellular cF was not crucial in the maintenance of culturability.

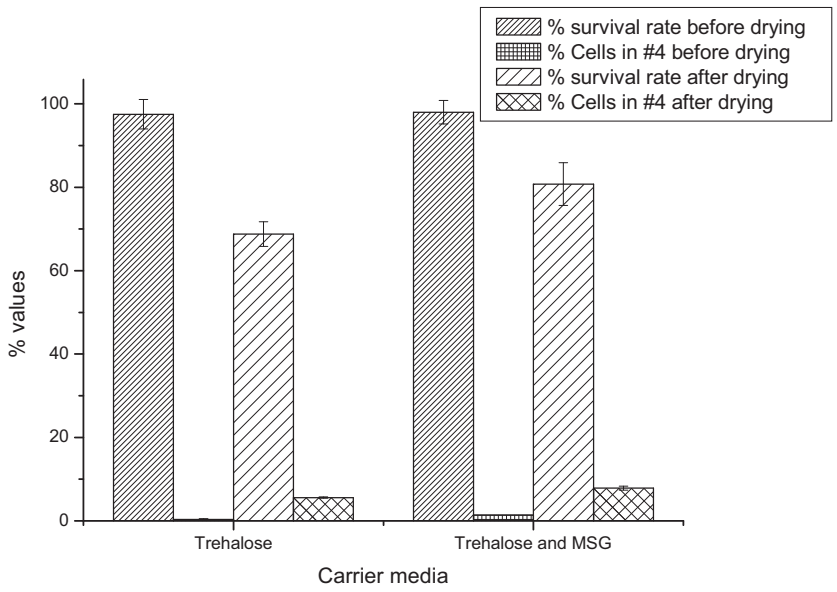


Figure 4.23: The survival rates of L. rhamnosus strain, spray dried in trehalose or trehalose supplemented with MSG at 65-70°C, by plating on MRS agar and the percentage of cF accumulating population in #4 of the flow cytometry density plots (Figure 4.22).
Results are means based on data from three drying experiments; error bars show standard deviations of the means.

After 10 weeks of storage of spray dried samples at 25°C, there was more than 7log reduction in the viability of *L. rhamnosus* in non-supplemented trehalose. Almost 90% of cell population was gated in #1 while less than 10% was gated in #3 or #4. On the other hand, FCM density plot of the strain in trehalose supplemented with MSG showed the gating of some populations (30%) in #4 with corresponding decrease in populations previously gated, before storage, in #1 or #3 (data not shown). This indicated that bacterial cells were able to recover

by repairing damaged/permeabilized membranes under storage conditions but this was not shown by culture techniques. An almost 5log reduction in viability was observed.

4.7.5 Scanning Electron Microscopy of spray dried cells

The Scanning Electron Microscopy images of spray-dried *L. rhamnosus* strain in skim milk powder and trehalose powder are as shown in Figure 4.24.

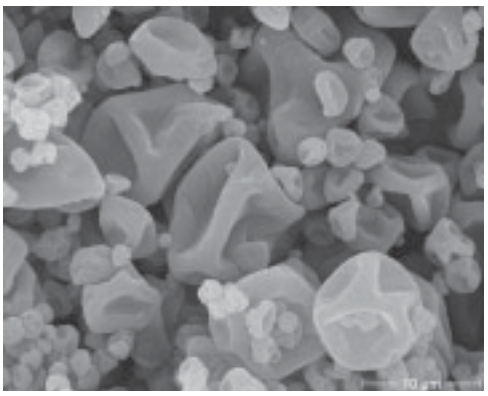
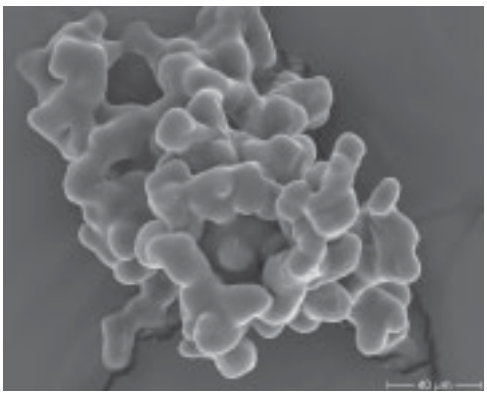
(a)  (b) 

Figure 4.24: Scanning Electron Microscopy images of a L. rhamnosus in (a) skim milk powder and (b) trehalose powder spray dried at 65-70°C.

The shapes and surfaces of all spray dried samples are as shown in Figure 4.24 but not without some characteristic dimples in skim milk powder, which are typical of spray dried materials. It was observed that the scanning electron microscopy of the spray-dried powders did not reveal that the probiotic cells were present. It was assumed that probiotics were encapsulated in the dried samples. Gardiner et al. (2000) had similar observation but also reported that further studies by CSLM technique revealed that the probiotic lactobacilli cells were encapsulated in the milk powder particles. Skim milk had individual particles but with uniform appearances and surfaces (Figure 4.24a). It could be possible that besides the individuality of spray-dried particles, the dimples in skim milk powder served as additional protective compartments for spray dried probiotics thus reducing cells' exposure to environmental stress. Spray-dried trehalose appeared in an irregular form (Figure 4.24b) therefore the created spaces must have exposed spray-dried cells to stress impacts thus lowering cells' viability. Therefore the reported differences in the viability of probiotics (Section 4.7.2) irrespective of the encapsulation could be related to the structural forms of powders. From another point of view, there was no difference in the structural form of trehalose when supplemented with MSG compared with trehalose alone (not shown), yet there was an improved

survival rates. This indicated that MSG protected the exposed dried cells from other environmental stress.

4.7.6 Survival kinetics of spray-dried probiotics at non-refrigerated storage conditions

Previous studies showed that temperature is critical for microbial survival during storage, and higher survival rates were obtained at lower storage temperatures. But it was apparent from these studies (Teixeira et al., 1995b; Gardiner et al., 2000; Corcoran et al., 2004) that the applications of the probiotics are more limited to refrigerated products. Refrigerated storage is expensive to both suppliers and retailers of probiotic products therefore survival of spray dried *Lactobacillus rhamnosus strains* at elevated temperatures necessitated investigation. Powders of the probiotic cultures produced, by spray-drying, at a constant air outlet temperature of 65-70°C were stored at 25°C and 37°C. Storage was kept at a relative humidity of 11% extracted from studies (Ananta et al., 2005; Highl et al., 2007) and this has been reported to be optimal for the maintenance of high viability level during the storage of dried bacteria (Castro et al., 1995). The viability of the *Lactobacillus rhamnosus* strains during a storage period of 6-8 weeks at these temperatures were evaluated (Figure 4.25 & 4.26).

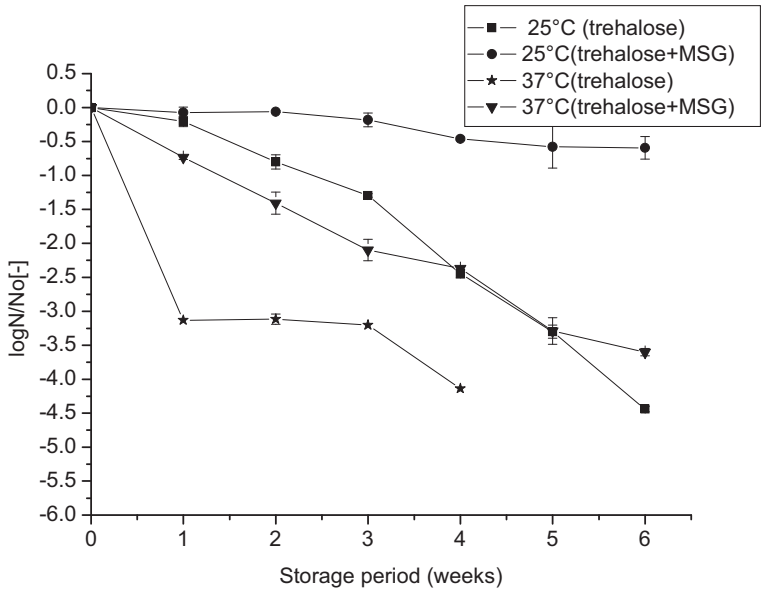


Figure 4.25: Survival of L. rhamnosus GG in spray-dried trehalose and trehalose supplemented with MSG during storage at 25°C and 37°C ,at a constant relative humidity of 11%, expressed as a logarithmic values of relative survival fraction (log N/N_o).
Results are means of two replicate spray drying trials and storage experiments. Error bars represent the standard deviations of the mean.

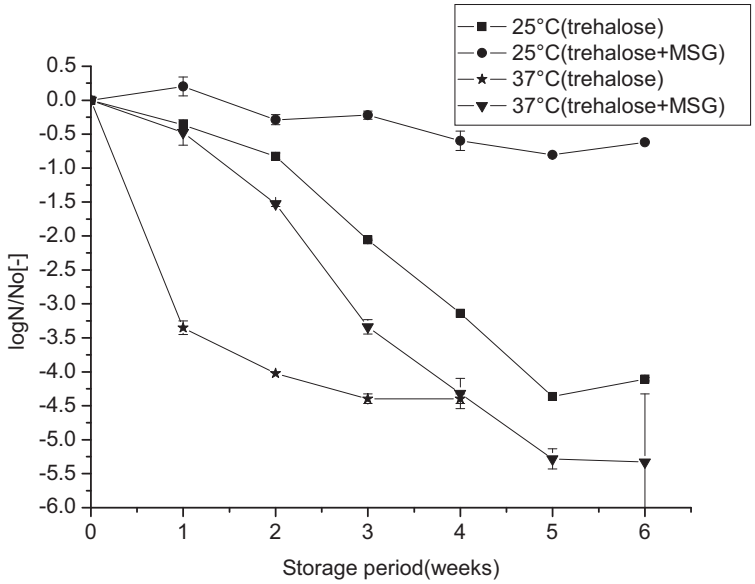


Figure 4.26: Survival of L. rhamnosus E800 in spray-dried trehalose and trehalose supplemented with MSG during storage at 25°C and 37°C, at a constant relative humidity of 11%, expressed as a logarithmic values of relative survival fraction (log N/N_o) .
Results are means of two replicate spray drying trials and storage experiments. Error bars represent the standard deviations of the mean.

Loss of viability was represented by the logarithmic values of the survival fractions at different storage periods. Within a period of 6 weeks, there was a decline in viability of both strains in spray-dried non-supplemented trehalose powders stored at both temperatures. At 25°C 2log reductions of bacterial strains were observed within a period of 4 weeks. This was equivalent to a viable count of ~10^7 cfu mL^{-1}. At 37°C the loss of viability increased significantly as revealed by the absence of survivors after 4 weeks which is in agreement with the report of Ananta (2005). The lower survival rates of *L. rhamnosus E800* (Figure 4.26) during storage may be related to the extensive damage that might have occurred as a result of spray drying. Teixeira et al. (1996) reported the evidence of lipid oxidation of *Lactobacillus bulgaricus* cells during drying and storage. Spray drying induced lesions in the cellular lipid-containing structures of the cells which reduced the ratio of unsaturated/saturated fatty-acids. Upon excessive contact with air, there was a possibility of lipid oxidation. This oxidative damage has at least two indirect consequences leading to cell death – the product of lipid peroxidation may lead to DNA damage and alteration of membrane lipid composition may cause dysfunction of membrane-associated enzymes, such as ATPase due to a decrease in membrane fluidity or weakening of hydrophobic interactions (Marnett et al., 1985; Castro et al., 1996).

4.7.6.1 Effect of monosodium glutamate (MSG) on survival of spray-dried cells during storage

At 25°C and 37°C, survival was higher ($p<0.05$) in spray-dried trehalose powders supplemented with MSG than in dried non-supplemented trehalose thus indicating the ability of MSG to suppress the oxidative damage that leads to membrane rupture and cell death. Over a shelf life of 4 weeks there were reductions of 0.45log unit corresponding to 41% survival rates in LGG (Figure 4.25) and 0.59log unit corresponding to 26% survival rates in *L. rhamnosus E800* (Figure 4.26).) under storage temperature of 25°C.and at the end of storage period, a high viable count ~10^8 cfu mL^{-1} was still obtained. On the contrary, there was a drastic decline in logarithmic values.at 37°C. However, *L. rhamnosus GG* still had a viable count of ~10^7 cfu mL^{-1} , by the fourth week (Figure 4.25) ,which could still meet the requirement of the level of viable cells in probiotic foods (IDF,1992). MSG was reported to increase the stability of freeze-dried lactobacilli during long term storage (Carvalho et al., 2003). MSG has a moderating effect in controlling the rate of ascorbate oxidation thus imparting stabilization during storage (Porubcan & Sellars, 1975). On the contrary, MSG was unable to protect *L. delbrueckii spp. bulgaricus* during storage at 20°C (Teixeira et al., 1995b). Spray-dried cells of *Lactobacillus sakei CTC 494* survived ca. 60% longer when cells were grown in the presence of MSG (Ferreira et al., 2005). Glutamate was shown to be accumulated by osmotically stressed *L. plantarum* (Kets and de Bont, 1997), in agreement with the general observations that glutamate levels are markedly increased as part of osmo-adaptive response. Glutamate is probably a counterion for K^+ to balance the intracellular charge accumulated by bacteria under osmotic stress (Kets et al., 1997). An alternative way of improving cell survival was by modifying the gas composition of the storage atmosphere (i.e. by replacing air with nitrogen or by application of vacuum) (Castro et al., 1995). However, this present finding proved the suitability of trehalose-MSG medium as a good carrier medium for a large-scale production of spray-dried probiotic bacteria with long storage stability. MSG was able to minimize oxidation of membrane lipids which might have impaired probiotics' viability during storage. Although MSG has been given a bad reputation as a suspicious additive that many consumers believe gives allergies but many studies have found that MSG does not cause ill effects (MacLaughlin, 2007).

Below is an overview of the protective abilities of trehalose on *Lactobacillus* strains during vacuum drying or spray drying.

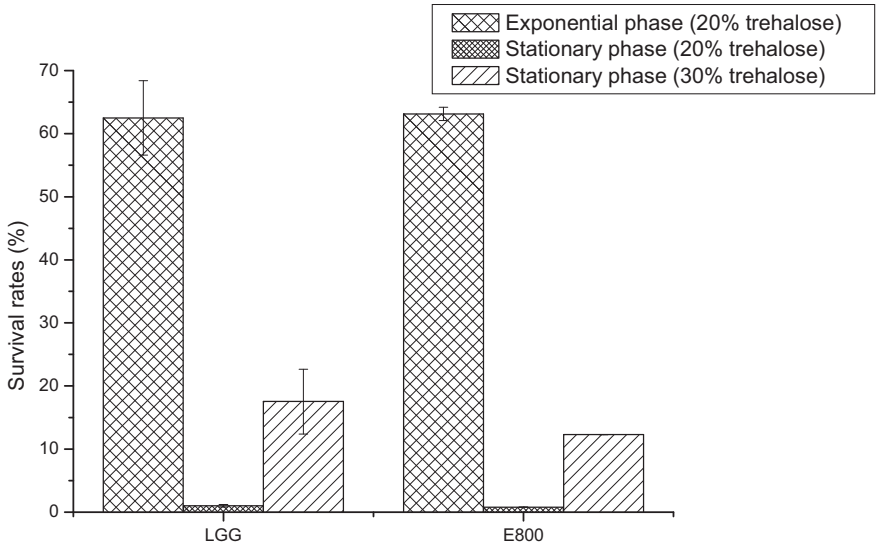


Figure 4.27(a): Survival of exponential phase and stationary-phase cells of LGG and L. rhamnosus E800 in different trehalose concentrations during drying in vacuum oven at 25°C.
Standard deviations are shown by vertical bars.

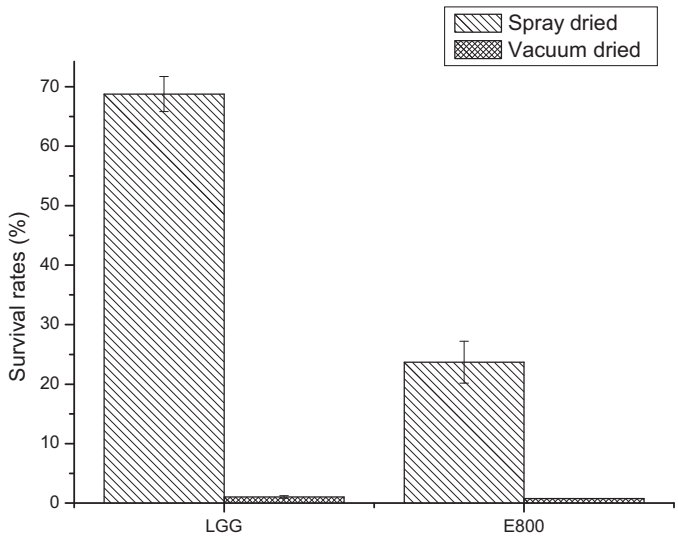


Figure 4.27(b): Survival of stationary-phase cells of LGG and L. rhamnosus E800 during vacuum and spray-drying in 20% (w/w) trehalose.
Results are means of triplicate experiments. Standard deviations are shown by vertical bars.

Figures 4.27(a, b) reveal that the level of performance of trehalose in protecting an organism during drying is dependent on the bacterial strains and growth phase, the concentration of trehalose used and the drying processes applied. This is in line with the review made by Meng et al. (2008). Though the bacteria under study are of same species, yet they vary in respect to tolerance during vacuum- or spray drying. This highlights the importance of strain selection as one of the important parameters for consideration prior to onset of drying proc-

esses. Stationary phase cells are known to develop a general stress resistance to various types of stresses. Thus they are taken as the optimal growth phase for dehydration survival. However, this may not be applicable in all dehydration cases. For example in Figure 4.27a bacterial cells at the exponential phase survived better than cells in the stationary phase when vacuum dried in 20% trehalose. Spray dried stationary phase cells performed better than vacuum dried cells in same concentration of carrier media (Figure 4.27b). Therefore besides the method of drying, survival of stationary phase cells may also be dependent on the concentration of carrier media being used.

4.7.7 Stability of spray-dried L. rhamnosus GG in dairy beverage powder and apple juice

Probiotic-containing products, whether they are dairy-based beverages, yoghurts, tablets or health snacks, present unique challenges to manufacturers. Many active milk cultures die even before the consumer receives any of the health benefits (i.e. during manufacturing, during storage or during transport of the finished product). The addition of probiotics to food systems is seen as a very effective means of restoring at least part of the initial value associated with microflora that are typically destroyed during such treatments as sterilization, pasteurization, disinfection, irradiation, washing and peeling (Suita-Cruce and Goulet, 2001). Because trehalose has mild sweetness, low cariogenicity, low hygroscopicity and it is stable during processing and storage it can be used in several food preparations e.g. beverages such as tea, fruit juices and dairy beverages.

Probiotic- containing spray-dried trehalose powders was mixed with a chocolate dairy powder or 100% apple juice to a final concentration of ~10^9 CFU g^{-1} or mL^{-1}.

There was a rapid decline in the viable counts of LGG (dried in non-supplemented trehalose) in dairy beverage powder over a period of 8 weeks (Table 4.3).

Table 4.3: Stability of *Lactobacillus rhamnosus GG* in a chocolate dairy beverage stored at 25°C.

Storage period (Weeks)	Viable counts of LGG			
	[a]Non - Supplemented trehalose		[a]Supplemented trehalose (with MSG)	
	Concentrations (CFU g^{-1})	Survival rates (%)	Concentrations (CFU g^{-1})	Survival rates (%)
0	$4.40\pm0.29\infty10^9$	100.0	$5.13\pm0.14\infty10^9$	100.0
1	$6.75\pm0.30\infty10^8$	15.3	$4.83\pm0.04\infty10^9$	94.1
2	$1.50\pm0.10\infty10^7$	0.3	$4.80\pm0.01\infty10^9$	93.7
3	$6.23\pm0.25\infty10^6$	0.1	$3.45\pm0.03\infty10^9$	67.3
4	0[b]	0.0	$3.03\pm0.19\infty10^9$	59.1
5	0	0.0	$2.63\pm0.06\infty10^9$	51.2
6	0	0.0	$2.15\pm0.13\infty10^9$	41.9
7	0	0.0	$7.64\pm0.20\infty10^8$	14.9
8	0	0.0	$4.60\pm0.22\infty10^7$	0.9

[a] carrier media . Initial bacterial concentration was ~10^9.

[b] Less than 10CFU g^{-1}

Values are results of duplicate experiments.

It was noted that LGG survival rates were maintained at a higher level in the beverage powder formulated with supplemented trehalose throughout the storage period. After 10 weeks of storage, the viable counts remained constant at ~10^7 CFU g^{-1} (data not shown).The composition of the chocolate powder includes cocoa, maltodextrin, sugar, salt, calcium carbonate and some vitamins. Besides the water activity (0.35±0.02) of this food product, it could be assumed that the protective abilities of some composition of this beverage powder, maltodextrin and calcium, (King and Su, 1993; Burin et. al., 2004) might have also contributed to the probiotic survival and recovery rates. However comparing these results in relation to supplementation, MSG played a significant role.

A daily dose of at least 10^8 cells was proposed to elicit the health promoting effect on consumers' health (Lourens-Hattingh and Viljoen, 2001). These high numbers achievable by consuming 100g or mL of food containing a minimal level of 10^6 CFU g^{-1} or mL^{-1} have been suggested to appropriately compensate for the possible loss in the numbers of probiotic organisms during passage through the stomach and intestine.

Spray- dried *Lactobacillus rhamnosus GG* powders was also mixed with sterile water samples and stored at 25°C over a period of 8 weeks as a control storage test (Table 4.4). There was a drastic loss in viable counts of LGG in both water samples, although it was only the-growth of *LGG* embedded in non-supplemented trehalose that was hardly recovered from water samples after one week of storage. In comparison, spray-dried LGG was more stable in chocolate dairy beverage powder than in water samples. This was due to the difference in

their available free water molecules. The water activity (a_w) of sterile water is 1.00 which is higher than a_w of beverage powder, 0.35.

Table 4.4: Stability of *Lactobacillus rhamnosus GG* in sterile water stored at 25°C.

Storage period (Weeks)	Viable counts of LGG			
	[a] Non - Supplemented trehalose		[a] Supplemented trehalose (with MSG)	
	Concentrations (CFU mL^{-1})	Survival rates (%)	Concentrations (CFU mL^{-1})	Survival rates (%)
0	$2.90\pm0.00\infty10^9$	100.0	$2.20\pm0.00\infty10^9$	100.0
1	$1.11\pm0.12\infty10^7$	0.6	$1.10\pm0.34\infty10^8$	4.9
2	0 [b]	0	$5.34\pm0.25\infty10^6$	0.3
3	0	0	$8.26\pm0.01\infty10^5$	0.0
4	0	0	$1.49\pm0.01\infty10^5$	0.0
5	0	0	$2.90\pm0.00\infty10^4$	0.0

[a] carrier media . Initial bacterial concentration was ~10^9.

[b] Less than 10CFU mL^{-1}

Values are results of duplicate experiments.

Therefore, the role of water activity (a_w) of food constituents on the level of viability and stability of added probiotics is considered as very important.One of the mechanisms that have been suggested to explain the protective effect of sugar matrix on biological systems is the ability of sugars to form a glassy structure in which biomolecules are embedded. When such dried glassy structure are exposed to increasing level of a_w there is a tendency of uptake of water and this can lead to lowering of the glass transition temperature (T_g). When the T_g reduces in such samples, the viability of the embedded microbes reduces, therefore T_g cannot be regarded as an absolute threshold of bacteria stability during storage (Higl et al., 2007).The anti-oxidant role of MSG pronouncedly differentiated the survival rates of LGG in both water batches.

The apple juice (Table 4.5) used in this study contained neither additives nor preservatives. It was proved to be of safe microbiological quality by sampling on microbial growth media such as Standard 1 Nutrient Agar, Orange Serum Agar, Oxytetracycline Glucose Yeast Agar (OGY Agar), Plate Count Agar, Dextrose Casein-Peptone Agar, Reinforced Clostridia Agar, ENDO Agar, Violet-Red Bile Dextrose Agar, PALCAM Listeria Selective Agar, and ROGOSA Agar. Thereafter the juice was mixed with spray dried *L. rhamnosus GG*, in supplemented or non-supplemented trehalose, to a final concentration of ~10^9 CFU mL^{-1} and stored under refrigeration (4°C) over a period of 14 days.

Table 4.5: Stability of *Lactobacillus rhamnosus* GG apple juice stored under refrigeration.

Storage period (Days)	Viable counts of LGG			
	[a] Non - Supplemented trehalose		[a] Supplemented trehalose (with MSG)	
	Concentrations (CFU mL^{-1})	Survival rates (%)	Concentrations (CFU mL^{-1})	Survival rates (%)
0	$1.75\pm0.01\infty10^{9}$	100.0	$4.04\pm0.05\infty10^{9}$	100.0
2	$6.41\pm0.12\infty10^{8}$	36.6	$2.01\pm0.10\infty10^{9}$	49.6
4	$6.15\pm0.23\infty10^{6}$	0.4	$5.55\pm0.23\infty10^{8}$	13.7
6	$5.92\pm0.07\infty10^{6}$	0.3	$5.24\pm0.12\infty10^{8}$	13.0
8	$2.25\pm0.05\infty10^{6}$	0.1	$4.34\pm0.34\infty10^{7}$	1.1
10	$3.07\pm0.28\infty10^{5}$	0.0	$1.22\pm0.20\infty10^{7}$	0.3
12	$6.05\pm0.22\infty10^{4}$	0.0	$6.18\pm0.25\infty10^{5}$	0.0

[a] carrier media . Initial bacterial concentration was ~10^{9}.

Values are results of duplicate experiments.

As storage period advanced, weak and small colonies were observed on MRS agar plates probably due to the continuous stress imposed on the cells by the acidity of the juice. There was no significant change in pH (3.2 ±0.2) throughout the storage period. In non-supplemented trehalose there were reductions in viable counts reaching 2.25 x 10^{6} CFU mL^{-1} and 4.34 x 10^{7} CFU mL^{-1} in supplemented trehalose after 8 days of storage. These decreases might be attributed to the antimicrobial activity, associated with pH and other antimicrobial compounds, in apple juice and/or oxidative stress. The acidity of apple juice is largely due to malic acid which has bacteriostatic actions. Other acids include citric acid which is bactericidal; and ascorbic acid. Though acids impair bacterial growth and viability but acid tolerant lactic acid bacteria allow their intracellular pH to drop (Siegumfeldt et al, 2000) in response to drop in external pH, therefore it may not be the maintenance of highest possible intracellular pH that is critical for the stability of *L. rhamnosus* in apple juice (Saarela et al., 2008). Reid et al. (2007) reported the viability of microencapsulated *L.rhamnosus ROII* in frozen vegetable juice to be 33.4 % after two weeks, which was almost of same value as 36.1% viable cells of spray-dried *LGG* (in trehalose supplemented with MSG) recovered from refrigerated apple juice after one week in this study. The storage temperatures coupled with the method of probiotic preparation made it impossible to give a better comparison. However the storage of apple juice in a frozen state may give an improved stability to *LGG* as well. Saarela et al. (2008) achieved a better performance of acid stressed and freeze-dried *L. rhamnosus* cells in apple juice (pH 3.4-3.8) stored at 4°C. Although apple juice used by these authors had a higher pH but this better performance was attributed to the adjustment of growth pH before drying process. According to them, fermentation at reduced pH may ensure a better performance of *L. rhamnosus* cells during subsequent acid stress. The higher

survival rates of LGG (contained in supplemented trehalose) in apple juice may, therefore, be attributed to the alleviation of oxidation by MSG.

Although there is variability in the minimum recommended levels for probiotics in foods (Charteris et al., 1998), an official standard requiring a minimum of 10^6-10^7 CFU g^{-1} have been introduced by several food organizations worldwide (Ishibashi and Shimamura,1993; IDF,1992; Talwalkar and Kailasapathy, 2004). However, a minimal level of ~10^5 cfu/g till expiry was considered as sufficient (Shah and Lankaputhra, 1997; Schillinger, 1999).

4.8 Influence of mild pressure pre-treatment on the thermotolerance of L. rhamnosus GG

Cross protective action of pressure against heat was investigated to allow a justification regarding its applicability in aiding probiotic production, where in case of spray drying the lethal effect of high temperature needed to be overcome (Ananta and Knorr, 2005).

This experiment was conducted simply to verify whether the increased thermo-tolerance reported in LGG (Ananta and Knorr, 2003) was not due to the effect of heat (37°C) applied during pressure pre-treatment. There was supposed to be clarity on the source of stress metabolites produced in the course of pressure pre-treatment. In order to clarify this, lower temperatures, 20 and 30°C, were used while other parameters were same.

The findings in this study were as presented in Figure 4.28.

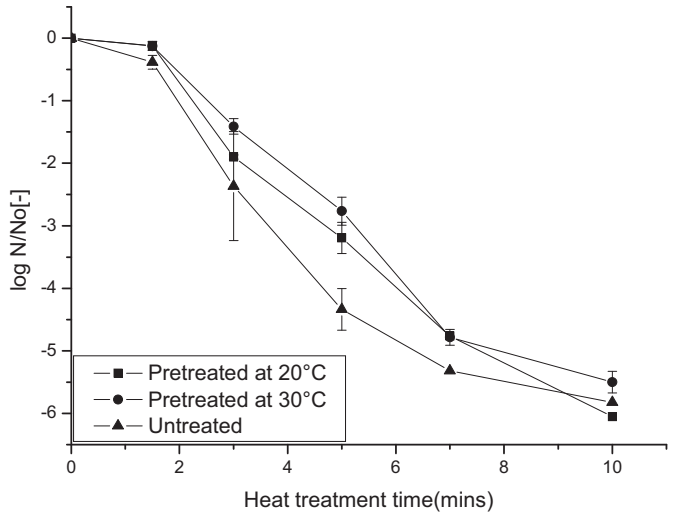


Figure 4.28: Heat inactivation of LGG at 60°C after previous pre-treatment at 100MPa for 10 min. Temperatures applied during pressure pre-treatment were 20°C and 30°C. Data shows means of two independent measurements.

In the course of pressure pre-treatment there was an increase in applied temperatures by +2 to +3°C but the comparison of results with that of Ananta and Knorr (2003) showed no significant difference. In this study, there was no significant viability loss upon exposure of cells to 60°C for 1.5min. In comparison to control cells, other groups survived well by ~1.6 and ~1.2 log cycles after being exposed to pressure pre-treatment for 10 min at 20°C and 30°C respectively. Similar result within this range was reported by these authors. This confirms that the improved heat tolerance was as a result of synthesized stress metabolites (Ananta and Knorr, 2004) by applied pressure and not by the temperature applied during pressure pre-treatment. These stress metabolites are defined as pressure induced proteins (PIP), which were identified as classical heat shock proteins acting as chaperons including GroEL, DnaK as well as proteins related to cold shock response and an unknown protein of 15.6kDa (Welch et al., 1993). High pressure pre-treatments were reported to induce microbial tolerance to various stresses such as high temperature, high pressure etc. (Tamura et al., 1998; Scheyhing et al., 2004).

Pressure increase to approximately 50MPa perturbed cell division, nucleotide structure and the total rate of protein synthesis of *E. coli*. However, pressure also initiated heat shock response, since both high pressure and high temperature destabilize the quaternary structure of proteins, and an increased proportion of dissociated subunits could induce a heat shock response. As a result, many proteins exhibited elevated rates of synthesis. Many of these pressure-induced proteins were also induced by heat or cold shock (Welch et al., 1993; Wemekamp-Kamphius, 2002; Ananta and Knorr, 2003).

It is hereby suggested that working at higher temperatures in inducing the thermotolerance of bacteria may not be necessary; working at lower temperatures is cost effective.

In support of the work of Ananta and Knorr (2004), this data shows the induction of thermotolerance by pressure pre-treatment thus giving the evidence of utilizing this mechanism of cross protection in protecting probiotics from environmental stresses during industrial production e.g. spray drying.

During the course of spray drying in this study, cells in the stationary phase were used thus the induction of thermotolerance was not investigated. Stationary phase cells had already developed resistance against various types of environmental stresses. Moreover, the carrier media trehalose and MSG proved to be good protectants.

However, the potential of high pressure pre-treatment in improving survival during subsequent treatment may be investigated. Accoding to Saarela et al. (2004) stationary phase cells had to be studied instead of log-phase cells as during the indusrial production of cultures high enough densities have to be reached before harvesting the cells.

5 Conclusion and Recommendation

Fermented and unfermented dairy products enriched with probiotic bacteria have developed into one of the most successful categories of functional foods (Meng et al., 2008); but during recent years, probiotics have been increasingly incorporated into non-dairy food systems e.g. vegetable juice, dried fruits and biscuits. However the availability of these probiotics in the dried viable forms for convenient functional food applications has been a great challenge in the scientific community. The production of dried probiotic powders should ensure that adequate numbers of living cultures are maintained in the powder following manufacture, and moreover the stability of the probiotic properties should be maintained throughout the shelf life and after ingestion.

Drying processes and storage conditions lead to the exposures of live probiotic bacteria to a variety of stresses such as heat, osmotic and cold, amongst others, which may lead to impaired functionality and loss of viability. It is well known that lactic acid bacteria, like other organisms, are subjected to potentially stressful environmental changes in nature, where they are able to evolve stress- sensing systems and defences against stress thus they withstand harsh conditions and sudden environmental changes (van de Guchte et al., 2002). Hence the subjection of same micro-organisms to stressful conditions is expected to make them develop pre- adaptation or resistance mechanisms to the environmental conditions they may encounter from the period of food processing till after consumption.

This study investigated the responses of *L. rhamnosus GG (LGG)* and *L. rhamnosus E-97800 (E800)* to osmotic stress induced by non-electrolytes (sucrose and trehalose) which are commonly used disaccharides in food processing. Physiological fluorescence dyes, cFDA or SYTO9 or PI were applied in flow cytometry measurements and analysis to examine process- induced changes in cellular integrity or metabolic activities of *L. rhamnosus* strains under osmotic stress.

When subjected to osmotic stress, FCM analyis showed that the responses of both strains were slightly different from one another. For example, cell membrane permeabilization, resulting to the leakage of cF out of the cells, occurred greatly in *E800* upon trehalose treatments. Leslie et al. (1995) achieved the penetration of sucrose and trehalose into cells when membranes had a higher permeability. Therefore besides the leakage of cF, the transient membrane permeabilization phase must have enhanced the flow and accumulation of trehalose into the cells. Hence there was a restoration of internal turgor pressure which was an important factor responsible for the survival of cells under osmotic stress. On the contrary cF

leakage was not observed in sucrose-treated cells but the survival of cells was an indication of accumulation of sucrose in order to maintain an osmotic balance. Under this circumstance, it was either the degree of membrane permeabilization might not be sufficient to allow the leakage of cF out of the cells (based on molecular weight) or there was no membrane permeabilization. The molecular weight of sucrose is 342.3g/mol while that of cF is 376g/mol therefore the difference in molecular weight could be taken as the major point for non-leakage of cF.

Survival rates according to culture techniques were not always in correlation to % esterase activity shown by FCM analysis. This indicated that the intracellular accumulation of cF and/or the occurrence of esterase activity may not necessarily reflect crucial metabolic activities, which are involved in the maintenance of reproductive growth. The improvement of cF efflux (made possible by a dye extrusion system most likely mediated by an ATP-driven transport system) rates by trehalose treatment signified its ability to enhance the resistance of probiotics to drugs or toxic compounds. From another point of view, it is hereby suggested that the use of trehalose in pharmaceuticals should be limited to the manufacture of drugs that are not meant for antimicrobial actions.

Moreover from the results of FCM analysis, E800 cells treated at 1.2M or 1.5M sucrose still possessed intact membranes and esterase activity but not all of them were culturable. The non-culturable population was not able to extrude cF completely. Therefore any cell inactivation which may likely occur under stressful conditions can be as a result of damages to membrane related defence mechanism.

Trehalose pre-treatment improved the tolerance of LGG to bile stress but reduced its heat tolerance. The heat tolerance of *E800*.was also reduced. This indicated that this phenomenon of improving cells' tolerance to some stressful conditions may not lead to a general improved tolerance to others. Moreover, from the microscopy pictures, tolerance to osmotic conditions displayed by morphological changes was strain dependent. Bile stressed *E800* cells pre-treated with trehalose had their membranes damaged while sucrose pre-treated cells had their membranes intact. This shows that the ability of dissacharides to protect bacterial membranes depends on the environmental conditions involved.

Trehalose has been demonstrated mostly as an effective protectant during freezing and freeze-drying (Meng et al., 2008) due to its high glass transition temperature, strong ion-dipole interactions and strong hydrogen bonding between biomolecule and trehalose (Patist and Zoerb, 2005). Freeze-drying is mostly employed currently in producing dried bacterial

populations due to the possibility of operating at mild conditions in order to minimise cell injury. But for effective costs and time management spray drying or vacuum drying, as alternative methods, to produce dried probiotic preparations in trehalose matrices were explored in this study. FCM analysis was also conducted to identify cellular injuries, which could not be revealed by culture techniques, but might have occurred during the drying processes. Dried powder was observed by Scanning Electron Microscopy.

The evaluation of spray-drying process in the production of probiotic powders reveals that the inclusion of MSG in the carrier component hindered higher rates of deteriorative reactions such as lipid oxidation; powders that contained not less than 10^9 cfu g^{-1} of *L. rhamnosus* strains were produced. This anti-oxidant property was also exhibited during storage period. Higher survival rates were obtained when compared with survival rates in non-supplemented trehalose. Moreover there was <10% population in #4 at the beginning of storage of spray-dried cells in trehalose supplemented with MSG but a gating of more populations in this quadrant on subsequent storage with no correlation to culturability. This depicted bacterial transformation into non-culturable state which is not devoid of probiotic effectiveness.
Since recent studies found out that MSG does not cause ill effects, many of the reported ill effects may be psychological. The large scale production of spray dried probiotics in trehalose-MSG media should be encouraged. Trehalose has mild sweetness so it can be used in many food formulations.

Drying of probiotics under vacuum resulted in severe bacterial inactivation, which was discovered when fluorescence pattern was compared with culturability. This transformation into non-culturable state is an adaptive response in coping with adverse environmental conditions. However since non-culturability does not necessarily reflect non-viability, probiotic effectiveness should not be placed on culturability. Non-culturable cells can elicit health benefits upon consumption. During storage, *L. rhamnosus* strains still remained inactivated; but they can still retain a functional cell membrane typical for viable cells (Ananta, 2005). Although spray dried trehalose was not characterized in terms of glass transition temperature but the mobility and rapid decline in culturability of stored vacuum-dried samples at 37°C was an assumption that the samples were stored in a ‘rubbery state’. Therefore maintenance of bacterial survival during storage would be well achieved at temperatures well below 37°C.
The viscous nature of probiotic samples produced may serve as a limitation to its use in food product development but the production of functional confections e. g. candy, jam from under-utilized crops such as groundnuts, coconuts, mango etc. may be considered. In developing countries, this will prevent economic losses due to food wastages and also enhance a

good way of utilizing these crops to produce functional products that are of health benefits. Indiscrimate use of medicaments will thereby be curbed.
Cellular sites of injury were also investigated by culture techniques, using some selective agents. Spray- and vacuum drying processes indicated similar sites of cells injury but at different intensities. Sensitivity to lysozymes indicated that they caused more damages to cell wall components of *E800* dried in trehalose (without MSG) matrices. Bacterial membranes were also identified as sites of injuries during both drying processes. The higher sensitivity of E800 to lysozyme after spray- or vacuum-drying is a limitation to its use as a probiotic in food products in which lysozyme is used as preservatives. MSG minimized this sensitivity therefore the use of MSG as a supplementary carrier component in drying may be necessary when a high probiotic survival is desired. Therefore, protective media employed in drying processes should target protecting bacterial cells from drying-related damages.
Since drying processes affect several components of bacterial cells, it is suggested that more detailed identification methods of cellular sites of injuries be put in place. This will assist in putting forward relevant solutions to be applied in minimizing or preventing cell damages.

During storage of food products and water in which spray-dried LGG was added, there was an influence of water activity (a_w) of food or water samples on the survival of the incorporated probiotics. Probiotic stabilization decreased at a fast rate with increasing water activity. This signified a decrease in the glass transition temperature (T_g) (though not determined), which is the parameter that describes the glassy state of amorphous material. Glass forming properties of trehalose is one of the factors responsible for the stabilization of biomaterials at low moisture level. Therefore the entrapment of these bacteria in glassy state appeared to have little effect on absolute stability.

In addition the reduced stability of spray dried LGG in apple juice within a period of 12 days might have been due to non- induction of proteins responsible for improving bacterial survival to a lethal acid challenge. During bacterial growth, there was no pH control before harvesting. It was therefore impossible for LGG to possess an acid pre-adaptation which would have initiated acid tolerance response to lethal pH challenge.
Above all, MSG played an important role in preventing higher loss of probiotic survival during the storage of food and water samples. Although *L. rhamnosus* strains are facultative anaerobes, data from this study indicated that when it comes to industrial processing or storage oxygen is detrimental to their survival.

Induction of thermotolerance by high pressure pre-treatment was conducted on exponential phase cells and it was discovered that even with low temperatures during pressure pre-

treatment, heat tolerance of *LGG* was improved. Spray drying of exponential phase cells was supposed to be employed as a heat challenge condition. Since cultures of high density were needed cells in the stationary phase were used in spray drying. Based on the general resistance of stationary phase cells, high pressure pre-treatment was therefore considered unnecessary. Higher survival rates obtained by trehalose pre-treatment after spray drying further justify the irrelevance of pressure pre-treatment. However, high pressure pre-treatment may improve survival during subsequent treatments. This may be investigated.

The survival rates, duration of production and also energy consumption are crucial parameters to be considered in evaluating the economy of production processes for industrial probiotic cultures. Therefore spray drying processes were seen to stand in a better position than the vacuum drying processes in light of the mentioned parameters. Furthermore the utilization of osmotic pre-treatment and the application of MSG improve the survival and stability of probiotics during industrial processing and storage.

Required further studies include:classification of the antimicrobial activity of dried probiotics; pilot scale plant trial and comparison with Ringer's solution alone; and examination of a multitude of combinations such as culture stage of growth, culture state of stress resistance and other carrier media (with or without trehalose/MSG) towards developing an ultimate alternative to a milk-based carrier.

References

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

Abe, F. and Horikoshi, K. (1995). Hydrostatic pressure promotes the acidification of vacuoles in S. cerevisiae. FEMS Microbiol. Lett. 130: 307-312.

Abe, F., Ishibashi, N. and Shimamura, S. (1995). Effect of administration of bifidobacteria and lactic acid bacteria to newborn calves and piglets. J. Diary Sci. 78: 2838-2846.

Abee, J. and Wouters, J.A.(1999) Microbial stress response in minimal processing. Int. J. Food Microbiol. 50: 65-91.

Aertsen, A., van Houdt, R., Vanoirbeek, K. and Michiels, C.W. (2004). An SOS Response induced by high pressure in Escherichia coli. J. Bacteriol 186(18): 6133-6141.

Agar, D.W., and Bailey, J.E. (1982). Cell cycle operation during batch growth of fission yeast populations. Cytometry 3: 123-128.

Alakomi, H.-L., Matto, J., Virkajarvi, I and Saarela, M. (2005) Application of a micro-plate scale fluorochrome-staining assay for the assessment of viability of probiotic preparation. J. Microbiol. Meth. 62: 25-35.

Alcon, A., Santos, V.E., Casas, J.A and Garcia-Ochoa, F. (2004). Use of flow cytometry for growth structured kinetic model development. Application to Candida bombicola growth. Enzyme and Microbial Technol. 34: 399-406.

Allison, S.D., Chang, B., Randolph, T.W. and Carpenter, J.F. (1999). Hydrogen bonding between sugar and protein is responsible for inhibition of dehydration-induced protein unfolding. Arch. Biochem. Biophys. 365: 289-298.

Allman, R., Hann, A.C., Phillips, K.L., Martin, K.L. and Lloyd, D. (1990). Growth of Azotobacter vinelandii with correlation of coulter size, flow cytometric parameters and ultrastructure. Cytometry 11: 822-831

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 pretreatment for the improvement of stress tolerance. PhD Thesis, Berlin University of Technology, Germany. pp 123-227.

Ananta, E. and Knorr, D (2004). Evidence on the role of protein synthesis in the induction of heat tolerance of Lactobacillus rhamnosus GG by pressure pre-treatment. Int. J. Food Microbiol. 96: 307-313.

Ananta, E. and Knorr, D. (2003). Pressure-induced themotolerance of Lactobacillus rhamnosus GG. Food Res. Int. 36: 991-997.

Ananta, E., Heinz, V. and Knorr, D. (2004). Assessment of high pressure induced damage on Lactobacillus rhamnosus GG. by flow cytometry. Food Microbiol. 21: 567-577.

Ananta, E., Volkert, M. and Knorr, D. (2005) Cellular injuries and storage stability of spray dried Lactobacillus rhamnosus GG. Int. Dairy J. 15, 399-409.

Andersson, H., Asp N-G, Bruce, A., Roos, S., Wadstrom, T. and Wold, A.E. (2001). Health effects of probiotics and prebiotics: A literature review on human studies. Scand. J. Nutr., 45: 58-75.

Ang, D., Libereck, K., Skowyra, D., Zylicz, M. and Georgopoulous, C. (1991). Biological role and regulation of the universally conserved heat shock proteins. J. Biol. Chem. 266:24233-24236.

Argüelles, J.C. (2000). Physiological roles of trehalose in bacteria and yeasts: a comparative analysis. Arch. Microbiol. 174: 217-224.

Attfield, P.V., Kletsas, S., Veal, D.A., van Rooijen, R. and Bell, P.J. (2000). Use of flow cytometry to monitor cell damage and predict fermentation activity of dried yeasts. J. Appl. Microbiol. 89: 207-214.

Auty, M.A.E., Gardiner, G.E., McBrearty, S.J., O'Sullivan, E.O.., Mulvihill, D.M., Collins, J.K., Fitzgerald, G.F., Stanton, C., and Ross, R.P. (2001) Direct in situ viability assessment of bacteria in probiotic dairy products using viability staining in conjunction with confocal scanning laser microscopy. Appl. Env. Microbiol. 67: 420-425.

Bailey, J.F., Fazel-Madjlessi, J., McQuitty, D.E., Lee, L., Allred, J. G: and Oro, J.A. (1978). Measurement of structured microbial population dynamics by flow microfluorometry. AIChE J. 24: 570-577.

Ballongue, J. (1998). Bifidobacteria and probiotic action p. 519-597. In. S. salminen and A. von Wright (ed.) Lactic acid bacteria: microbiology and functional aspects. Marcel Dekker Inc., New York, N.Y.

Balny, C., Masson, P. and Heremans, K. (2002). High Pressure effects on biological macromolecules: from structural changes to alteration of cellular processes. Biochim. Biophys. Acta 1595: 3-10.

Barer, M,.R and Harwood C. R. (1999). Bacterial viability and culturability. Adv. Microb. Physiol. 41: 93-137.

Bayrock, D. and Ingledew, W.M. (1997). Mechanism of viability loss during fluidised bed drying of baker's yeast. Food Res. Int. 30:417-425

Beales, N. (2004). Adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH and osmotic stress: A review. Comprehensive Reviews in Food Science and Food Safety 3: 1-26.

Ben Amor, K., Breeuwer, P., Verbaarschot, P., Rombouts, F.M, Akkermans, A.D.L., De Vos, W.M and Abee, T. (2002). Multiparametric flow cytometry and cell sorting for the assessment of viable, injured and dead Bifidobacterium cells during bile salt stress. Appl. Environ. Microbiol. 68 (11): 5209-5216.

Berg, R.D. (1998) Probiotics, prebiotics or 'conbiotics'. Trends in Microbiology 6(3): 89-92.

Bernet, F.M., Brassart, D., Neeser, J.R. and Servin, A. (1993). Adhesion of human bifidobacterial strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interaction. Appl. Environ. Microbiol. 59: 4121-4128.

Betoret, N., Puente, L., Diaz, M.J., Pagan, M.J., Garcia, M.J., Gras, M.L., Martinez-Monzo, J., and Fito, P. (2003). Development of Probiotic enriched dried fruits by vacuum impregnation. J. Food Eng. 56: 273-277.

Bianchi, G., Gamba, A., Limiroli, R., Pozzi, N., Elster, R., Salamini, F., and Bartels, D. (1993). The unusual sugar composition in leaves of the resurrection plant Myrothamus flabellifolia. Physiol. Plant 87: 223-226.

Bielecka, M. and Majkowska, A. (2000). Effect of spray drying temperature of yoghurt on survival of starters, moisture content and sensory properties of yoghurt powder. Nahrung/Food 44:257-260

Blakeley, D., Tolliday, B., Colaco, C. and Roser, B. (1990). Dry instant blood typing plate for bedside use. Lancet 336; 854-855.

Bogosian, G., Aardema, N.D., Bourneuf, E., Morris, P.J.L. and O'Neil, J.P. (2000). Recovery of Hydrogen Peroxide – Sensitive culturable cells of Vibrio vulnificus gives the appearance of resuscitation from a viable but non culturable state. J. Bacteriol. 182(8): 5070-5075.

Booth, I.R., Pourkomailian, B., Mc Laggan, D., Koo, S. – P. (1994). Mechanisms controlling compatible solute accumulation: a consideration of the genetics and physiology of bacterial osmoregulation. J. Food Eng. 22: 381-397.

Bouvier, J., Bordes, P., Romeo, Y., Fourcans, A., Bouvier, I. and Gutierrez, C. (2000). Characterization of OpuA, a glycine-betaine uptake system of Lactobacillus lactis. J. Mol. Microbiol. Biotechnol. 2: 199-205.

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

Breeuwer, P. and Abee, T. (2000). Assessment of viability of microorganisms employing fluorescence techniques. Int. J. Food Microbiol. 55. 193-200.

Breeuwer, P., Drocourt, J.L., Rombouts, F.M. and Abee, T. (1994). Energy dependent, carrier-mediated extrusion of carboxyfluorescein from Sacharomyces cerevisiae allows rapid assessment of cell viability by flow cytometry. Appl. Environ. Microbiol. 60:1467-1472.

Brennan, M., Wanismail, B., Johnson, M.C. and Ray, B. (1986). Cellular damage in dried Lactobacillus acidophilus. J. Food Prot. 49: 47-53.

Brown, A.D. (1976) Microbial water stress. Bacteriol. Rev. 40: 803-843.

Brul, S., Nussbaum, J. and Dielbandhoesing, S.K. (1997). Fluorescent probes for wall porosity and membrane integrity in filamentous fungi. J. Microbiol. Meth. 28:169-183.

Buera, M.P., Rossi, S., Moreno, S., and Chirife, J. (1999). DSC confirmation that vitrification is not necessary for stabilization of the restriction enzyme EcoRI dried with saccharides. Biotechnol. Prog.. 15: 577-579.

Bunthof, C.J. (2002). Flow cytometry, Flourescent Probes and Flashing Bacteria. Thesis Wageningen University, Wageningen, The Netherlands 160p.

Bunthof, C.J., Bloemen, K., Breeuwer, P., Rombouts, F.M. and Abee, T. (2001). Flow cytometric assessment of viability of lactic acid bacteria. Appl. Environ. Microbiol. 67(5): 2326-2335.

Bunthof, C.J., van den Braak, S., Breeuwer, P., Rombouts, F.M. and Abee, T. (2000). Fluorescence assessment of Lactococcus lactis viability. Int. J. Food Microbiol. 55:291-294.

Bunthof, C.J., van den Braak, S., Breeuwer, P., Rombouts, F.M. and Abee, T. (1999). Rapid flourescence assessment of the viability of stressed Lactococcus lactis. Appl. Environ. Microbiol. 65 (8):3681-3689.

Burin, C., Jouppila, K., Roos, Y.H., Kansikas, J., and Buera, M.P. (2004). Retention of ß-galactosidase activity as related to Maillard reaction, lactose crstallization, collapse and glass transition in low moisture whey system. Int. Dairy J. 14: 517-525.

Caffrey, M., Fonseca, V. and Leopold, C.A. (1988). Lipid-sugar interactions. Plant Physiol. 86: 754-758.

Cardona, S., Schebor, C., Buera, M.P., Karel, M. and Chirife, J. (1997). Thermal stability of invertase in reduced-moisture amorphous matrices in relation to glassy state and trehalose crystallization. J. Food Sci. 62: 105-112.

Carpenter, J.F. and Crowe, J.H. (1988). Modes of stabilization of a protein by organic solutes during desiccation. Cryobiology 25: 459-470.

Carpenter, J.F. and Crowe, J.H. (1989). An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. Biochemistry 28: 3916-3922.

Carpenter, J.F., Crowe, L.M. and Crowe, J.H. (1987). Stabilization of phosphofructokinase with sugars during freeze-drying: characterization of enhanced protection in the presence of divalent cations. Biochim. Biophys. Acta 923:109-115.

Carvalho, A.S., Silva, J., Ho, P., Teixeira, P., Malcata, F.X. and Gibbs, P. (2003). Protective effect of sorbitol and monosodium glutamate during storage of freeze-dried lactic bacteria. Lait, 83: 203-210.

Castro, H., Teixeira, P., and Kirby, R. (1995). Storage of lyophilized cultures of Lactobacillus bulgaricus under different relative humidities and atmospheres. Appl. Microbiol. Biotechnol. 44: 172-176.

Castro, H.P., Teixeira, P.M., and Kirby, R. (1996). Changes in the cell membrane of Lactobacillus bulgaricus during storage following freeze-drying. Biotechnol. Lett. 18: 99-104

Cerrutti, P., de Huergo, M.S., Galvagno, M., Schebor, C. and del Pilar Buera, M. (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.

Champagne, C.P, Gardiner, N., Brochu, E. and Beaulieu, Y. (1991). The freeze drying of lactic acid bacteria. A review. Canadian Institute for Science and Technology Journal 24: 118-125.

Chang, G. (2003). Multidrug resistance ABC transporters. FEBS Lett. 555: 102-105.

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

Charteris, W.P., Kelly, P.M. , Morelli, M. and Collins, J.K. (1998). Ingredient selection criteria for probiotic microorganisms in functional dairy foods. Int. J. Dairy Technol. 51: 123-136.

Cheftel, J.C. (1995). Review: high pressure, microbial inactivation and food preservation. Food Sci. Technol. Int. 1: 75-90.

Chen, T., Acker, J.P., Eroglu, A., Cheley, S., Bayley, H., Fowler, A. and Toner, M. (2001). Beneficial effect of intracellular trehalose on the membrane integrity of dried mammalian cells. Cryobiology, 43: 168-181.

Christiansen, P.S. Edelsten, D., Kristiansen, J.R., and Nielsen, E.W. (1996). Some properties of ice cream containing Bifidobacterium bifidum and Lactobacillus acidophilus. Milchwissenschaft 51:502-504.

Chrzanowski, T.H., Crotty, R.D., Hubbard, J.G. and Welch, R.P. (1984). Applicability of the fluorescein diacetate method of detecting active bacteria in fresh water. Microbial Ecology 10: 179-185.

Clark, D. and Parker, J. (1984). Proteins induced by high osmotic pressure in Escherichia coli. FEMS Microbiol. Lett. 25:81-83.

Clegg, J.S., Seitz, P., Seitz, W. and Hazlewood, C.F. (1982). Cellular responses to extreme water loss. The water-replacement hypothesis Crybiology 19: 306-316.

Colaco, C., Sen, S., Thangavelu, M., Pinder, S. and Roser, B. (1992). Extraordinary stability of enzymes dried in trehalose: simplified molecular biology. Bio/Technology 10:1.

Comas, J. and Vives-Rego, J. (1998). Enumeration, viability and heterogeneity in Staphylococcus aureus cultures by flow cytometry. J. Microbiol. Meth. 32: 45-53.

Comas-Riu, J. and Vives-Rego, J. (1999). Use of calcein and SYTO-13 to assess cell cycle phases and osmotic shock effects on E.coli and S. aureus by flow cytometry. J. Microbiol. Meth. 34: 215-221.

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

Corcoran, B.M., Ross, R.P., Fitzgerald, G.F. and Stanton, C. (2004). Comparative survival of probiotic lactobacilli spray-dried in the presence of prebiotic substances. J. Appl. Microbiol. 96:1024-1039.

Corcoran, B.M., Stanton, C., Fitzgerald, G.F., and Ross, R.P. (2005). Survival of probiotic lactobacilli in acidic environments is enhanced in the presence of metabolized sugars. Appl. Environ. Microbiol. 71 (6): 3060-3067.

Crissman, H.A., Darzynkiewiez, Z., Tobey, R.A. and Steinkamp, J.A. (1985). Correlated measurements of DNA, RNA and protein in individual cells by flow cytometry. Science, 228: 1321-1324.

Crittenden, R., Latila, A., Forssel, P., Matto, J., Saarela, M., Mattila-Sandholm, T.and Myllärinen, P. (2001). Adhesion of Bifidobacteria to granular starch and its implications in probiotic technologies. Appl. Environ. Microbiol. 67(8): 3469-3475

Crowe, J.H. and Clegg, J.S. (1973). Anhydrobiosis (eds.) Dowben, Hutchinson & Ross, Stroudsburg, PG.

Crowe, J.H. and Clegg, J.S. (1978). Dry Biological Systems. Academic Press, New York.

Crowe, J.H. and Crowe, L.M. (1992). Preservation of liposomes by freeze drying. In “Liposome Technology” (G. Gregoriadis, Ed.) 2nd ed. CRC Press, Boca Raton, FL.

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

Crowe, J.H., Carpenter, J.P. and Crowe, L.M. (1998). The role of vitrification in anhydrobiosis. Ann. Rev. Physiol. 60: 73-103.

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

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

Crowe, J.H., Crowe, L.M. and Chapman, D. (1984a). Preservation of membranes in anhydrobiotic organisms: the role of trehalose. Science 223: 701-703.

Crowe, J.H., Crowe, L.M. and Chapman, D. (1984b). Infrared spectroscopic studies on interactions of water and carbohydrates with a biological membrane. Arch. Biochem. Biophys. 232: 400-407.

Crowe, J.H., Crowe, L.M., Carpenter, J.F. and Aurell Winstrom, C.A. (1987). Stabilization of dry phospholipids bilayers and proteins by sugars. Biochem. J. 242: 1-10.

Crowe, J.H., Crowe, L.M., Carpenter, J.F., Prestrelski, S., Hoekstra, F.A., de Araujo, P. and Panek, A.D.(1997a). Anhydrobiosis: Cellular adaptation to extreme dehydration pp. 1445-1477 in Dantzler, W.H. (Ed.). Handbook of physiology. Vol. II) Oxford, Oxford University Press.

Crowe, J.H., Oliver, A.E., Hoekstra, F.A. and Crowe, L.M. (1997b). Stabilization of Dry Membranes by mixtures of hydroxyethyl starch and glucose. The Role of Vitrification. Crybiology 35: 20-30.

Crowe, J.H., Crowe, L.M., Carpenter, J.F., Rudolph, A.S., Anrell Winstrom, C., Spargo B.J. and Anchordoguy, T.J. (1988). Interactions of sugars with membranes. Biochem. Biophys. Acta 947: 367-384.

Crowe, J.H., Crowe, L.M., Oliver, A.E., Tsvetkova, N., Wolkers, W., and Tablin, F. (2001). The trehalose myth revisited: Introduction to a symposium on stabilization of cells in the dry state. Cryobiology 43: 89-105.

Crowe, J.H., Crowe, L.M. and Hoekstra, F.A.(1989a). Phase transitions and permeability changes in dry membranes during rehydration. Mini review. J. Bioenerg. Biomemb. 21, 77-91.

Crowe, J.H., Hoekstra, F.A. and Crowe, L.M. (1989b). Membrane phase transitions are responsible for inhibitional damage in dry pollen. Proceedings of the National Academy of Sciences, USA 86: 520-523.

Crowe, J.H., Hoekstra, F.A., Crowe, L.M., Archordoguy, T.J. and Drobnis, E. (1989c) Lipid phase transitions measured in intact cells with Fourier transform infrared spectroscopy. Cryobiology 26: 76-84.

Crowe, J.H., Hoekstra, F.A., Nguyen, K.H.N. and Crowe, L.M. (1996). Is vitrification involved in depression of the phase transition temperature in dry phospholipids? Biochima et Biophysica Acta. 1280: 187-196

Crowe, J.H., Leslie, S.B. and Crowe, L.M. (1994). Is vitrification sufficient to preserve liposomes during freeze-drying? Cryobiology 31: 355-366.

Crowe, L.M. and Crowe, J.H. (1982). Hydration-dependent hexagonal phase lipid in a biological membrane. Arch. Biochem. Biophys. 217: 582-587.

Crowe, L.M. and Crowe, J.H. (1988). Trehalose and dry dipalmitoyl-phosphatidylcholine revisited. Biochim. Biophys. Acta 946: 193-201.

Crowe, L.M. and Crowe, J.H. (1995). Freeze-dried liposomes. pp. 237-272 in Peuisieux, F.: Couvreur, P., Delattre, J., Devissaguet, J.P.(Eds) Liposomes, new systems and new trends in their applications. Paris, Editions de Sante.

Crowe, L.M., Crowe, J.H., Rudoph, A., Womersley, C. and Appel, L. (1985). Preservation of freeze-dried liposomes by trehalose. Arch. Biochem. Biophys. 242: 240-247.

Csonka, L.N, and Hanson, A.D. (1991). Prokaryotic osmoregulation: genetics and physiology. Ann. Rev. Microbiol. 45:569-606.

Csonka, L.N. (1989) Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. 53: 121-147.

Csonka, L.N. and Epstein, W. (1996). Osmoregulation. In: Neidhardt, F.C. et al. (Ed.), Escherichia coli and Salmonella, ASM Press, Washington pp. 1216-1223.

D'Souza–Ault, M.R., Tombras, S.L. and Smith, G.M. (1993). Roles of N-acetylglutaminylglutamine and glycine betaine in adaptation of Pseudomonas aeruginosa to osmotic stress. Appl. Environ Microbiol. 59: 473-478.

da Silveira, M.G., San Romao, M. V., Loureiro-Dias, M.C., Rombouts, F.M., and Abee, T. (2002). Flow cytometric assessment of membrane integrity of ethanol-stressed Oenococcus oeni cells. Appl. Environ. Microbiol. 68: 6087–6093.

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

Dave, R.I. and Shah, N.P. (1997). Viability of yoghurt and probiotic bacteria in yoghurts made from commercial starter culture. Int. Dairy J. 7: 31-41.

Davey, H.M., Jones, A., Shaw, A.D. and Kell, D.B. (1999). Variable selection and multivariate methods for the identification of microorganisms by flowcytometry. Cytometry 35:162-168.

De Angelis, M., and Gobetti, M. (2004). Environmental stress responses in Lactobacillus. A review. Proteomics 4: 106-122.

Desmond, C., Fitzgerald, G.F., Stanton, C., and Ross, R.P. (2004). Improved stress tolerance of GroESL-overproducing Lactococcus lactis and probiotic Lactobacillus paracasei NFBC 338. Appl. Environ. Microbiol. 70:5929-5936.

Desmond, C., Ross, R.P., O´Callaghan, E., Fitzgerald, G., and Stanton, C. (2002). Improved survival of Lactobacillus paracasei NFCB 338 in spray-dried powders containing gum acacia. J. Appl. Microbiol. 93: 1003-1011.

Desmond, C., Stanton, D., Fitzgerald, G.F., Collins, K., and Ross, R.P. (2001). Environmental adaptation of probiotic lactobacilli towards improvement of performance during spray drying. Int. Dairy J. 12: 183-190.

Diaper, J.P., Thither, K. and Edwards, C. (1992). Rapid assessment of bacterial by flow cytometry.Appl. Microbiol. Biotechnol. 38: 268-272.

Diniz-Mendes, L., Bernardes E., de Araujo, P.S., Panek, A.D. and Paschoalin, V.M.F. (1999). Preservation of frozen yeast cells by trehalose Biotechnol. Bioeng. 65: 572-578.

Dinnbier, U., Limpinsel, E., Schmid, R. and Bakker, E.P. (1988). Transient accumulation of potassium glutamate and its replacement by trehalose during adaptation of growing cells of Escherichia coli K-12 to elevated sodium chloride concentrations. Arch Microbiol. 150: 348: 357.

Donnelly, C.W. and Baigent, G.J. (1986) Method for flow cytometic detection of Listeria monocytogenes in milk. Appl. Environ Microbiol. 52: 689-695.

Earnshaw, R.G., Appleyard, J. and Hurst, R.M. (1995). Understanding physical inactivation processes: combined preservation opportunities using heat, ultrasound and pressure. Int. J. Food Microbiol. 28: 197-219.

Eleutherio, E.C.A., de Araujo, P.S. and Panek, A.D. (1993). Role of the trehalose carrier in dehydration resistance of Saccharomyces cerevisiae. Biochim. Biophys. Acta 1156: 263-266.

Erijman, L. and Clegg, R.M. (1998). Reversible stalling of transcription elongation complexes by high pressure. Biophys. J. 75: 453-462.

Espina, F and Paclard, V.S. (1979). Survival of Lactobacillus acidophilus in a spray drying process. J. Food Prot. 42:149-152

FAO/WHO (2001). Evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Cordoba, Argentina pp 1-34.

Farias-Rodriguez, R., Mellor, R., Arias, C. and Pena-Cabrales, J.J. (1998).The accumulation of trehalose in nodules of several cultivars of common – bean (Phaseolus vulgaris) and its correlation with resistance to drought stress. Physiol Plant 102: 353-359.

Fay, A.C. (934). The effect of hypertonic sugar solution on the thermal resistance of bacteria. J. Agric Res. 48: 453-468.

Ferreira, V., Soares, V., Santos, C., Silva, J., Gibbs, P.A., and Teixeira, P. (2005). Survival of Lactobacillus sakei during heating, drying and storage in the dried state when growth has occurred in the presence of sucrose or monosodium glutamate. Biotechnol. Lett. 27: 249-252.

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

Franks, F. (1993). Solid aqeous solutions. Pure Appl. Chem. 65: 2527-2537.

Franz, C..M.A, and von Holy, A. (1996). Thermotolerance of meat spoilage lactic acid bacteria and their inactivation in vacuum-packaged Vienna sausages. Int. J. Food Microbiol. 29: 59-73.

Frezard, F. (1999). Liposomes: from biophysics to the design of peptide vaccines. Brazilian J. Med. Biol. Res. 32: 181-189.

Fu, W.-Y. and Etzel, M.R.(1995). Spray drying of Lactococcus lactis ssp. lactis C2 and cellular injury. J. Food Sci. 60:195-200.

Fuller, R. (1989). Probiotics in man and animals. J. Appl. Bacteriol. 66:365-378.

Fuller, R. (1992). History and development of probiotics. In "Probiotics: The Scientific Basis, "ed. R. Fuller pp. 1-9. Chapman and Hall, London.

Gadd, G.M., Chalmers, K. and Reed, R.H. (1987). The role of trehalose in dehydration of Saccharomyces cerevisiae FEMS Microbiol. Lett. 48: 249-254.

Galinski, E. A. and Herzog, R. M. (1990). The role of trehalose as a substitute for nitrogen-containing compatible solutes (Ectothiorhodospira halochloris). Arch. Microbiol. 153: 607-613.

Gänzle, M.G. and Vogel, R.F. (2001). On-line fluorescence determination of pressure mediated outer membrane damage in Escherichia coli. Syst. Appl. Microbiol. 24: 477-485.

Gänzle, M.G., Ulmer, H.M. and Vogel, R.F. (2001). High pressure inactivation of Lactobacillus plantarum in a model beer system J. Food Sci. 66: 1174-1181.

Garcia de Castro, A. and Tunnacliffe, A. (2000). Intracellular trehalose improves osmotolerance but not desiccation tolerance in mammalian cells. FEBS Letters 487:199-202.

Garcia-Graells, C., Masschalck, B. and Michiels, C.W. (1999). Inactivation of Escherichia coli in milk by high hydrostatic pressure treatment in combination with antimicrobial peptides J. Food Prot. 62: 1248-1254.

Garcia-Ochoa, F., Santos, V.E., and Alcon, A. (1998) Intracellular compounds quantification by means of flow cytometry in bacteria: application of xanthan production by Xanthomonas campestris Biotech. Bioeng 51: 87-94.

Gardiner, G., Ross, R.P., Collins, J.K., Fitzgerald, G. and Stanton, C. (1998). Development of a probiotic cheddar cheese containing human-derived Lactobacillus paracasei strains. Appl. Environ. Microbiol. 64 (6): 2192-2199.

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

Gibson, G.R. and Roberfroid, M.B. (1995). Dietary modulation of the human colonic microbiota: Introducing the concept of probiotics. J. Nutr. 125: 1401-1412.

Gilliland, S.E. and Lara, R.C. (1988). Influence of storage at freezing and subsequent refrigeration temperatures on β– galactosidase activity of Lactobacillus acidophilus. Appl. Environ. Microbiol. 54: 898-902.

Gilliland, S.E., Nelson, C.R., and Maxwell, C. (1985) Assimilation of cholesterol by Lactobacillus acidophilus. Appl. Environ. Microbiol. 49: 377-381.

Glaasker, E., Konings, W.N. and Poolman, B. (1996). Osmotic regulation of intracellular solute pools in Lactobacillus plantarum. J. Bacteriol. 178: 575-582.

Glaasker, E., Tjan, F.S.B., Ter Steeg, P.F., Konings, W.N. and Poolman, B. (1998). Physiological response of Lactobacillus plantarum to salt and non-electrolyte stress. J. Bacteriol 180 (17): 4718-4723.

Goldin, B.R., and Gorbach, S.L. (1984). The effect of milk and lactobacillus feeding on human intestinal bacterial enzyme activity. Am. J. Clin. Nutr. 39: 756-761.

Gordon-Kann, W.J. and Steponkus, P.L. (1984). Lamellar-to-hexagonal II phase transitions in the plasma membrane of isolated protoplasts after freeze-induced dehydration. Proc. Natl. Acad. Sci. USA 81: 6373-6377.

Gouesbet, G., Jan, G and Boyaval, P. (2001). Lactobacillus delbrueckii spp. bulgaricus thermoleranance. Lait 81: 301-309

Gouffi, K., Pichereau, V., Rolland, J., Thomas, D., Bernard, T., and Blanco, C. (1998). Sucrose is a non-accumulated osmoprotectant in Sinirhizobium meliloti. J. Bacteriol. 180 (19): 5044-5051.

Gould, G.W., Abee, T., Granum, P.E. and Jones, M.V.(1995). Physiology of food poisioning microorganisms and the major problems in food poisoning control. Int. J. Food Microbiol. 28: 121-128.

Guarner, F. and Schaafsman, G.J. (1998). Probiotics. Int. J. Fod Microbiol. 39: 237-238.

Guzzo, J., Cavin, J. and Divies, C. (1994). Induction of stress proteins in euconostic oenis to perform direct inoculation of wine. Biotechnol. Lett. 16: 1189-1194.

Hansen, M.C., Nichsen, A.K., Molin, S., Hammer, K., Kilstrup, M., Palmer Jr. R.J. Udsen, C. and White, D.C. (2001). Changes in rRNA levels during stress invalidate results from mRNA blotting: fluorescence in situ rRNA hybridization permits renormalization for estimation of cellular mRNA levels. J. Bacteriol. 183: 4747-4751.

Hartke, A., Giard, J.C., Laplace, J.M. and Auffray, Y. (1998). Survival of *Enterococcus faecalis* in an oligotropic microcosm:changes in morphology, development of general stress resistance, ans analysis of protein synthesis. Appl. Environ. Microbiol. 64:4238-4245.

Härnulv, B.G., Johansson, M., and Snygg, B.G. (1977). Heat resistance of Bacillus stearothermophilus spores at different water activities. J. Food Sci. 42: 91-93.

Haugland, P. (1999). Molecular Probes-Handbook of fluorescent probes and research chemical. 7th ed. (online) Molecular probes. Inc. Eugene, oreg. CD-ROM and www. probes.com.

Haugland, R.P. (2002). Handbook of fluorescent probes and research products. 9th Ed., Eugene, OR, USA; Molecular probes, Inc.

Havenaar, R. and Huis in't Veld, J.H.J. (1992) Probiotics. A general view In: Wood BJB: The Lactic Acid Bacteria, Vol. 1: The Lactic Acid Bacteria in Health and Disease, Chapman and Hall, New York, NY: 209-224.

Hecker, M., Schumann, W. and Volker, U. (1996). Heat shock and genetic stress response in Bacillus subtilis. Mol. Microbiol. 19: 417-428.

Hekmat, S. and McMahon, D.J. (1992). Survival of Lactobacillus acidophilus and Bifidobacterium bifidum in ice cream for use as a probiotic food. J.Dairy Sci. 75:1415-1422.

Hewitt, C.J. and Nebe-von-Caron, G. (2001). An industrial application of multi-parameter flow cytometry: assessment of cell physiological state and its application to the study of microbial fermentations. Cytometry 44:179-187.

Higl, B., Kurtmann, L., Carslen, C.U., Ratjen, J., Först, P., Skibsted, L.F., Kulozik, U., and Risbo, J. (2007). Impact of water activity, temperature, and physical state on the storage stability of Lactobacillus paracasei ssp. paracasei freeze-dried in a lactose matrix. Biotechnol. Prog. 23: 794-800.

Hirons, G.T., Fawcett, J.J. and Crissman, H.A. (1994). TOTO and YOYO: new very bright fluorochromes for DNA content analyses by flow cytometry. Cytometry 15: 129-140.

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

Holzapfel, W.H., Haberer, P., Snel, J., Schillinger, U.and Huis in't Veld, J.H.J. (1998). Overview of gut flora and probiotics. Int. J. Food Microbiol. 41: 85-101.

Homma, N. (1988). Bifidobacteria as a resistance factor in human beings- Bifidobacteria Microflora 7: 35-43.

Horlacher, R., and Boos, W. (1997). Characterization of TreR, the major regulator of the Escherichia coli trehalose system. J. Biol. Chem. 272: 13ß26-13032.

Hounsa, C.G., Brandt, E.V., Thevelein, J., Hohmann, S. and Prior, B.A. (1998). Role of Trehalose in survival of Saccharomyces cerevisiae under osmotic stress. Microbiology 144: 671-680.

Hughey, V.L. and Johnson, E.A. (1987). Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease. Appl. Env. Microbiol. 53(9): 2165-2170.

IDF (1992). General standard of identity for fermented milks. Int. Dairy Fed. 163.

International Dairy Federation (1993). Dried milk and dried cream. Determination of water content. International Dairy Federation Standard. 26A. International Dairy Federation, Brussels, Belgium.

Ishibashi, N. and Shimamura, S. (1993). Bifidobacteria: research and development in Japan. Food Technol. 47: 126-135.

Jebbar, M., Tabbart, R., Gloux, K., Bernard, T. and Blanco, C. (1992). Osmoprotection of Escherichia coli by ectoine: uptake and accumulation characteristics. J. Bacteriol. 174: 5027-5035.

Jepras, R.I., Paul, F.E., Pearson, S.C. and Wilkinson, M.J.(1997). Rapid assessm,ent of antibiotic effects of Escherichia coli by bis-(1,3-dibutylbarbituric acid) trimethine oxonol and flow cytometry. Antimicrob. Agents Chemother. 41: 2001-2005.

Kamaya, T. (1970). Lytic action of lysozyme on Candida albicans. Mycopathologia et Mycologia applicata 42 (3-4): 197-207.

Kaprelyants, A.S. and Kell, D.B. (1992). Rapid assessment of bacterial viability and vitality by rhodamine 123 and flow cytometry. J. Appl. Bacteriol. 72: 410-442.

Kaprelyants, A.S., Mukamolova. G.V., Davey, H.M. and Kell, D.B. (1996). Quantitative analysis of the physiological heterogeneity within starved cultures of Micrococcus luteus by flow cytometry and cell sorting. Appl. Env. Microbiol. 62: 1311-1316.

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

Keer, J.T. and Birch, L. (2003). Molecular method for the assessment of bacterial viability. J Microbiol. Meth. 53: 175-183.

Kell, D.B., Kaprelyants A.S,, Weichart D.H, Harwood C.R., and Barer M.R (1998). Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. Antonie Leeuwenhoek 73:169-187.

Kell, D.B., Rhyder, H.M., Kaprelyants, A.S and Westerhoff, H.Y. (1991). Quantifying heterogeneity: Flow Cytometry of bacterial cultures. Antonie van Leeuwenhoek 60: 145-158.

Kesarcodi-Watson, A, Kaspar, H., Lategan, M.J., and Gibson, L. (2008). Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. Aquaculture 274. 1-14.

Kessler, U. (1993). Experimentelle Untersuchung und Modellierung der Überlebensrate von Milchsäurebakterien bei der thermischen Trocknung. Münich: Verlag A. Kessler.

Kets, E.P.W, Teunissen, P.J.M & de Bont, J.A.M (1996). Effect of compatible solutes on survival of lactic acid bacteria subjected to drying. Appl. Environ. Microbiol. 62: 259-261

Kets, E.P.W., and de Bont, J.A.M. (1994). Protective effect of betaine on survival of L. plantarum subjected to drying. FEMS Microbiol. Lett. 116:251-256

Kets, E.P.W., and de Bont, J.A.M. (1997). Effect of carnitine on Lactobacillus plantarum subjected to osmotic stress. FEMS Microbiol. Lett. 146:205-209.

Kets, E.P.W., Galinski, E.A. and de Bont, J.A.M (1994). Carnitine: a novel compatible solute in L. plantarum. Archiv. Microbiol. l62: 243-248.

Kets, E.P.W., Groot, M.N., Galinski, E.A. and de Bont, J.A.M. (1997). Choline and acetylcholine novel cationic osmolytes in Lactobacillus plantarum. Appl. Microbiol. Biotechnol. 48:94-98.

Khalil, A.H., and Mansour, E.H. (1998). Alginate encapsulated Bifidobacteria survival in mayonnaise. J. Food Sci. 63: 702-705.

Kilstrup, M., Jacobson, S., Hammer, K. and Vogensen, F.K. (1997). Induction of heat shock proteins Dnak, GroEL and GroES by salt stress in Lactococcus lactis. Appl. Environ. Microbiol. 63: 1836-1837.

Kim, W.S, Perl, L., Park J.H, Tandianus J.E and Dunn, N.W (2001). Assessment of Stress response of the probiotic Lactobacillus acidophilus. Curr. Microbiol. 43: 346-350.

King, V. and Su, J. (1993). Dehydration of Lactobacillus acidophilus. Process Biochem. 28: 47-52.

King, V.A.E. and Lin, H. -J. (1995). Studies on the effect of protectant on Lactobacillus acidophilus strain dehydrated under controlled low-temperature vacuum dehydration and freeze-drying by using response surface methodology. J. Sci. Food Agric. 68:191-196.

Klaver, F.A.M. and Meer, R.V.D. (1993). The assumed assimilation of cholesterol by lactobacilli and Bifidobacterium bifidum is due to their bile salt deconjugating activity. Appl. Environ. Microbiol. 59: 1120-1124.

Klein, G., Pack, A., Bonnaparte, C.and Reuter, G. (1998) Taxonomy and physiology of lactic acid bacteria. Int. J. Food Microbiol. 41:103 – 125.

Klionsky, D.J., Nelson, H. and Nelson, N. (1992). Compartment acidification is required for efficient sorting of proteins to the vacuole in S. cerevisiae. J. Biol. Chem. 267: 3416-3422.

Knorr, D. (1998). Technology aspects related to microorganisms in functional foods. Trends Food Sci. Technol. 9: 295-306.

Knorr, D., Schlüter,O . and Heinz, V. (1998). Impact of high hydrostatic pressure on phase transitions of foods. Food Technol. 52:42-45.

Kobori, H., Sato, M., Tameike, A., Hamada, K., Shimada, S. and Osumi, M. (1995). Ultrastrucultural effects of pressure stress to the nucleus in Saccharomyces cerevisiae: a study of immunoelectron microscopy using frozen thin sections. FEMS Microbiol. Lett. 132: 253-258.

Lankaputhra, W.E.V. and Shah, N.P. (1995). Survival of L. acidophilus and Bifidobacterium spp. in the presence of acid and bile salts. Cult. Dairy Prod. J. 30: 2-7.

Lankaputhra, W.E.V. and Shah, N.P. (1998a) Adherence of probiotic bacteria to human colonic cells. Biosci. Microflora 17: 105-113.

Lankaputhra, W.E.V. and Shah, N.P. (1998b). Antimutagenic properties of probiotic bacteria and of organic acids. Mutation Res. 397: 169-182.

Laplace, J.M., Sauvageot, N., Hartke, A., and Auffray, Y. (1999). Characterization of Lactobacillus collinoides response to heat, acid and ethanol treatments. Appl. Microbiol. Biotechnol. 51: 659-663.

Larsen, P.I., Sydnes, L.K., Lanfald, B. and Strom, A.R. (1987) Osmoregulation in Escherichia coli by accumulation of organic osmolytes: betaines, glutamic acid and trehalose. Arch. Microbiol. 147: 1-7.

Lee, J.C. and Timasheff, S.N. (1981). The stabilization of proteins by sucrose. J Biol. Chem. 256: 7193-7201.

Lee, Y.K. and Salminen, S. (1995). The coming of age of probiotics. Trends Food Sci. Technol. 6: 241-245.

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

Leslie, S.B., Teter, S.A., Crowe, L.M. and Crowe, J.H. (1994). Trehalose lowers membrane phase transitions in dry yeast cells. Biochim. Biophys. Acta 1192: 7-13.

Leverrier, P. Dimova, D., Pichereau, V., Auffray, Y., Patrick, B. and Jan, G. (2003). Susceptibility and Adaptive Response to Bile Salts in Propionibacterium freudenreichii: Physiological and Proteomic Analysis. Appl. Environ. Microbiol. 69 (7): 3809-3818.

Levine, H. and Slade, I. (1992). Another view of trehalose for drying and stabilizing biological materials. BioPharm 5: 36-40.

Lilly, D.M. and Stillwell, R.H. (1965) Probiotics: Growth promoting factors produced by microorganisms. Science, 147: 747-748.

Linders, L.J.M. Meerdink, G., and Van't Riet, K (1997a). Effect of growth parameters on the residual activity of Lactobacillus plantarum after drying. J. Appl. Microbiol. 82: 683-688.

Linders, L.J.M., Meerdink, G., de Jong, G.I.W. and Van't Riet, K. (1997b). Carbohydrates and the dehydration inactivation of Lactobacillus plantarum- The importance of moisture distribution and water activity. J. Food Eng. 31(2): 237-250.

Linders, L.J.M., Wolkers, W.F., Hoekstra, F.A. and van 't Riet, K.(1997c). Effect of added carbohydrates on membrane phase behaviour and survival of dried Lactobacillus plantarum. Crybiology 35: 31-40.

Lippert, K. and Galinski, E.A. (1992) Enzyme stabilization by ectoine-type compatible solutes: protection against heating, freezing and drying. Appl. Microbiol. Biotech. 37: 61-65.

Liu, S. -Q, Asmundson, R.V., Gopal, P.K.., Holland, R. and Crow, V.L. (1998). Influence of reduced water activity on lactose metabolism by Lactococcus lactis subsp. cremoris at different pH values. Appl. Environ. Microbiol. 64: 2111-2116.

Lopez-Amoros, R, Castel, S., Comas-Riu, J. and Vives-Rego, J. (1997). Assessment of E.coli and Salmonella viability and starvation by confocal laser microscopy and flow cytometry using rhodamine 123, DiBAC4(3), propidium iodide, and CTC. Cytometry 29: 298-305

Lopez-Amoros, R., Comas, J. and Vives-Rego, J. (1995) Flow cytometric assessment of Escherichia coli and Salmonella typhimurium starvation survival in seawater using rhodamine 123, DiBAC4(3), propidum iodide, and oxonol. Appl. Environ. Microbiol. 61: 2521-2526.

Lopez-Diez, E.C. and Bone, S. (2004). The interaction of trypsin with trehalose: an investigation of protein preservation mechanisms. Biochim. Biophys. Acta. 1673: 139-148.

Louis, P., Trüper, H.G. and Galinski, E.A. (1994). Survival of Escherichia coli during drying and storage in the presence of compatible solutes. Appl. Microbiol. Biotechnol. 41: 684-688.

Lourens-Hattingh, A. and Viljoen, B.C. (2001). Yoghurt as probiotic carrier food. Int. Dairy J. 11:1-17.

Ludovico, P., Sansonetty, F. and Corte-Real, M. (2001). Assessment of mitochondrial membrane potential in yeast cell populations by flow cytometry. Microbiology 147: 3335-3343.

Madin, K.A.C. and Crowe; J.H. (1975). Comparative physiology and biochemistry anhydrobiosis in nematodes: Carbohydrate and lipid metabolism during dehydration. J. Exp. Zool. 193: 335.

Maftah, A., Huet, O., Gallet, P.F., and Ratinaud, M-H. (1993). Flow cytometry's contribution to the measurement of cell functions. Biol. Cell: 78: 85-93.

Manas, P. and Mackey, B.M. (2004). Morphological and physiological changes induced by high hydrostatic pressure in exponential- and stationary-phase cells of Escherichia coli: Relationship with cell death. Appl. Environ. Microbiol. 70(3): 1545-1554.

Mann, G.V. and Spoerry, A. (1974). Studies of a surfactant and cholesterolemia in the Messai. Am. J. Clin. Nutr. 27 : 464 – 469.

Marnett, L.J, Hund, H., Hollstein, M., Levin, D.E. Esterbauer, H. , and Ames, B.N. (1985). Naturally occuring carbonyl compounds are mutagens in Salmonella tester strain TA 104. Mutation Res. 148: 25-34

Marteau, P., Minekus, M., Havenaar, R. and Huis in't Veld, J.H.J. (1997). Survival of lactic acid bacteria in a dynamic model of the stomach and small intestine: validation and effects of bile. J. Dairy Sci. 80:1031-1037

Martin, M.C., Diaz, L.A., Manzanal, M.B. and Hardisson, C. (1986).Role of trehalose in the spores of Streptomyces. FEMS Microbiol Lett 35: 49-54.

Mason, D.J., Allman, R., Stark, J.M. and Llyod, D. (1994). Rapid estimation of bacterial antibiotic susceptibility with flow cytometry J. .Microsc. 176: 8-16.

Mason, D.J., Lopez-Amoros, R. Allman, R. Stark, J.M. and Llyod, D. (1995). The ability of membrane potential dyes and calcafluor white to distinguish between viable and non-viable bacteria. J. Appl. Bacteriol. 78: 309-315.

Masters, K. (1985). Analytical methods and properties of dried dairy products. In R. Hansen (Ed.) Evaporation, membrane filtration and spray drying in milk powder and cheese production (pp. 393-403) Vanlose Denmark: North European Dairy Journal.

Masters, K. (1991). Spray Drying. Essex, UK: Longman Scientific & Technical and John Wiley & Sons Inc.

Matar, C., Goulet, J., Bernier, R. and Brochu, E. (2000). Bioactive peptides from fermented foods: their role in the immune system. In "Probiotics 3: Immunomodulation by the Gut Microflora and Probiotics. "ed. R. Fuller and G. Perdigon, pp. 193-212. Kluwer Academic Publ. Dordrecht, Netherlands.

Mathys, A., Heinz, V., Schwartz, F.H. and Knorr, D. (2007). Impact of agglomeration on the quantitative assessment of Bacillus stearothermophilus heat inactivation. J. Food Eng. 81: 380-387.

McLaughlin, K. (2007) A New Taste Sensation. www.WSJ.com

Meng, X.C., Stanton, C., Fitzgerald, G.F., Daly, C., and Ross, R.P. (2008). Anhydrobiotics: The challenges of drying probiotic cultures. Food Chem. 106: 1406-1416.

Mermelstein, N.H. (2001). Spray drying. Food Technol. 55:92-95.

Midgley, M. (1987). An efflux system for cationic dyes and related compounds in E. coli. Microbiol. Sci. 4: 125-128.

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

Miller, K.J., Zelt, S.C. and Bae, J. (1991) Glycine betaine and proline are the principal compatible solutes of Staphylococcus aureus. Curr. Microbiol. 23: 131-137.

Misra, A.K. and Kuila, R.K. (1991). Intensified growth of Bifidobacterium and preparation of Bifidobacterium bifidum for a dietary adjunct. Cult. Dairy Prod. J. 26:4-6.

Mitsuoka, T. (1989). Microbes in the intestine. Yakult Honsha Co. Ltd. Tokyo, Japan.

Molenaar, D., Bolhuis, H., Abee, T., Poolman, B. and Konings, W.N. (1992). The efflux of a fluorescent probe is catalyzed by an ATP-driven extrusion system in Lactococcus lactis. J. Bacteriol. 174:3118-3124.

Molenaar, D., Hagting, A., Alkema, H., Driessen, A.J.M. and Konings W.N. (1993) Characterisitcs and osmoregulatory roles of uptake systems for proline and glycine betaine in Lactococcus lactis. J. Bacteriol. 175: 5438-5444.

Molina-Gutierrez, A., Stippl. V., Delgado, A., Gänzle, M.G. and Vogel, R.F. (2002). In situ determination of intracellular pH of Lactococcus lactis and Lactobacillus plantarum during pressure treatment. Appl. Environ. Microbiol. 68: 4399-4406.

Nebe-von Caron, G. and Badley, R.A. (1995). Viability assessment of bacteria in mixed populations using flow cytometry. J. Microsc. 179:55-66.

Nebe-von Caron, G., Stephens, P. and Badley, R.A. (1998). Assessment of bacterial viability status by flow cytometry and single cell sorting. J. Appl. Microbiol. 84:988-998.

Nebe-von Caron, G., Stephens, P.J., Hewitt, C.J. Powell, J.R. and Badley, R.A. (2000). Analysis of bacterial function by multicolour fluorescence flow cytometry and single cell sorting. J. Microbiol. Meth. 42: 97-114.

Neidhardt, F.C., Van Bogelen, R.A., and Vaughan, V. (1984). The genetics and regulation of heat-shock proteins. Annu. Rev. Genet. 18:295-329-

Newman, D.K. and Banfield, J.F. (2002). Geomicrobiology: how molecular scale interactions underpin biogeochemical systems. Science 296: 1071-1077.

Newman, Y.M., Ring, S.G. and Colaco, C. (1993). The role of trehalose and other carbohydrates in biopreservation. Biotechnol. Genet. Eng. Rev. 11: 263-294.

Nexmann Jacobsen, C., Rasmussen, J. and Jakobsen, M (1997). Viability staining and flow cytometric detection of Listeria monocytogenes. J. Microbiol. Meth. 28: 35-43.

Niven, G.W., Miles, C.A., and Mackay, B.M. (1999). The effect of hydrostatic pressure on ribosome conformation in Escherichia coli: an in vivo study using differential scanning calorimetry. Microbiology 145: 419-425.

Noh, D.O., and Gilliland, S.E. (1993). Influence of bile on cellular integrity and beta-galactosidase activity of Lactobacillus acidophilus. J.Dairy Sci. 76: 1253-1259.

Novo, D., Perlmutter, N.G., Hunt, R.H. and Shapiro, H.M.(1999) Accurate flow cytometric membrane potential measurement in bacteria using diethytoxacarbocyanine and a ratiometric technique. Cytometry 35: 55-63.

Obis, D., Guillot, A., Gripson, J.C.,Renault, P., Boltin, A. and Mistou, M.Y. (1999). Genetic and biochemical characterization of a high affinity betaine uptake system (BusA) in Lactoccus lactis reveals a new functional organization within bacterial ABC transporters J. Bacteriol. 181: 6238-6246.

Olutiola, P.O., Famurewa, O. and Sonntag, H.G. (1991). An Introduction to General Microbiology. A practical approach. Heidelberger Verlagsanstalt und Drukerei GmbH Heidelberg.

Onwulata, C.I., Ramkishan Rao, D. and Vankineni, P. (1989). Relative efficiency of yoghurt, sweet acidophilus milk, hydrolysed – lactose milk and a commercial lactase tablet in alleviating lactose maldigestion. Am. J. Clin. Nutr. 49: 1233-1237.

Ouwehand, A.C., Kurvinen, T., and Rissanen, P. (2004). Use of a probiotic Bifidobacterium in a dry food matrix, an invivo study. Int. J. Food Microbiol. 95: 103-106.

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

Pagan, R., Jordan, S., Beito, A. and Mackey, B.M. (2001). Enhanced acid sensitivity of pressure-damaged Escherichia coli 0157 cells. Appl. Environ. Microbiol. 67: 1983-1985.

Palmfeldt, J., and Hahn-Hagerdal, B. (2000). Influence of culture pH on survival of Lactobacillus reuteri subjected to freeze drying. Int. J. Food Microbiol. 55: 235-238.

Panoff, J.M., Thammavong, B. and Gueguen, M. (2000). Cryoprotectants lead to phenotypic adaptation to freeze-thaw stress in Lactobacillus delbrueckii ssp. bulgaricus. Ctp 101027T. Cryobiology 40: 264-269.

Park, H.-K., So, J.-S. and Heo, T.-R. (1995).Acid adaptation promotes survival of Bifidobacterium breve against environmental stresses. Foods and Biotechnol. 4: 226-230.

Patist, A. and Zoerb, H. (2005). Preservation mechanisms of trehalose in food and biosystems. Colloids and Surfaces B: Biointerfaces 40: 107-113.

Patterson, M. (1999). High – pressure treatment of foods, p. 1059-1065 In. R.K. Robertson, A. Batt and P.D. Patel (ed.). The encyclopedia of food microbiology. Academic Press, London, United Kingdom.

Patterson, M.F., Quinn, M., Simpson, R. and Gilmour, A. (1995). Sensitivity of vegetative pathogens to high hydrostatic pressure in phosphate – buffered saline and foods. J. Food Prot. 58:524-529.

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

Pilon-Smits, E.A.H., Ebskamp, M.J.M., Paul, M.P., Jeuken, M.J.W., Weisbeek, P.J., and Seekems, S.C.M. (1995). Improved performance of transgenic fructan-accumulating tobacco under drought stress. Plant Physiol. 107:125-130.

Pinder, A.C., Purdy, P.W., Poulder, S.A.G. and Clark, D.C. (1990); Validation of flow cytometry for rapid enumeration of bacterial concentrations in pure cultures. J. Appl. Bacteriol. 69: 92-100.

Piper, P.W. (1993). Molecular events associated with acquisition of heat tolerance by the yeast Saccharomyces cerevisiae. FEMS Microbiol. Rev. 11: 339-355

Pocard, J.A., Smith, L.T., Smith, G.M. and Le Rudulier, D. (1994). A prominent role for glucosy lglycerol in the adaptation of Pseudomonas mendocina SKB 70 to osmotic stress. J. Bacteriol. 176: 6877-6884.

Poolman, B. and Glaasker, E. (2009). Compatible solute accumulation in bacteria. Mol. Microbiol. (in press).

Poolman, B. (1993). Energy transduction in lactic acid bacteria. FEMS Microbiol. Rev. 12: 125-147.

Poolman, B. and Glaasker, E. (1998). Regulation of compatible solute accumulation in bacteria. Mol. Microbiol. 29: 397-407.

Porter, J., Deere, D., Pick-up, R. and Edwards, C. (1996) Fluorescent probes and flow cytometry: new insights into environmental bacteriology. Cytometry 23: 91-96.

Porter, J., Edwards, C. and Pickup, R.W. (1995). Rapid assessment of physiological status in Escherichia coli using fluorescent probes. J. Appl. Bacteriol. 79: 399-408.

Porubcan, R.S. (2006). Formulations to increase in vivo survival of probiotic bacteria and extend their shelf life. Retrieved October 17, 2006 from www.patentstorm.us/patents/7122370.html

Porubcan, R.S. and Sellars, R.L. (1975). Spray drying of yoghurt and related cultures. J. Dairy Sci. 58 (5): 787.

Prasad, J., McJarrow, P. and Gopal, P. (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.

Prestrelski, S.J., Tedeschi, N., Arakawa, T., and Carpenter, J.F. (1993). Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. Biophys. J. 65: 661-671.

Rahman, D.(2005). Introduction to Flow Cytometry. Serotec Ltd. 22 Bankside, Kidlington, UK pp.4-7.

Ratinaud, M.H. and Revidon, S. (1996). Flow cytometric method to assess functional state of the Listeria membrane. J. Microbiol. Meth. 25: 71-77.

Re, M.I. (1998). Microencapsulation by spray drying. Drying Technol. 16: 413-425.

Reed, R.H., Richardson, D.L., Warr, S.R. and Stewart, W.D. (1984). Carbohydrate accumulation in cyanobacteria. J. Gen. Microbiol. 130: 1-4.

Reid, A.A., Champagne, C.P., Gardner, N., Fustier, P., and Vuillemard, J.C. (2007). Survival in food systems of Lactobaciilus rhamnosus R011 microentrapped in whey protein gel particles. J. Food Sci. 72 (1) : M31-M37.

Richards, A.B.., Krakowka, S., Dexter, L.B., Shmid, H., Wolterbeek, A.P. Waalkens-Berendsen, D.H., Shigoyuki, A. and Kurimoto, M. (2002). "Trehalose: a review of properties, history of use and human tolerance, and results of multiple safety studies. Food Chem. Toxicol. 40(7): 871-98.

Ritz, M., Pholozan, J.L, Federighi, M., and Pilet, M.F, (2001). Morphological and physiological characterization of Listeria monocytogenes subjected to high hydrostatic pressure. Appl. Environ. Microbiol. 67: 2240-2247.

Roberfroid, M.B. (1998) Prebiotics and Synbiotics: Concepts and nutritional properties. British J. Nutr. 80: 197-202.

Roos, Y. and Karel, M. (1990). Differential scanning calorimetry study of phase transitions affecting the quality of dehydrated materials. Biotechnol. Prog. 6: 159-163.

Roos, Y., and Karel, M. (1991). Plasticizing effect of water on thermal behaviour and crystallization of amorphous and food models. J. Food Sci. 34: 324-329.

Roos, Y.H. (2002). Importance of glass transition and water activity to spray drying and stability of dairy powders. Lait. 82: 475-484.

Rosin, M.P. and Zimmerman, A.M. (1977). The induction of cytoplasmic peptide mutants of Saccharomyces cerevisiae by hydrostatic pressure. J. Cell. Sci. 26: 373-385.

Ross, R.P., Desmond, C., Fitzgerald, G.F. and Stanton, C. (2005). Overcoming the technological hurdles in the development of probiotic foods. J. Appl. Microbiol. 98: 1410-1417.

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

Russell, N.J. and Fukanaga, N. (1990). A comparison of thermal adaptation of membrane lipids in psychrophilic and thermophilic bacteria. FEMS Microbiol. Rev. 75: 171-182.

Saarela, M., Rantala, M. Hallamaa K., Nohynek, L., Virkajarvi, I and Matto, J. (2004). Stationary-phase acid and heat treatment for improvement of viability of probiotic lactobacilli and bifido bacteria. J. Appl. Microbiol. 96 (6): 1205-1214.

Saarela, M., Virkajärvi, I., Alakomi, H.-L, Mattila-Sandholm, T., Vaari, A., Suomalainen, T., and Mätto, J. (2005). Influence of fermentation time, cryoprotectant and neutralization of cell concentrate on freeze-drying survival, storage stability, and acid and bile exposure of Bifidobacterium animalis ssp. lactis cells produced without milk-based ingredients. J. Appl. Microbiol. 99: 1330-1339.

Saarela, M.H., Alakomi, H.-L, Puhakka, A., and Mätto, J. (2009). Effect of the fermentation pH on the storage stability of Lactobacillus rhamnosus preparations and suitability of in vitro analyses of cell physiology fuctions to predict it. J. Appl. Microbiol. 106:1204-1212.

Salminen, S, Ouwehand, A.C. and Isolauri, E. (1998). Clinical applications of probiotic bacteria. Int. Diary J. 8:563-572.

Sandine W. E. (1996). Commercial production of dairy starter cultures, p191-206 In T.M. Cogan and J. -P. Accolas (ed.), Dairy Starter cultures. VCH publishers Inc., New York, N.Y.

Sato, T., Ohsumi, Y., Anraku, Y. (1984). Substrate specificities of active transport systems for amino acids in vacuolar-membrane vesicles of S. cerevisiae. J. Biol. Chem. 259: 11505-11508.

Schebor, C., Buera, M.P. and Chirife, J. (1996). Glassy state in relation to the thermal inactivation of enzyme invertase in amorphous dried matrices of trehalose, maltodextrin and polyvinylpyrolidone J.Food Eng. 30: 269-282.

Schebor, C., Burin, L., del Pilar Beura, M. and Chirife, J. (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.

Scheyhing, C.H., Hörman, S., Ehrmann, M.A., and Vogel, R.F. (2004). Barotolerance is inducible by preincubation under hydrostatic pressure, cold-, osmotic and acid-stress conditions in Lactobacillus sanfranciscensis DSM 20451. Lett. Appl. Microbiol. 39: 284-289.

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.

Segal, G. and Ron, E.Z. (1998). Regulation of heat-shock response in bacteria. Ann. N.Y. Acad. Sci. 851:147-151

Seo, J.H. and Bailey, J.E. (1987). Cell cycle analysis of plasmid containing Escherichia coli HB101 populations with flow cytometry. Biotech. Bioeng. 30: 297-305.

Severin, J., Wohlfahrt, A. and Galinski, E.A. (1992). The predominant role of recently discovered tetrahydropyrimidines for the osmoadaptation of halophilic eubacteria. J. Gen. Microbiol. 138:1629-1638.

Shah, N.P. (2001). Functional Foods from probiotics and prebiotics Food Technol. 55(11): 46-53.

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

Shapiro, H.M. (1995). Practical flow cytometry. 3rd ed. Wiley-Liss, Inc. New York, N.Y.

Shapiro, H.M. (2001). Multiparameter flow cytometry of bacteria: Implication for diagnostics and therapeutics. Cytometry 43:223-226.

Shin, H.S., Lee, J.H., Pestka, J.J., and Ustunol, Z. (2000). Growth and viability of commercial Bifidobacterium ssp. in skim milk containing oligossacharides and inulin. J. Food Sci. 65:884-887.

Siegumfeldt, H., Rechinger, K.B., and Jakobsen, M. (2000). Dynamic changes of intracellular pH in individual lactic acid bacterium cells in response to a rapid drop in extracellular pH. Appl. Environ. Microbiol. 66:2330-2335.

Silva, A., Carvalho, A.S., Teixeira, P. and Gibbs, P.A. (2002). Bacteriocin production by spray-dried lactic acid bacteria. Lett. Appl. Microbiol. 34: 77-81.

Silva, J., Carvalho, A.S., Ferreira, R., Vitorino, R., Amado, F., and Domingues, P. (2005). Effect of the pH of growth on the survival of Lactobacillus delbrueckii subsp. bulgaricus to stress conditions during spray drying. J. Appl. Microbiol. 98: 775-782.

Simpson, R.K. and Gilmour, A. (1997). The effect of high hydrostatic pressure on the activity of intracellular enzymes of Listeria monocytogenes. Lett. Appl. Microbiol. 25: 48-53.

Slade, L., and Levine, H. (1991a). A food polymer science approach to structure – property relationships in aqueous food systems: non-equilibrum behaviour of carbohydrate – water systems. In water relationships in Foods; Levine H., Slade, L. Eds.: Plenum Press: New York pp. 29-101.

Slade, L. and Levine, H. (1991b). Beyond water activity: recent advances based on an alternative approach to the assessment of food quality and safety. Crit. Rev. Food Sci. Nutr. 30:115-360.

Slade, L. and Levine, H. (1994). Glass transitions and water-food structure interactions. In Advances in Food and Nutrition Research: Kinsella, J. E., Ed.; Academic Press: San Diego, CA. Vol. 38.

Sola-Penna, M. and Meyer-Fernandes, J.R. (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.

Somero, G.N. (1995). Adaptations to high hydrostatic pressure. Ann. Reviews Physiol. 54: 537-577.

Spargo, B.J., Crowe, L.M., Ioneda, T., Beaman, B.L. and Crowe, J.H. (1991). Cord factor (α,α – trehalose 6, 6'dimycolate) inhibits fusion between phospholipids membranes. Proc Natl Acad Sci. 88: 737-740.

Steen, H.B; Boye, E; Skarstad, K; Bloom, B; Godal, T; Mustafa, S.(1982). Applications of flow cytometry on bacteria: cell cycle kinetics, drug effects and quantification of antibody binding. Cytometry 2: 249-260.

Stockall, A.M. and Edwards, C. (1985) Changes in respiratory activity during encysment of Azotobacter vinelandii. J. Gen. Microbiol. 131: 1403-10.

Strom, A.R. and Kaasen, I. (1993). Trehalose metabolism in Escherichia coli: Stress protection and stress regulation of gene expression. Mol. Microbiol 8: 205-210.

Stumbo, C.R. (1965). Death of bacteria subjected to moist heat p. 55-78. In: M.L. Anson, C.O. Chichester, E.M. Mrak and G.F. Stewart (ed.) Thermobacteriology in food processing. Acaemic Press Inc. New York., N.Y.

Stute, R., Klingler, R.W., Boguslawski, S., Esthiaghi, M.N. and Knorr, D. (1996). Effects of high pressures treatment on starches. Starch 48: 399-408.

Suita-Cruce, P. and Goulet, J. (2001). Improving probiotic survival rates: Microencapsulation preserves the potency of probiotic microorganisms in food systems. Food Technol. 55 (10):36-42

Sunny-Roberts, E.O. and Knorr, D. (2007) Physiological analysis of Lactobacillus rhamnosus VTT E-97800 adaptive response to osmotic stress induced by trehalose. British Food J. 109(9): 735-748.

Sunny-Roberts, E.O. and Knorr, D. (2008) Evaluation of the response of Lactobacillus rhamnosus VTT E-97800 to sucrose-induced osmotic stress Food Microbiol. 25 (1):183-189.

Sunny-Roberts, E.O., Ananta, E. & Knorr. D. (2007). Flow cytometry assessment of Lactobacillus rhamnosus GG (ATCC 53103) Response to Non-electrolytes stress. Nurt. Food Sci. 37 (3): 184-200.

Talibart, R., Jebbar, M., Gouesbet, G., Himdi-Kabba, S., Wroblewski, H., Blanco, C. and Bernard, T. (1994). Osmoadaptation in rhizobia: ectoine-induced salt tolerance. J. Bacteriol. 176: 5210-5217.

Talwalker, A. and Kailasapathy, K. (2004). A review of oxygen toxicity in probiotic yoghurts. Influence on the survival of probiotic bacteria and protective techniques. Comp. Rev. Food Sci. & Food Safety 3:117-124.

Tamura, K., Miyashita, M., and Iwahashi, H. (1998). Stress tolerance of pressure shocked Saccharomyces cerevisiae. Biotechnol. Lett. 20: 1167-1169.

Taneja, S. and Ahmed, F. (1994). Increased themal stability of proteins in the presence pf amino acids. Biochem. J. 303: 147-153.

Teixeira, P., Castro, H. and Kirby, R. (1994). Inducible thermotolerance in Lactobacillus bulgaricus. Lett. Appl. Microbiol. 18: 218-221.

Teixeira, P., Castro, H. and Kirby, R. (1996). Evidence of membrane lipid oxidation of spray dried Lactobacillus bulgaricus during storage. Lett. Appl. Microbiol. 22: 34-38.

Teixeira, P., Castro, H., Mohacsi-Farkas, C. and Kirby, R. (1997). Identification of sites of injury in Lactobacillus bulgaricus during heat stress. J. Appl. Microbiol. 83: 219-226.

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

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

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

Terebiznik, M.R., Buera, M.P., and Pilosof, A.M.R. (1997). Thermal stability of dehydrated a-amylase in trehalose matrices in relation to its phase transitions. Lebensmittel-Wissenschaft und -Technologie (lwt). 30: 513-518.

Thanassi, D.G., Cheng, L.W., and Nikaido, H. (1997). Active efflux of bile salts by Escherichia coli. J. Bacteriol. 179: 2521-2518.

Tharmaraj, N., and Shah, N.P. (2004). Survival of Lactobacillus acidophilus, Lactobacillus paracasei, Lactobacillus rhamnosus, Bifidobacterium animalis, and Propionibacterium in chees-based dips and the suitability of dips as effective carriers of probiotic bacteria. Int. Dairy J. 14: 1055-1066.

Thevelein, J.M. (1984). Regulation of trehalose mobilization in fungi. Microbiol. Rev. 48: 42-59.

Thevelein, J.M. (1996). Regulation of trehalose metabolism and its relevance to cell growth and function. In: Marzluf G.A. (eds). The mycota vol. 3. Springer, Berlin Heideberg New York, pp. 395-414.

Todar, K. (2009). Online textbook of Bacteriology. www.textbookofbacteriology.net pp.1-10.

Turner, K., Porter, J., Pick-up. R and Edwards, C. (2000). Changes in viability and macromolecules content of long term batch cultures of Salmonella typhimurium measured by flow cytometry. J. Appl. Microbiol. 89: 90-99.

Tymczyszyn, E. E., Gomez-Zavaglia, A., and Disalvo, E.A. (2007). Effect of sugars and growth media on the dehydration of Lactobacillus delbrueckii subsp. bulgaricus. J. Appl. Microbiol. 102: 845-851.

Ueckert, J., Breeuwer, P., Abee, T., Stephens, P., Nebe-von-Caron, G. and ter Steeg, P.F. (1995). Flow cytometry applications in physiology study and detection of food borne microrganisms. Int. J. Food Microbiol. 28: 317-326.

Uguen, P., Hamelin, J., Le Pennec, J-P. and Blanco C. (1999). Influence of osmolarity and the presence of an osmoprotectant on Lactococcus lactis growth and bacteriocin production. Appl. Environ. Microbiol. 65 (1):291-293.

Ulmer, H.M., Gänzle, M.G. and Vogel, R.F. (2000). Effects of high pressure on survival and metabolic activity of L. plantarum TMWI.460. Appl. Environ. Microbiol. 66: 3966-3973.

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

Van de Guchte M., Serror, P., Chervaux, C.,Smokvina, T., Ehrlich, S.D. and Maguin, E. (2002). Stress responses in lactic acid bacteria. Antonie van Leeuwenhoek 82: 187-226

Van Eck, J.H., Prior, B.A. and Brandt, E.V. (1993). The water relations of growth and polyhydroxy alcohol production by ascomyscetous yeasts. J. Gen. Microbiol. 139: 1047-1054.

Van Laere, A. (1989). Trehalose, reserve and/or stress metabolite? FEMS Microbiol. Review 63: 201-210.

Visick, J. and Clark, S. (1995). Repair, refold, recycle: how bacteria can deal with spontaneous and environmental damage to proteins. Mol. Microbiol. 16: 835-845

Vives-Rego, J., Lebaron, P. and Nebe-von Caron, G. (2000). Current and future applications of flow cytometry in aquatic microbiology. FEMS Microbiol. Rev. 24: 429-448.

Webb, S.J. (1965). Bound water in Biological Integrity. Thomas, Spring-field, III.

Weitzel, G., Pilatus, U., and Rensing, L. (1987). The cytoplasmic pH, ATP content and total protein synthesis rate during heat shock protein inducing treatments in yeast. Exp. Cell. Res. 170: 64-79

Welch, T.J. and Bartlett, D.H. (1988). Identification of a regulatory protein required for pressure-responsive gene expression in the deep-sea bacterium Photobacterium species strain. SS9. Mol. Microbiol. 27:977-985.

Welch, T.J., Farewell, A., Neidhardt, F.C. and Bartlett, D.H. (1993). Stress response of Escherichia coli to elevated hydrostatic pressure. J. Bacteriol. 175: 7170-7177.

Welsh, D.T. and Herbert, R.A. (1993). Identification of organic solutes accumulated by purple and green sulphur bacteria during osmotic stress using natural abundance 13C nuclear magnetic resonance spectroscopy. FEMS Microbiol. Ecol. 13:445-449.

Welsh, D.T. and Herbert, R.A. (1999). Osmotically induced intracellular trehalose but not glycine betaine accumulation promotes dessication tolerance in Escherichia coli. FEMS Microbiol. Lett. 174:57-63.

Welsh, D.T., Guyoneaud, T. and Caumette, P. (1998) Utilization of the compatible solutes sucrose and trehalose by purple sulphur and non-sulphur bacteria. Can. J. Microbiol. 4: 974-979.

Welsh, D.T., Reed, R.H. and Herbert, R.A. (1991). The role of trehalose in the osmoadaptation of Escherichia coli NCIB 9484: interaction of trehalose, K+ and glutamate during osmoadaptation in continuous culture. J. Gen. Microbiol. 137: 745-750.

Wemekamp-Kamphuis, H. H., Karatzas, A. K., Wouters, J.A. and Abee, T. (2002). Enhanced levels of cold shock proteins in Listeria monocytogenes L028 upon exposure to low temperature and high hydrostatic pressure. Appl. Environ. Microbiol. 68:456-463.

Whatmore, B.M. and Reed, R.H. (1990). The effects of osmotic upshock on the intracellular solute pools of Bacillus subtilis J. Gen. Microbiol. 136:2527-2535.

Xie, G.F. and Timasheff, S.N. (1997). The thermodynamic mechanism of protein stabilization by trehalose Biophys. Chem. 64: 25-43.

Zaragoza, A., Aranda, F.J., Espuny, M.J., Teruel, J.A., Marques, A., Angeles, M., and Antonio, O. (2009). Mechanism of membrane permeabilization by a bacterial trehalose lipid biosurfactant produced by Rhodococcus sp. Langmuir (in press).

Zarate, G., Chaia, A.P., Gonzalez, S., and Oliver, G. (2000). Viability and beta-galactosidase activity of dairy propionibacteria subjected to digestion by artificial gastric and intestinal fluids. J. Food Prot. 63: 1214-1221.

Zayed, G., and Roos, Y.H. (2004). Influence of trehalose and moisture content on survival of Lactobacillus salivarius subjected to freeze-drying and storage. Process Biochem. 39: 1081-1086.

Zimmerman, A.M.(1971). High pressure studies in cell biology. Int. Rev. Cytol. 30: 1-47.

Curriculum vitae

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