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).

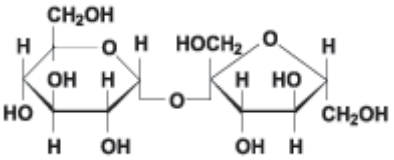
The disaccharide trehalose is widely distributed in nature and can be found in many organisms, including bacteria, fungi, plants, invertebrates and mammals. It consists of two subunits of glucose bound by a α: $1 \rightarrow 1$ linkage (α-D-glucopyranosil α-D-glucopyranoside) and is thus non-reducing. 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) 

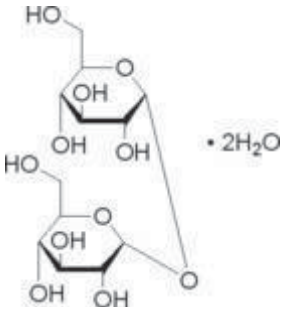
(b) 

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)*.