Studies on the stability of probiotic bacteria during long term storage

A thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Food Technology at Massey University, Palmerston North, New Zealand

Arup Nag

2019

Massey University

Abstract

According to the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), probiotics are defined as ''live microorganisms which when administered in adequate amounts confer a health benefit for the host'' (FAO/WHO, 2001). Lactobacilli and bifidobacteria are two major group of organisms considered to have probiotic properties. The primary objective of this project was to develop a novel stabilization technology for probiotic bacteria, through which a range of probiotic bacterial strains could potentially be delivered to the host through shelf stable dry and intermediate moisture foods. For preliminary experiments (reported in Chapter 4.0), *Lactobacillus casei* 431, a commercial strain from Chr Hansen, Denmark, was chosen as the experimental strain and milk powders (both skimmed and full-fat) were chosen as the principal supporting agent while stabilizing the bacterial cells.

Stabilization efficiency in terms of long term ambient temperature storage viability was compared using freeze and fluidized bed drying techniques. Fluidized bed drying was able to retain 2.5 log cfu/g higher viability after 52 weeks of storage at 25 °C. A combination of fluidized bed drying and osmotic stress adaptation to the probiotic cells yielded further improvement of 0.83 log cfu/g higher viability compared to the unstressed cells. The findings were validated with other two lactobacilli and two bifidobacterium strains with probiotic characteristics and significant improvements in storage stability over freeze-dried samples were observed. Fortification of vitamin E in the stabilization matrix as an antioxidant improved the stability by 0.18 log cfu/g during

20 weeks storage period at 25 °C, whereas any similar benefit of fortifying inulin as a prebiotic was not observed. Incubation in simulated gastric fluid and intestinal fluid (*in vitro*) revealed that the *L. casei* 431 cells were better protected within the stabilized matrix than in the free form. The survival of the stabilized cells were 5.0 and 2.1 log cycles higher than free cells in gastric juice and bile salt solution respectively. Physical characterization of the probiotic ingredient showed very good flow-ability and solubility, with 470 Kg/m3 bulk density, water activity of 0.27 and agglomerated particles of 125.6 µm mean diameter.

Thereafter, the project aimed to understand the underlying mechanism of the processes responsible for gradual decay in cell viability of another probiotic strain (*Lactobacillus reuteri* LR6) during long term storage at 37 °C (Chapter 5.0 onwards). Vacuum drying of sorbitol- or xylitol-coated *Lactobacillus reuteri* LR6 cells and fluidized bed drying of the same coated cells with different excipients were compared for the cell viability post drying. LR6 cells coated with xylitol and desiccated in unsupported form or together with skim milk powder as an excipient were found to be better protected when exposed to moderate as well as high drying temperatures. In Chapter 6.0, a closer examination of the protein and polypeptide components of the cell envelopes (amide regions) via Fourier transform infrared spectroscopy revealed different degrees of structural deformation in individual samples, which correlated well with the residual cell viability.

It was also important to understand the underlying mechanisms responsible for the loss of viability of stabilized probiotic cells when stored at non-refrigerated temperatures. In Chapter 7.0, the stabilized *Lactobacillus reuteri* LR6 cells were stored at 37 °C and at

two water activity (a_w) levels. Superior storage stability was recorded in a lower a_w environment, supported by a stronger glassy matrix when skim milk powder was used as the excipient. Fourier transform infrared spectroscopic examination of the cell envelopes revealed substantial dissimilarities between samples at the beginning and at the end of the storage period. In milk powder-based matrices, adjusting the a_w to 0.30 resulted in a weaker or no glassy state whereas the same matrices had a high glass transition temperature at a_w 0.11. This strong glassy matrix and low a_w combination was found to enhance the bacterial stability at the storage temperature of 37 °C. During storage of the stabilized cells for 121 days at 37 °C, the measured T_g for all the samples was slightly lower than what was recorded at the beginning. Scanning electron microscopy revealed the formation of corrugated surfaces and blister-type deformations on the cell envelopes during the stabilization process whereas the freshly harvested cells were found to be with a smooth surface and undamaged membrane. Inspection of the cell bodies via transmission electron microscopy showed freshly harvested cells with normal shapes with no damage in the inner membrane structure. An almost intact but slightly waved outer membrane structure was observed. The findings emphasize the importance of protecting the integrity of the membrane of probiotic cells by using suitable protecting agents to enhance their stability during long term storage.

The stabilized cell matrix samples were segregated into 4 groups based on the average particle diameter by passing through sieves of different mesh sizes. The degree of agglomeration had a very important role in offering physical protections to the LR6 cells during the desiccation process. The viable cell populations in the higher particle size groups (above 500µm and 1000µm) were between 9.5 to 9.9 log cfu/g whereas the same

for the lower particle size (below 500µm but above 250µm) group was only 7.8 log cfu/g. The minimum viable cell concentration was recorded (7.3 log cfu/g) in the finer particles having less than 250µm diameter but having the maximum mass fraction. In case of stored samples, it was found that the bacterial cells adhered to the finest particles suffered the maximum loss in viability (41.4%) whereas the minimum loss (14.9%) was within the particles with average diameter above 500µm.

In order to assess the effect of stabilization and storage (12 weeks, 37 °C) on the common probiotic attributes of the LR6 cells, an *in vitro* study on acid, bile salts tolerance and surface hydrophobicity was conducted. The results showed considerable reductions in cell viability for the desiccated as well as stored cells when incubated in simulated gastric (acid tolerance) and intestinal (bile salts tolerance) environments. A coating of xylitol over the cell bodies during desiccation was found to be marginally protective against these stresses. High a_w storage was found to be more detrimental to the cells in terms of their ability to survive in the acid or bile environments. The cell surface hydrophobicity towards various hydrocarbons was also found to be adversely affected due to desiccation and non-refrigerated storage. Considerable degradation in hydrophobicity was found to be occurring in the cells stored at a_w 0.30, a trend similar to the acid and bile resistance properties.

To my wife Tuli, my son Antariksh, and my parents

For your love and support

Acknowledgements

Table of Contents

Contents

Contents

Chapter 4.0 Improving ambient temperature stability of probiotics with stress adaptation and fluidized bed drying………………....……….99

Contents

Contents

List of tables

Tables

List of figures

Figures

Figures

Figures

Chapter 1.0. Introduction

At the beginning of the 20th century, the importance of lactobacilli for human health was first hypothesized by the famous Russian biologist Ilya Metchnikoff. He considered that the total community of gut microbes may be detrimental and suggested that the desirable effects can be obtained if they are supplemented with the yoghurt bacteria (Holzapfel *et al*., 1998). Since then a lot of research efforts and commercial practices have been guided towards improving the human and animal health status by modulating the gut microbiota with live microbial adjuncts, also known as probiotics.

According to the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), probiotics are defined as ''live microorganisms which when administered in adequate amounts confer a health benefit for the host'' (FAO/WHO, 2001). Probiotic bacteria are known for conferring beneficial effects on human gut health. However, the successful delivery of these bacteria to the human intestine via proper food matrices is challenging because the stresses encountered by the probiotics during processing, storage and gastric transition cause major loss of viability (Semyonov *et al*., 2012). Major stresses involved in the food manufacturing processes are heat, sub-zero processing, and osmotic pressure. Considerable loss of cell viability also takes place during storage of the longer shelf life products, particularly in case of non-refrigerated storage and in presence of light, oxygen, and high moisture (Dianawati *et al*., 2013).

Following ingestion with food or in the form of capsules (dietary supplement), the bacteria are exposed to the very harsh acidic environment in the stomach where the pH ranges from 1.0 to 2.0 and thereafter again to the detrimental bile salts present in the intestinal pathway. Probiotics are also exposed to milder acidic environments when added to fermented food, juice or beverages. The exact mechanism of bile salt toxicity is yet to be completely understood but it has been reported that bile salts being surface active amphipathic molecules, possess some antimicrobial properties and their detergent-like nature aids in disassembling bacterial cell membranes (De Angelis and Gobbetti, 2004). Osmotic stress is induced to probiotic bacteria when the solute concentrations of the surrounding media changes suddenly. It is important to maintain the cytoplasmic solute concentration at a constant level for the bacteria to survive. An increase in media osmolarity results in diffusion of water from cell inside to outside, thereby causing changes in cell volume and subsequent cell deaths (Poolman and Glaasker, 1998). Lactobacillus and bifidobacterium, the major two probiotic species are anaerobic in nature and therefore exposure to oxygen during processing and storage creates another stressful environment to them (Guerzoni *et al.*, 2001). Overall, it is clear that a number of challenges are involved in the process of successful delivery of viable probiotic cultures right from the collection and growth stage up to the human intestinal walls where they are expected to colonize in order to impart the expected health benefits.

Probiotic bacteria are natural inhabitants of the human gastrointestinal tract but due to the action of antibiotic compounds (e.g. treatment of infections with antibiotics), the presence of the microflora is badly affected. Therefore, it is important to restore their balance in the gut, by supplying from an outside source in the form of dietary

supplements or functional foods containing live probiotic cultures. In doing so, the cultures need to be grown, stored and applied to food products. During the manufacturing process used in food products, the probiotic bacteria are subjected to various kinds of stresses such as heat, cold, osmotic pressure and oxygen. The most detrimental among them is heat stress (e.g. during the spray drying process). A huge loss of cell viability takes place during storage as an ingredient or as a part of any food product.

The choice of ingredients for the protective matrix is an important factor in increasing the stability of the probiotic bacteria. A variety of food materials including carbohydrates, polysaccharides, plant-hydrocolloids, bacterial exopolysaccharides, plant and milk proteins, and cellulose have been used in the encapsulation matrix, the most common among them being sodium alginate gels in the presence of divalent cations (Anal and Singh, 2007). The use of milk proteins in the encapsulation of probiotics has been studied with great interest due to its well-known functional properties (Rosenberg and Sheu, 1996; Chen and Subirade, 2007). Some of the functional properties of milk proteins, for example, bland flavor, high solubility, emulsifying and film forming nature and low viscosity in solutions have made them very attractive for use as one of the shell materials in the microencapsulation process (Madene *et al.*, 2006; Heidebach *et al.*, 2009). Food proteins easily meet GRAS standards, have high nutritional value and excellent gelation, foaming, and water binding capacity, thus making them highly suitable for probiotics or any kind of neutraceutical carrier material, which can be orally administered (Chen *et al.*, 2005).

Probiotic-containing food products are one of the major contributors to the growing functional food markets around the world. Probiotics are found in traditional fermented dairy products, such as fermented milk, yoghurts, and cheese. However, it is now also common for them to be in a stabilized form and fortified into powdered foods or nutraceutical formulations. At the end of their shelf life (typically 6 months to 1 year for dried products), the products need to contain cell viability of at least 10^6 colony forming units (cfu) per gm to confer the desired health benefits (FAO/WHO, 2003). Unfortunately, maintaining the viability of probiotics in non-refrigerated foods over long shelf life is difficult. To date, non-refrigerated foods have generally failed to meet the required viability target at the end of shelf-life and probiotic foods outside the chilled dairy area remain scarce. Where they are included in dried foods (for example, infant formula), a considerable degree of "overage" is required (that is, the addition of much higher viable counts at the start of shelf life in order to account for losses in viability during storage). Probiotic cultures are expensive ingredients (usually costing > USD $ 1400 per kg) and the need to add up to 10-100 fold overages to account for 1-2 $\log_{10}$ declines in viability adds considerable cost to the final product. Hence, there is a strong industry demand for technologies that can improve probiotic survival in foods outside the traditional chilled, fermented dairy sector.

This project was initiated with the primary objective of developing a novel encapsulation or stabilization technology for probiotic bacteria, through which a range of probiotic bacterial strains could potentially be delivered in shelf-stable dry and intermediate moisture foods. The first part of this thesis (Chapter 4.0) describes the development of this technology to encapsulate a probiotic strain *Lactobacillus casei*

CRL431, the achievements and shortcomings. The developed technology (named as Probiolife) was patented and now going through the process for commercial applications. However, the technology does have some limitations. It is able to successfully maintain the required level of probiotic cell viability at 25 ºC storage for at least 9 months, but this is a lower temperature and duration than would be required for certain applications and locations. Thereafter the project aimed to address these limitations by understanding the underlying mechanism of the processes responsible for gradual decay in cell viability of another probiotic strain (*Lactobacillus reuteri* LR6) during long term storage at increased storage temperature (Chapter 5.0 onwards). It is believed that proper understanding of the responsible factors would help further development of the technology to achieve the stated goal. *Lactobacillus reuteri* LR6 is one of the well characterized and clinically validated strains from National Dairy Research Institute (NDRI), India but need to be stabilized and delivered through a shelf stable food matrix ideal for the tropical climate of India.

The importance of ambient temperature stable probiotic products in India is very high considering its tropical climate and the absence of any established cold chain distribution system throughout the country. Poor digestive health conditions (due to lack of hygienic practice and poor quality drinking water in many parts of India) and compromised overall immunity, especially among children in the rural sectors of India, are of major concern. The most popular probiotic strains with proven health benefits available from commercial manufacturers are mostly of European origin. However, India is currently developing guidelines that may mean probiotic strains sold there will have to be of Indian origin, and the health claims backed by clinical trials conducted on

Indian populations if they are to gain market approval. This is primarily driven by concern about the inherent differences in gut microbiota of Indian population compared to their European counterparts. Therefore, the isolation, selection, and characterization of probiotic strains of Indian origin (*Lactobacillus reuteri* LR6) and the development of a suitable stabilization technology to enable these strains to be delivered to the Indian population via the available distribution channels while retaining the required level of activity offer significant commercial opportunity.

The key research questions of this project and the corresponding objectives can be summarised into the following parts:

1)   Established methods of freeze-drying probiotic cells are not ideal for subsequent ambient temperature storage and the commercial manufacturers do not recommend this. What alternative mode of stabilizing probiotics can maximize the viability during ambient storage?

Objective 1: To optimize the fermentation process and combine with the most suitable drying technology in order to achieve the maximum quantity of residual viable cells following long-term storage at elevated temperature.

2)   During the stabilization process, if the probiotic cells are directly exposed to a range of drying temperatures, what type of changes take place in the cell composition, particularly in the cell protein secondary structures? Secondly, what is the most critical and differentiating factor responsible for any

superior protection available to the probiotic cells when desiccated in the presence of different supporting agents?

Objective 2: To identify and explain the changes related to the cell composition of the experimental probiotic strain during desiccation in unsupported form and in the presence of common supporting excipients.

3)   Is the loss of probiotic cell viability only predominant during the desiccation process or does it continue during storage as well? What causes such loss in viability in a stabilized state during storage at elevated temperature?

Objective 3: To analyze the periodical cell viability and explain any gradual loss in viability by identifying any conformational changes in the cell composition.

4)   Whether the probiotic attributes remain unaltered due to processing and exposure to adverse storage environment? Should viability be the only criterion in assuring the quality of probiotic cells present in a product at any time within its shelf life?

Objective 4: To compare the already validated probiotic attributes (*in vitro*) of the experimental strains such as, tolerance to gastric juice, duodenal juice and bile environments, adhesion properties, with the stabilized cells after the processing and moderate temperature storage period.

Chapter 2.0. Literature review

2.1 Introduction

The literature review is divided into 4 parts. Part 1 provides an introduction to probiotic bacteria, their selection criterion and the challenges associated with handling them. This part also briefly discusses the health benefits of the probiotic strains and associated regulatory aspects.

Part 2 describes the importance of stabilizing probiotic bacteria, the processes involved and the consequences of applying such processes on the physiological state of the bacteria. It also identifies the most suitable processing techniques that could be used for stabilizing the experimental probiotic strain keeping the project objectives in mind. A detailed comparison of the benefits and shortcomings of each process has been done.

Part 3 discusses the problems associated with delivering probiotics in a stabilized format which should be shelf stable as well for a considerable period of time. It identifies the most relevant previous approaches to address these problems and takes clues from them to progress our understanding and insight into the stabilization of probiotics.

Part 4 of this review identifies the advanced analytical techniques which could potentially be applied for our purpose based on their usefulness as found by previous researchers. This section also gives a detailed description of the characteristics of the

experimental probiotic strain and its potential health benefits based on previous validation studies.

2.2 What are probiotic bacteria?

2.2.1 Definitions

Probiotic bacteria have been defined in many ways but perhaps the most widely used and accepted definition is proposed by Fuller (1992) according to which probiotics are living microorganisms which when ingested have beneficial effects on the host by improving the physiological functions of the intestinal microflora. Another definition had been provided by Havenaar and Huis In't Veld (1992), which explains probiotics as "a preparation of a product containing viable, defined microorganisms in sufficient numbers, which alter the microbiota (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host". Probiotics have also been defined as "live microbes which transit the gastro-intestinal tract and in doing so benefit the health of the consumer" (Tannock *et al.*, 2000), which contradicts the earlier definitions focusing only on the interactions between the probiotic organisms and the original intestinal microflora (Fuller, 1989). Another definition can be found in The Food and Agricultural Organization of the United Nations and the World Health Organization's documents which define probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). But all of these definitions agree on one point that probiotics must deliver some

kind of health benefit and should be living in nature, and able to form colonies on the intestinal wall.

2.2.2 Characteristics of probiotic bacteria

A particular bacterial strain is considered as probiotic in nature only when it fulfills certain criteria. These include the physiological and manufacturing demands through which the bacterial strain must survive and also they should have all the capabilities to exert the beneficial effects on the host as per their basic definition (O'Grady and Gibson, 2005). One of the conditions namely, colonization, was however challenged by Tannock (2003), according to whom the transit time of the intestinal tract allows smooth multiplication of bacteria even if there is cell loss with fecal material. This becomes important while considering the nature of allochthonous microorganisms which exerts effect from their luminal habitat and this proves colonization is not an essential condition for a probiotic strain (O'Grady and Gibson, 2005).

Probiotic dairy products are categorized as 'novel type' and the European market study shows a steadily increasing range of yoghurt like products claiming to contain probiotic strains (Reuter, 1997). A probiotic microorganism is expected to promote the health of the host in general but to critically identify a particular strain as probiotic, the following three categories of key criteria have been identified. (Havenaar and Huis In't Veld, 1992; Holzapfel *et al*., 1998).

2.2.2.1 General microbiological criteria

The factors to be considered in this case are safety or non-pathogenicity, survival strength through the human defense system in the upper part of GIT (containing saliva, gastric juice, and bile juice), moreover, it should be of human origin and must have genetic stability.

2.2.2.2 Technological aspects

The strain should be adaptive to the most common manufacturing stresses and should impart an acceptable shelf life and sensory qualities in terms of color, aroma, taste, and texture. Viability throughout the period of claimed shelf life is also an important factor.

2.2.2.3 Functional benefits of probiotic bacteria

This is the most important and a particular strain is considered as probiotic if it is able to confer certain beneficial traits to the human body.

The important functional benefits obtained in general from a single or group of probiotic strains include immune modulation and improving the strength of gut mucosal barrier. These are achieved by modification of the gut microflora, the capability to adhere with intestinal mucosa which in turn helps to prevent pathogen adherence or activation, by modifying the dietary proteins and bacterial enzyme capacity and by influencing the gut mucosal permeability (Holzapfel *et al.*, 1998).

According to Kailasapathy and Chin (2000), "probiotics have been reported to play a therapeutic role by modulating immunity, lowering cholesterol, improving lactose tolerance and preventing some cancers." The probiotic health benefits are also associated with digestive and respiratory functions, prevention and restriction of infectious disease in children and other high-risk populations (FAO/WHO, 2001).

2.2.3 Probiotics in human gastro-intestinal tract

The human gastrointestinal tract (GI) is a very complex and sophisticated ecosystem comprising of more than 400 different species and out of them, about 40 species predominate. The GI tract starts from the oral cavity, which is made up of mouth, nose, and throat and a complex microbiota exists here. It has been reported that a relatively high number of bacteria can be found on the posterior and anterior tongue, sub and supragingival plaque, buccal mucosa and vestibular mucosa (Vandenplas *et al*., 2015). The acidic environment inside the stomach provides a highly effective barrier against pathogenic and benign invading microorganisms and here the cell count falls below 10^3 cfu/ml of the gastric contents. The small intestine does not provide a healthy environment for the growth of bacteria due to rapid transit time and the presence of bile. The same holds true for duodenum which also has low microbial population and secrets intestinal fluid, creating a hostile environment (Sarkar, 2013). However, along the jejunum and ileum the numbers, as well as the variety of bacterial species, increase progressively. The large intestine is favorable for the growth of these bacteria due to factors like slow transit time, a favorable pH environment and availability of nutrients

(Pace *et al.*, 2015). The small intestine contains species such as enterococci, enterobacteria, lactobacilli, Bacteroides and clostridia in the range of $10^4 - 10^6$ cfu/ml, and this population goes up to $10^{11} - 10^{12}$ cfu/ml in the large intestine (Vinusha *et al.*, 2018).

The human gastrointestinal tract remains sterile until the birth of a newborn and then the microbial colonization process begins during the delivery process. The inoculum is mainly obtained from the mother's vaginal or fecal flora in case of conventional birth or from the environment when there is caesarean delivery. This probiotic population gradually evolves in a fashion which can best cope with the physiological and microbiological pressure encountered within the ecosystem. This resistance, also known as 'barrier effect', helps the indigenous microbiota to fight against invading pathogens and to compete for nutrients (Alderbeth *et al.*, 2000). In the very beginning, *E. coli* and *Streptococcus* dominate but gradually along with breastfeeding, Bifidobacterium population starts to increase with a decrease of the other two bacteria. There is a strong indication that the type of diet greatly influences the diversity of the intestinal microflora of a newborn (Jazayeri *et al.*, 2017).

The healthy intestinal epithelium having a good intestinal flora can create a barrier against the uptake of pathogens, antigens and other harmful compounds from the gut lumen. The intestinal mucosa also is very efficient in assimilating antigens and evoking specific immune responses (Heyman *et al.*, 1982; Holzapfel *et al.*, 1998). Baba *et al.* (1991) showed that intestinal probiotics challenge the invading microorganisms and prevent diseases through competitive exclusion and this was best demonstrated by

showing that germ-free animals were more susceptible to infection and diseases. It is, therefore, understood that the composition of intestinal microflora greatly influences the gastrointestinal health and affects the host health (O'Grady and Gibson, 2005).

Figure 2.1: *Microbial colonization of the human gastro-intestinal tract (Simon and Gorbach, 1982; Holzapfel et al., 1998)*

The intestinal flora has a wide range of beneficial activities, such as degrading certain food components, producing certain B vitamins, stimulating the immune system and producing digestive and protective enzymes. It also helps in metabolizing some carcinogenic substances. The colonic flora produces short-chain fatty acids (SCFA) which are passively absorbed by the enterocytes (Venema, 2018).

2.2.4 Some common probiotic species and strains

Nowadays a large number of species have been recognized as having probiotic characteristics. Major probiotic bacteria, mainly the lactobacilli and bifidobacterium are

of high importance to the researchers and the majority of studies have been carried out on these strains. Consequently, they have been used widely in the food industry as probiotic organisms. Probiotics are commercially marketed either in a lyophilized form or as a part of the fermented food products. *L. acidophilus*, *L. casei* strain *Shirota*, *L. rhamnosus* and *L. reuteri* are the most popular choices and have a long application history followed by some bifidobacterium spp. and also a few non-lactic bacterial species which are mainly used in pharmaceutical applications (Williams, 2010). Table 2.1 shows a list of popular probiotic strains.

Table 2.1: *Microorganisms used or considered for use as probiotics in human (Williams, 2010)*

2.2.4.1 Lactobacillus species

Lactobacilli are one of the most abundant probiotic organisms and are characterized by these most common properties, such as gram-positive, non-sporulating, catalase-negative and oxidase-negative, devoid of cytochromes, non-aerobic in nature but can tolerate oxygen to some extent, are fastidious and strictly fermentative. The end product of their sugar fermentation is lactic acid (Axelsson, 1993; Anal and Singh, 2007). They have beneficial effects on the gut health in the form of easy digestion, immune stimulation and inhibition of pathogens (Stanton *et al.*, 2003). Lactobacilli are not the major species present in the gastrointestinal tract but normally they are very easily cultivated there and they have been used safely for a long time in various food applications (Stanton *et al.*, 2003).

The most common among the all species found in the human gut is *Lactobacillus acidophilus* complex, *Lactobacillus salivarius* and *Lactobacillus casei* complex. *Lactobacillus acidophilus* complex group comprises of 6 similar phenotype traits but *L. acidophilus* is most commonly used in probiotic preparations due to its acid and bile resistant qualities, antimicrobial activity and survivability during the gastro-intestinal transit (Quigley and Eamonn, 2019). *Lactobacillus salivarius* is known for its resistant property to acid and bile, good adherence quality to gastric epithelial cells and smooth transportability through the intestinal tract. *Lactobacillus casei* complex group includes *Lactobacillus casei/paracasei*, *Lactobacillus rhamnosus GG* and *Lactobacillus casei shirota* (Quigley and Eamonn, 2019).

2.2.4.2 Bifidobacterium species

Bifidobacteria are classified as Gram-positive, non-sporulating, catalase-negative, non-acid fast bacilli. They are strict anaerobes and are difficult to cultivate in milk and other food preparations (Vuyst *et al*., 2004). They have a low tolerance to acid, only tolerant to pH 4.5 and above, and are unable to grow in a medium with high oxidative potential (Stanton *et al*., 2003). The carbohydrate fermentation end product of bifidobacteria are mainly acetic acid and lactic acid but not carbon dioxide, butyric acid or propionic acid (Anal and Singh, 2007). Bifidobacteria are the predominant variety in infant's colon, with the *Bifidobacterium longum*, *B. infantis* and *B. breve* dominating and they are the third most populous in the adult colon with the maximum presence of *Bifidobacterium longum* and *B. adolescentis* and therefore are frequently used as probiotic cultures (Vuyst *et al*., 2004).

2.2.5 Health benefits and human clinical trials with probiotic bacteria

There is a huge body of research publications on human clinical trials to determine the efficacy of probiotic bacteria. This subject is not in the scope of the current review, but a short glimpse of the important and common studies is mentioned below. For more details see recent reviews by Fijan (2014), Basso *et al*. (2019) and Perez *et al*. (2019).

Idiopathic inflammatory bowel disease is the most common disease associated with disturbed gut microflora and two major forms of this are Crohn's disease and ulcerative

colitis. Several attempts have been made to treat these two diseases (Scarpato *et al*., 2017; Ganji-Arjenaki and Rafieian-Kopaei, 2018; Basso *et al*., 2019) with the application of probiotics.

Pouchitis is a type of chronic inflammation of the ileal pouch which follows the colectomy in ulcerative colitis patients. The effectiveness of probiotics in treating this disease has been investigated in a number of studies, the most interesting one being that of Gionchetti *et al*. (2000), who used a randomized controlled trial among 40 patients and found that a significant percentage of people receiving probiotics showed improvement in pouchitis condition in comparison to those taking only maize starch placebo (O'May and Macfarlane, 2005).

The other important gastro-intestinal diseases on which clinical trials have been conducted with probiotics and found successful are irritable bowel syndrome (Hod *et al*., 2018; Maity and Gupta, 2019), antibiotic-associated diarrhoea and colstridium difficile associated diarrhoea (Maragkoudaki *et al.*, 2018), traveller's diarrhoea (Muller, 2016), infant diarrhoea (Gaon *et al*., 2003; McFarland *et al*., 2018).

Among the extra-gastrointestinal conditions in which probiotics application have been widely tested are atopic dermatitis and bacterial vaginosis (Ibanez, 2018; Pramanick *et al.*, 2018). In this context, another summarized and published table from Fijan (2014) is thought to be relevant and therefore reproduced in Table 2.2.

Table 2.2: *Recently published claimed health benefits of probiotic microorganisms (Fijan, 2014)*

Genus	**Species**	**Recently published health claims**
Lactobacillus	*L. rhamnosus*	Reduction of viral-associated pulmonary damage (*L. rhamnosus* CRL1505); prevention and reduction of severity of atopic dermatitis in children (*L. rhamnosus* GG); reduction of risk for developing allergic disease (*L. rhamnosus* GG), (*L. rhamnosus* HN001; anti-diabetic potential (various strains from human infant faecal samples); prevention of necrotizing enterocolitis in newborns (*L. rhamnosus* GG); prevention or treatment of bacterial vaginosis (*L. rhamnosus* GR-1); aid in weight loss of obese women (*L. rhamnosus* CGMCC1.3724); treatment of acute gastroenteritis in children (*L. rhamnosus* GG); reduction of risk for rhinovirus infections in preterm infants (*L. rhamnosus* GG and *L. rhamnosus* ATCC 53103); protection of human colonic muscle from lipopolysaccharide-induced damage (*L. rhamnosus* GG)
	L. acidophilus	Treatment of travellers' diarrhoea; reduction of hospital stay of children with acute diarrhoea; antifungal activity (*L. acidophilus* ATCC-4495); prevention or treatment of bacterial vaginosis; treatment of *C. difficile*-associated diarrhoea; reduction of incidence of febrile urinary tract infections in children; reduction of irritable bowel syndrome symptoms.
	L. plantarum	Prevention of endotoxin production; antifungal activity *(L. plantarum* NRRL B-4496) reduction of irritable bowel syndrome symptoms.
	L. casei	Treatment of functional constipation in adults (*L. casei* Lcr35 and *L. casei* Shirota); treatment of *C. difficile*-associated diarrhoea; restoration of vaginal flora of patient with bacterial vaginosis (*L. casei* Lcr35); reduction of irritable bowel syndrome symptoms; reduction of diarrhoea duration of antibiotic-associated diarrhoea in geriatric patients (*L.*

Genus	Species	Recently published health claims
		casei Shirota); immunomodulatory mechanisms (*L. casei* Shirota); improvement of rheumatoid arthritis status (*L. casei* 01); protection against *Salmonella* infection (*L. casei* CRL-431); prevention of *Salmonella*-induced synovitis; treatment of intravaginal staphylococcosis (*L. casei* IMV B-7280).
	L. delbrueckii subsp. *bulgaricus*	Antibiotic resistance of yogurt starter culture; enhancement of systemic immunity in elderly (*L. delbrueckii* subsp. *bulgaricus* 8481); antibacterial action against *E. coli*; modulation of brain activity.
	L. brevis	Protective role in bile salt tolerance (*L. brevis* KB290); reduction in plague acidogenicity (*L. brevis* CD2).
	L. johnsonii	Impact on adaptive immunity for protection against respiratory insults; reduction of occurrence of gastritis and risk of *H. pylori* infection (*L. johnsonii* MH-68); inhibition of *S. sonnei* activity (*L. johnsonii* F0421); treatment of perennial allergic rhinitis in children together with levocetirizine (*L. johnsonii* EM1).
	L. fermentum	Prevention or treatment of bacterial vaginosis (*L. fermentum* RC-14); blockage of adherence of pathogenic microorganisms on vaginal epithelium; antistaphylococcal action (*L. fermentum* ATCC 11739); potential for reduction of insulin resistance and hypercholesterolemia (*L. fermentum* NCIMB 5221).
	L. reuteri	Reduction of low-density lipoprotein cholesterol (*L. reuteri* NCIMB 30242); treatment of acute gastroenteritis in children; reduction of diarrhoea duration in children (*L. reuteri* ATCC 55730); management of infant colic (*L. reuteri* ATCC 55730 and *L. reuteri* DSM 17938); reduction of onset of gastrointestinal disorders in infants (*L. reuteri* DSM 17938); reduction of frequency of proven sepsis, feeding intolerance and duration of hospital stay in preterm infants (*L. reuteri* DSM 17938).
Bifidobacterium	*B. infantis*	Reduction of irritable bowel syndrome symptoms;

Genus	Species	Recently published health claims
		reduction of necrotizing enterocolitis in preterm infants.
	B. animalis subsp. *lactis*	Treatment of functional constipation in adults (*B. animalis* subsp. *lactis* DN-173 010); reduction of incidence of febrile urinary tract infections in children; modulation of brain activity; reduction of necrotizing enterocolitis in preterm infants; reduction of total microbial counts in dental plaque (*B. animalis* subsp. *lactis* DN-173 010); reduction of total cholesterol (*B. animalis* subsp. *lactis* MB 202/DSMZ 23733); reduction of risk of upper respiratory illness (*B. animalis* subsp. *lactis* BI-04).
	B. bifidum	Reduction of hospital stay of children with acute diarrhoea; reduction of necrotizing enterocolitis in preterm infants; reduction of total cholesterol (*B. bifidum* MB 109/DSMZ 23731).
	B. longum	Prevention and treatment of necrotizing enterocolitis in newborns; reduction of radiation induced diarrhoea; reduction of necrotizing enterocolitis with Bifidobacteria cocktail (*B. breve*, *B. infantis*, *B. bifidum*, *B. longum*); reduction of irritable bowel syndrome symptoms; treatment of gastrointestinal diseases (*B. longum* CMCC P0001); perinatal intervention against onset of allergic sensitization (*B. longum* CCM 7952).
	B. breve	Prevention and treatment of necrotizing enterocolitis in newborns; reduction of necrotizing enterocolitis with Bifidobacteria cocktail (*B. breve*, *B. infantis*, *B. bifidum*, *B. longum*); reduction of cholesterol (*B. breve* MB 113/DSMZ 23732).
Saccharomyces	*S. boulardi*	Treatment of travellers' diarrhoea; treatment and reduction of diarrhoea duration regardless of cause; treatment of irritable bowel syndrome; treatment of moderate ulcerative colitis; treatment and reduction of recurrent pseudomembrane colitis infection caused by *C. difficile*; treatment of acute gastroenteritis in children.

Genus	Species	Recently published health claims
Lactococcus	*L. lactis* subsp. *lactis*	Treatment of antibiotic-associated diarrhoea; adhesion to vaginal epithelial cells (*L. lactis* subsp. *lactis* KLDS4.0325); nisin production (*L. lactis* subsp. *lactis* CV56); modulation of brain activity; antimicrobial activity against *C. difficile*; antimicrobial and probiotic properties (*L. lactis* subsp. *lactis* ATCC 11454).
Enterococcus	*E. durans*	Antibiotic and antioxidant activity (*E. durans* LAB18s), adherence to colonic tissue and anti-inflammatory activity.
	E. faecium	Treatment of antibiotic-associated diarrhoea; efficient animal probiotic.
Streptococcus	*S. thermophilus*	Reduction of irritable bowel syndrome symptoms; antibiotic resistance of yogurt starter culture; reduction of necrotizing enterocolitis in preterm infants.
Pediococcus	*P. acidilactici*	Pediocin production with antimicrobial and probiotic properties (*P. acidilactici* UL5); bacteriocin production; elimination of *H. pylori* infections (*P. acidilactici* BA28).
Leuconostoc	*L. mesenteroides*	Leucoin production, probiotic profile (survival at low pH, in presence of bile salts, in presence of pepsin) (*L. mesenteroides* B7).
Bacillus	*B. coagulans*	Treatment of antibiotic-associated diarrhoea, treatment of bacterial vaginosis (*B. coagulans* ATCC PTA-11748); immunological support (*B. coagulans* GandenBC30); prevention of caries in children.
	B. subtilis	Efficient animal probiotic; treatment of diarrhoea and aiding in *H. pylori* eradication (*B. subtilis* R0179); production of nitric oxide.
	B. cereus	Efficient animal probiotic (*B. cereus* NVH75/95).
Escherichia	*E. coli* Nissle 1917	Treatment of functional constipation in adults; treatment of inflammatory bowel disease; treatment of gastrointestinal disorders; pro-inflammatory potential; prevention of surface ocular diseases; reduction of *Salmonella enterica* Typhimurium intestinal colonization by iron competition.

2.2.6 Regulations governing foods containing probiotics

Although the concept of using beneficial bacteria in food products originated at the very beginning of the 20th century, the term 'probiotic' appeared in the documents only in the 1960s. From a legal perspective, a number of definitions have appeared in the scientific literature but no internationally recognized legal definition has been published so far. However, since the products containing probiotic cultures are becoming very popular, risk is always there that consumers will be misled in terms of health claims or number of viable probiotic cells present in the product (Hicky, 2005). In the European Union (EU), the proposed regulation defines probiotic bacteria as "live food supplements which benefit the health of consumers if consumed regularly in sufficient quantities". It also proposes that any kind of health claim should be supported by sufficient scientific data and a product label should contain the quantity of food to be consumed necessarily to obtain the claimed health benefit, the persons who should avoid the product and if applicable, a warning showing the possible bad effects of consuming the product in excess of the recommended quantity (Hicky, 2005). According to an experts' report from United Nations Food and Agriculture Organization (FAO) and World Health Organization (WHO), a product claiming to have a probiotic culture in it should contain at least $10^6 – 10^7$ cfu of live viable bacteria per gram of the product (FAO/WHO, 2001).

The United Kingdom, being a member state of the EU, is expected to implement the same community legislation. Even before the EU came into existence, in order to protect consumers and to promote fair trade, a Functional Food Initiative was taken up by the

Ministry of Agriculture, Food and Fisheries (MAFF) in 1994 to get an overview of the marketplace and thereafter a Joint Health Claim Initiative (JHCI) was established in 1997 which involved consumer groups, enforcement authorities, and industry representatives. The JHCI came up with some regulations which clearly mentioned the health claim requirements (Hicky, 2005).

In the USA, however, there is no specific legislation on probiotic or functional foods. The Food and Drug Administration (FDA) has classified all the possible foods into four categories, viz. conventional foods, dietary supplements, foods for special dietary use and medicinal foods. In theory, probiotic products can be placed into any of the above categories but mostly the commercial products have been marketed as a dietary supplement only (Hicky, 2005).

The Codex Alimentary Commission (CAC) has adopted a new standard after its 26th session in July 2003 which is expanded to a broad range of fermented products including yogurt, sweetened/flavored yogurt, kefir, acidophilus milk, and koumiss. Although this standard does not mention probiotics but includes a minimum level of starter culture organisms of 1×10^{7} cfu g^{-1} and if a specific microorganism other than the normal starter culture is claimed to be present then the minimum level should be 1×10^{6} cfu g^{-1} (FAO/WHO, 2003; Hicky, 2005).

2.2.7 Mechanism of probiotic functionalities

The microbiota of the intestine is mainly involved in the fermentation process of various endogenous and exogenous growth substrates, the metabolic end products of which are beneficial to human health (O'Grady and Gibson, 2005; Diether & Willing, 2019). It is believed that one of the mechanisms utilized by the beneficial probiotics present in the gut is by colonic fermentation of soluble fibre, which is resistant to the action of pancreatic amylases and also by saccharolysis of oligosaccharides, which results in the production of various short-chain fatty acids (SCFA), such as, butyrate, acetate, propionate and lactate. These SCFA's finally result in a lower pH of the lumen which protects against invading pathogenic microorganisms and also reduces the transformation of primary bile acids into secondary pro-carcinogenic bile acids (Diether & Willing, 2019).

The mechanism of the beneficial traits offered by probiotic microorganisms to human host has been well described (Dunne *et al*., 1999; Kailasapathy and Chin, 2000; Naidu and Clemens, 2000; Andersson *et al.*, 2001; Isolauri and Salminen, 2005). By reinforcing the body's natural defenses and keeping out harmful pathogens by competitive exclusion, probiotics make their way to grow and colonize in the intestinal wall (Anal and Singh, 2007). This anti-pathogenic mechanism consists of several antagonist properties, including (a) reduction of luminal pH by producing a series of volatile short-chain fatty acids (SCFA); (b) making specific nutrients unavailable to the pathogenic organisms; (c) decreasing the redox potential thus suitable for only anaerobic

probiotic growth; (d) production of hydrogen peroxide in anaerobic condition; and/or (e) production of pathogen inhibitory compounds known as bacteriocins (Naidu and Clemens, 2000; Hoover and Chen, 2005).

2.2.7.1 Reduction of luminal pH

The fermentation process for carbohydrates in the lumen of the colon uses lactate dehydrogenase to convert pyruvate and thereby produces lactic acid and other short chain fatty acids such as acetic, propionic and butyric acid (Kailasapathy and Chin, 2000). These acids reduce the intestinal lumen pH which is unfavorable for both Gram-positive and Gram-negative bacteria.

Lipophilic acids such as, lactic and acetic acid are present in undissociated forms in the colon. These acids possibly penetrate through the pathogenic cell membranes and inhibit their growth (Naidu and Clemens, 2000). Acetic and lactic acids produced by bifidobacteria have been shown to have inhibitory effects against *Salmonella* spp., *E. coli* and *Staphylococcus aureus* (Naidu and Clemens, 2000).

Apart from pH reduction, probiotics have been reported to produce phenols, ammonia, steroid metabolites and bacterial toxins which are also helpful in pathogenic bacterial growth restriction (Hidaka and Eida, 1988; Naidu and Clemens, 2000).

2.2.7.2 Production of hydrogen peroxide

Lactic acid bacteria (LAB), such as bifidobacterium and lactobacilli, are able to produce hydrogen peroxide (H_2O_2) by using the electron transport chain. The strong oxidizing property of H_2O_2 affects the permeability of the pathogenic cell membranes. Specifically, the actions on cell components such as nucleic acids are responsible for the antimicrobial activity of LAB (Naidu and Clemens, 2000).

2.2.7.3 Production of bacteriocins

Bacteriocins are defined as a variety of antimicrobial proteins including metabolic products, antibiotic-like substances and bactericidal proteins produced by probiotic bacteria (Naidu and Clemens, 2000). Bacteriocins act through adsorption to receptors on the surface of target bacteria and they are species specific. The destruction of the target bacteria is the result of biological, morphological and metabolic changes caused by the bacteriocins.

Bacteriocins are mainly of 4 major types – antibiotics, heat-stable peptides, large heat-labile proteins and complex proteins whose activity require the association of carbohydrate or lipid moieties (Naidu and Clemens, 2000; Hoover and Chen, 2005). The major bacteriocins documented are nisin, pediocin, sakacin and reuterin, all produced by LAB. Nisin, produced by *L. lactis* has a specific antagonistic activity to *L. monocytogenes* through the formation of pores in cell membranes (Hoover and Chen, 2005). Similarly, reuterin produced by *L. reuteri* is a broad spectrum bacteriocin capable

of inhibiting Gram-negative species such as, salmonella and shigella and Gram-positive species such as, clostridia and listeria (Hoover and Chen, 2005).

2.2.7.4 Fermentative production of carbon dioxide

The production of carbon dioxide by lactobacilli is well described by Naidu and Clemens (2000). Hexose fermentation by heterofermentative lactobacilli results in the production of carbon dioxide. The production of carbon dioxide displaces oxygen in the colon and also decreases the pH. Thereby, an anaerobic environment is created where strict aerobic organisms find difficulty to proliferate.

2.2.7.5 Diacetyl production by fermentation

By the fermentation of pyruvates, lactobacilli are able to produce diacetyl and acetaldehyde. Diacetyl interferes with arginine utilization in the arginine-binding protein of Gram-negative species, thereby making them very susceptible (Jay, 1991; Naidu and Clemens, 2000). *Escherichia coli,* for example is one of the most sensitive species to diacetyl but some other species also reported to be sensitive are *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Salmonella anatum* (Motlagh *et al*., 1991; Naidu and Clemens, 2000).

2.2.7.6 Deconjugation of bile acids

Bile acids in the form of conjugates with glycine or taurine are secreted in the duodenum. Bifidobacterium can reduce the cholesterol reabsorption by deconjugating bile acids and by hydrolysis of sodium taurocholate and glycocholate in the colon (Ballongue, 2004).

It has been shown that certain lactic acid bacteria (LAB) have specific immune-regulating properties and as a result, they interact with mononuclear phagocytes and endothelial cells of the host (Tiscornia *et al*., 2012; Munoz-Atienza *et al*., 2015). Some particular strains of LAB showed adjuvant properties after infection with pathogenic microorganisms and this is achieved by stimulation of specific antibody response (Mokoena, 2017).

2.2.8 Challenges involved in incorporating probiotics into foods

2.2.8.1 Food processing factors

Common food processing parameters are designed to prevent pathogenic contaminations as well as to kill the existing harmful microbial population to make the food safe for consumption. Therefore, incorporating probiotic bacteria into processed foods has become difficult if the established processing conditions are followed. For example, heat treatment and drying are the two common techniques for enhancing the shelf life of food

but both of them are detrimental to some extent for the probiotic bacteria. Another big challenge is to restrict the multiplication of probiotic bacterial cells once they are added into a food, otherwise, instead of adding value to it, such bacterial growth would lead to spoilage of the product itself.

Other detrimental conditions for the survival of any probiotic culture in fermented dairy products reported are titratable acidity, pH, hydrogen peroxide, storage temperature, presence of other species and strains, lactic and acetic acid concentration and presence of certain food components (Kailasapathy and Supriadi, 1996; Lankaputhra *et al*., 1996; Dave and Shah, 1997; Anal and Singh, 2007).

Also, some physiological traits such as the susceptibility of probiotic lactobacilli and bifidobacteria towards acid, bile and oxygen stresses have made their incorporation difficult in dairy foods (Stanton *et al*., 2003). Each of these factors is described below in detail.

2.2.8.2 Exposure to gastric acid in the stomach

Acid tolerance is an important quality a probiotic strain should possess because the gastric pH frequently falls below 2.0 (McLauchlan *et al*., 1998; Stanton *et al*., 2003). *L. acidophilus* has been able to show a good resistance around pH 2.0 and maintain near neutral cytoplasmic pH (Kashket, 1987; Stanton *et al*., 2003). Acid tolerance can be improved in a number of ways such as up-regulation of the gene responsible for stress protection and by acidic environment adaptation (Kullen and Klaenhammer, 1999; Shah,

2000; Stanton *et al*., 2003). Similarly, the acid and bile tolerance in case of bifidobacteria also vary a lot among different strains. Species, such as *B. longum*, *B. pseudolongum*, and *B. animalis* are highly aciduric in nature with *B. longum* has been reported to survive up to 4.0% bile concentration (Clark and Martin, 1994; Lankaputhra and Shah, 1996; Stanton *et al*., 2003).

2.2.8.3 Exposure to bile salts present in the intestinal fluid

An essential condition to select a particular probiotic strain is their ability to survive the transit through the small intestine and the tolerance towards bile salts present there (Charteris *et al*., 1998; Stanton *et al*., 2003). Haller *et al.* (2001) reported that lactobacilli of intestinal origin are more bile resistant than those obtained from fermented foods. It was estimated that only 1.3 to 1.5% of the orally ingested *L. acidophilus* cells via fermented foods ultimately survived through the intestinal transit. Some strains of lactobacilli have been reported as bile stress tolerant and hence they are a good choice of selecting as a probiotic culture. There are some strains capable of deconjugating bile acids using the bile salt hydrolase enzyme, though the importance of this activity has not been completely understood (Marteau *et al*., 1993; Stanton *et al.*, 2003).

2.2.8.4 Oxygen intolerance of probiotic strains

The oxygen content and the redox potential of the environment containing probiotics play very important roles in deciding their viability. The anaerobic probiotic strains are

directly affected by the presence of oxygen in both intestinal microbial ecosystem and exogenous oxidative stress conditions (Stanton *et al*., 2003). The survival of *L. acidophilus* in yogurt was shown directly related to the oxygen permeability of the packaging materials (Dave and Shah, 1997). An anti-oxidative strain of *Lacobacilli*, identified as *L. fermentum*, have been found more viable in the presence of oxygen compared to other non-oxidative strains (Kullisaar *et al*., 2002; Stanton *et al*., 2003). The future research towards understanding the molecular mechanisms responsible for this particular anti-oxidative stress behaviour could lead to the development of more aero-tolerant lactobacilli strains (Stanton *et al*., 2003). Since bifidobacterium is highly anaerobic, oxygen sensitivity is very important in selecting a strain. They are more or less affected in several ways due to the presence of oxygen and it was reported that *B. longum* in an oxygenated environment experienced an extended lag phase and growth was limited, the cell morphology altered, with the cells becoming longer in size and also the cellular fatty acid profiles changed (Ahn *et al*., 2001; Stanton *et al*., 2003).

Therefore, it is apparent that the probiotic strains when administered along with a food preparation have to pass through many adverse conditions as discussed above. To increase their resistance against these adverse environments, several approaches have been proposed such as, selection of acid and bile resistant strains, packaging in oxygen protected materials, double-step fermentations, pre-adaptation to various stress conditions, adding micronutrients in the form of amino acids and peptides and most importantly, microencapsulation (Gismondo *et al*., 1999; Anal and Singh, 2007).

2.2.9 Selection criteria of a probiotic strain for microencapsulation and subsequent food applications

Several probiotic strains available commercially may have been proven clinically beneficial to human host but all of them may not be suitable for food applications because of the challenges, as discussed in section 2.2.8. Though the purpose of microencapsulation is to provide adequate protection to the strains against such adverse environment, it is always preferable to select strains which are already resistant. In a study, Kotikalapudi *et.al.* (2010) tested 4 commercial probiotic strains against *in vitro* acid tolerance, bile salt exposure, and resistance against anti-microbial compounds. It was reported that each strain acted differently against such challenges and finally *L. acidophilus* ATCC 11975 was found to be most suitable for encapsulation purpose. The importance of selecting acid and bile tolerant strains has also been stressed upon in a review by Sarkar (2010) where many strains of lactobacillus and bifidobacterium spp. have been found to be vulnerable to acid and bile stress. It was reported that *B. longum* survived better than *B. infantis*, *B. adolescentis* and *B. bifidum* in an acidic incubation test. Among the lactobacilli strains, *L. acidophilus* ATCC 4962, *L. casei* ASCC 290 and *L. casei* ASCC 292 were found to be very robust under acidic conditions.

2.3 Microencapsulation overview

2.3.1 Definition

A general definition of the microencapsulation technique is given by Champagne and Fustier (2007), according to which, it is a method of packaging a desired food material in miniature sealed capsules, made up of a suitable food grade matrix, which is able to release their contents at controlled rates and under controlled environmental triggers, such as shear, temperature, enzymes, pH etc. The size range of the microcapsules generally varies from submicrometer to a few millimeters depending upon the materials and technology adapted to produce them. Microencapsulation of probiotic cultures basically involves applying a physical barrier around the living cells which should ideally be capable of providing shelter against all the adverse external conditions, which it might face during processing, storage, and gastro-intestinal passage. Earlier, immobilized or entrapped cells were mainly used in biotechnological applications, the basic purpose is to keep the cells separate from metabolites. But in relatively recent times, the refined processes of microencapsulation have been applied to stabilize the cells, enhance the viability and stability during the production, storage and handling of lactic acid cultures etc. (Kailasapathy, 2002).

Immobilization and encapsulation are two terms that are often used interchangeably but in fact immobilization refers to entrapping the core material within or throughout a whole matrix whereas encapsulation is the process of making a continuous coating

around the core particle so that it is completely surrounded by the wall material, thus leaving a little chance of surfacing out (Kailasapathy, 2002; Simo *et al*., 2017). Microencapsulation has many advantages over the immobilization technique. They are semi-permeable, thus allowing the metabolites to pass through but made of strong shell materials retaining the bacterial cells inside. The encapsulated core material could be control released either by the mechanical breaking of the matrix, dissolution of the wall material or melting of the wall, by diffusion or by pressure (Kailasapathy, 2002; Ye *et al*., 2018).

2.3.2 Purpose of microencapsulation

Microencapsulation process was developed approximately 30 years ago to protect certain compounds or biological cells from the surrounding environment which could be destructive to the core. The process generally allows smaller molecules to enter or exit the capsules. Examples of encapsulation seen in nature are bird's egg, plant seed, bacterial spore-wall, skin, and seashells (Gibbs *et al*., 1999). Lactic acid bacteria (LAB) were first immobilized in 1975 on Berl saddles and later in alginate beads for the purpose of continuous yogurt fermentation (Linko, 1985; Gibbs *et al*., 1999). Microencapsulation improves the flow properties of the core material and protects them from moisture, oxygen, and heat. The process can also improve the nutritional quality of certain oxygen sensitive ingredients such as, vitamins and lipids. In both food and pharmaceutical industries, controlled release of flavors, aroma, perfumes, drugs, detoxicants etc. plays a very important role and this is achieved by microencapsulation

techniques. In case of effective drug delivery, microencapsulation helps in proper time release, improved stability of medical formulations and flavor masking of obnoxious compounds (Gibbs *et al*., 1999).

2.3.3 Structure of microcapsules

A microbead or microcapsule is made up of a single or combination of natural food ingredients such as sugars, gums, proteins, polysaccharides, lipids and synthetic or modified polymers coated around the core component. They are produced either in soft gel form (sometimes referred to "gel beads") or in dried powder form. The surface morphology of the beads can be smooth or irregular, with or without the presence of pores. Presence of pores is responsible for reduced encapsulation efficiency (Mortazavian *et al*., 2007). Two examples are shown in Figure 2.2 and Figure 2.3 to demonstrate the microencapsulation process and the type of microcapsules produced via industrial applications.

The most common classifications are based on matrix materials such as, with single wall material (sodium alginate) or a mix of materials (xanthan and gellan gums, alginate and chitosan, alginate and whey protein etc.) and microcapsule shape formation. The capsule shapes can be of regular and spherical with smooth surfaces or irregular shapes with uneven surface morphology. Sodium alginate microcapsules are generally regular in shape with smooth surface properties (Muthukumaraswamy *et al*., 2006). On the other hand, the presence of milk proteins in the wall material composition and their slow

gelling properties generally lead to irregularly shaped capsules (Ainsley-Reid *et al.*, 2005). It was also reported that same wall materials produce different shaped and sized capsules depending upon the microencapsulation techniques adapted to produce them. In a study, almost perfectly spherical particles with very smooth surfaces were produced with sodium alginate in extrusion process but emulsion technique resulted into irregular shaped, rough-surfaced particles (Muthukumaraswamy *et al.*, 2006). Another way to classify microencapsulation is by the manufacturing techniques, such as extrusion, emulsion, drying etc., which are discussed in more details in the following section. Any relationship between the microcapsule structures and the functionality of the probiotic bacteria entrapped therein could not be found in the published literature.
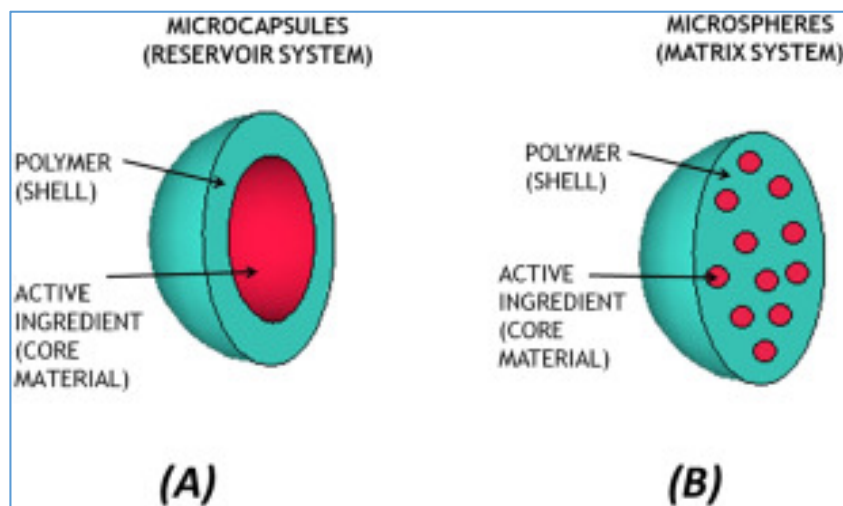
Figure 2.2: *Schematic diagram of two representative types of microcapsules, A: Matrix type and B: Aggregate type capsule*

Figure 2.3: *Schematic diagram of the encapsulation process and the end products*

2.3.4 Selection criteria for choosing encapsulating matrix

The release of any entrapped core material is controlled by the capsule wall properties (matrix control) or the coating material over the wall (membrane control). Releasing of encapsulated core materials is dependent upon the wall materials response towards the environmental triggers such as pH, temperature, shear stress etc. The permeability through the matrix, the solubility of the core component and the vapor pressure (in case of a volatile compound) are also important factors in choosing the right ingredient combination of a microencapsulation process (Gibbs *et al*., 1999). For example, it was reported that calcium alginate beads produced by ionotropic gelation are found to be porous, facilitating easy transport of the core materials out of the encapsulating matrix

(Anal and Singh, 2007). A range of biodegradable polymers has been used by several researchers to microencapsulate probiotic bacteria. A detailed list of such materials can be found in a review by Anal and Singh (2007). Each of these materials or combinations thereof has some unique advantages and disadvantages. κ-Carrageenan alone or in combination with locust bean gum was tried in many studies (Audet *et al*., 1988; 1990; 1991; Doleyres *et al*., 2002; 2004) to encapsulate probiotic bacteria but the gelling agent involved in the process was potassium chloride, which was found to have an inhibitory effect on *S. thermophilus* and *L. bulgaricus* (Audet *et al*., 1988, Anal and Singh 2007). The choice of wall ingredients also depends upon the objective of encapsulation. A certain material mix may be good in providing good protection against gastric passage but the idea of incorporating the same materials in food products may not be attractive. For example, enteric polymer coatings in the form of mainly cellulose derivatives are used extensively in the pharmaceutical industry for tablet coating purpose. One such polymer, cellulose acetate phthalate, was used to encapsulate *L. acidophilus* cells and excellent protection in the gastric environment was noted (Rao *et al*., 1989).

The goodness of milk components as food ingredients is accepted universally and therefore may be considered as a good choice for encapsulating probiotic bacteria. Microparticles made of milk proteins are finding increasing applications in the food industry because of their flexibility to encapsulate any type of hydrophilic, hydrophobic or biological substances, such as probiotic bacteria.

2.4 Drying of probiotic cultures and related physiological stresses

Among several techniques available, freeze drying is the most popular and widespread technology for drying probiotics. Though milder processing conditions in freeze-drying have been proven to be less detrimental than spray drying, the freezing step involved causes maximum injury to the cells. It was found that 60 to 70% of the cells which survived the freezing step could easily live through the desiccation step (To and Etzel, 1997). Freezing leads to extracellular ice crystal formation and consequent increase in extra-cellular osmolality, which in turn causes dehydration of bacterial cells (Meng *et al*., 2008). Disintegration of cell membranes and macromolecular denaturation are other detrimental effects of freezing and responsible for viability loss (De Angelis and Gobbetti, 2004). Bacterial cell size also plays an important role here. Fonseca *et al*. (2000) demonstrated that higher surface area of the cell leads to higher damage of the cell membranes during the freezing process and shown that spherical-shaped *Enterococci* were more resistant than rod-shaped lactobacilli during the freeze-drying process. Other factors responsible for the degree of cryopreservation, as stated in a review by Hubalek (2003) are temperature, pH and composition of the growth medium, osmolarity and aeration, cell water and lipid composition, cooling rate during freezing etc. Different researchers have taken different approaches to control cell damage during the freeze-drying process. Some of them are discussed in the following sections.

2.4.1 Use of cryoprotective agents in the freeze-drying media

The technology to protect the bacterial cells during freeze-drying is known as cryopreservation and the compounds used to achieve this protection are called cryoprotectants. Addition of cryoprotecting compounds prior to fermentation or drying helps in the adaptation of probiotic cells in the changed environment. The added compounds slowly start accumulating inside the bacterial cells, which helps in reducing the osmotic difference between inside and outside of cells (Kets *et al.*, 1996; Meng *et al.*, 2008). The cryoprotecting agent or CPA can either be added to the growth or the drying media and their action varies with different strains. But certain general compounds such as non-fat milk solids, lactose, trehalose, glycerol, betaine, adonitol, sucrose, glucose, dextran etc. have been regarded as suitable protectants for many species (Hubalek, 2003; Morgan *et al.*, 2006).

The protection mechanism is better understood by classifying the CPA's into two broad groups namely, (i) amorphous glass forming and (ii) eutectic crystallizing salts. The first group comprises of carbohydrates, proteins, and polymers and they act by imparting very high viscosity at the glass transition phase and thereby restricting the molecular mobility of the cells. Most successful cryoprotectants for probiotic bacteria fall into this group. The other group contains certain eutectic salts which tend to crystallize as the temperature approaches near the freezing point but instead of providing protection, they have been sometimes reported as detrimental to the cell membranes (Orndorff and Mackenzi, 1973; Morgan *et al.*, 2006).

The CPAs are sometimes classified in a slightly different manner into three groups namely, (i) highly permeable compounds, such as monovalent alcohols, amides, and sulfoxides, (ii) slowly permeable compounds, such as glycerol and (iii) non-permeable compounds, such as mono-oligo-polysaccharides, sugar alcohols, proteins, polyalcohols etc. Permeable compounds bind the intracellular water and prevent dehydration. Non-permeable protectants form a layer on the cell surface, allow partial outflow of water from the cell body, reduce the toxic effect of salts, stop excessive growth of ice crystals and maintain their structures (Hubalek, 2003; Saarela *et al.*, 2005).

Apart from offering protection during the drying process, compatible solutes have been shown to improve the acid tolerance property for probiotic cultures. The presence of glucose as a compatible solute was reported to increase the viability of cells by 6.0 log during simulated gastric juice trial (Corcoran *et al.*, 2005).

2.4.2 Improving viability by proper cell harvesting

All the probiotic species do not show equal vulnerability towards drying. The technological robustness of a species or strain depends upon several factors such as the age of the culture, cell size and shape, concentration of cells in the drying media, stress preventive mechanism of the cell and chemical structure of the cell membranes. For example, gram-positive cells have been found to be more robust than the gram-negative ones (Heckly, 1985; Carvalho *et al.*, 2004). The survivability during drying can also be improved by proper and timely harvesting of the cells. Generally, cells at their stationary

growth phase have been found to be most robust towards drying stresses (Mary *et al*., 1986; Rees *et al*., 1995; Corcoran *et al*., 2004). The explanation for such behavior has been cited as the physiological state during the stationary phase where starvation of carbon and other nutrients induce stress response to the cells and make them more adaptive towards subsequent drying stresses (Morgan *et al*., 2006).

2.4.4 Use of additives in the growth media for enhanced protection during drying

Manipulating the culture growth media with various additives has been proven to be beneficial for the cells. The factors responsible for this can be identified as compatible solutes accumulation inside the cells, exopolysaccharides production and change in the cell membrane fatty acid profile (Carvalho *et al*., 2004). Various sugars in the form of compatible solutes help to regain the osmotic balance of the bacterial cells during the low water activity stage of freezing and subsequent drying. This osmoregulation is performed by movement of the solutes through the cell membranes, which helps in the adaptation of the cells towards osmotic stress (Bayles and Wilkinson, 2000; Carvalho *et al*., 2004). Compatible solutes are able to accumulate at high levels into the cell cytoplasmic fluid. This process needs adequate time and adding them to the growth media instead of drying media helps in obtaining better results (Carvalho *et al*., 2004). Few examples of such compatible solutes are peptone, tryptone, yeast and meat extracts and various sugar substrates. Though the exact mode of action of these solutes against drying stress is quite complex, Ko *et al*. (1994) suggested that it is very similar to that by

cryoprotectants, i.e. prevention of aggregation of cell proteins and changing the cell membrane physical properties. A wide range of sugar substrates has been used by various researchers as supplements in the growth media, which has been summarized in a review paper by Carvalho *et al*. (2004). This includes glycine betaine, proline betaine, acetylcarnitine, carnitine, gamma-butyrobetaine and 3-dimethylsulphoniopropionate (Bayles and Wilkinson, 2000), non-fat skim milk and sucrose (Costa *et al*., 2000), adonitol (Devaldez *et al*., 1983), mannitol (Kets *et al*., 1996), etc.

2.5 Stabilization of microorganisms

2.5.1 Definition

The stabilized state of bacteria is defined as an inert state that is achieved by desiccation and the process is also referred to anhydrobiosis. In this state, the bacterium stops its metabolism for a temporary period of time (Garcia, 2011). This stabilized state is known by the maximum possible reduction of mensurable metabolism (Rebecchi *et al.,* 2007). In a more specific definition, the anhydrobiosis or stabilized state is believed to be achieved when the free water content is reduced to less than 0.1 g for every gm of dry cell mass of the bacterium (Alpert, 2005). However, this quantified definition only holds good for pure cell bodies isolated in a laboratory experiment when there is no water being held by the environment or carrier excipients of the cells. It is impossible to differentiate and quantify these two different water contents when bacterial cells are

desiccated in a natural environment and the surrounding chemical or biological environment is also going through the same desiccation process (Potts *et al*., 2005).

Desiccation is not a bacteria-friendly process and imparts a variety of stresses (eg. heat, freezing, osmotic) on the cells which will be discussed further in the following section. Therefore, a term often used to characterize a bacterial genus or species in the study of anhydrobiotics is 'desiccation tolerance' and sometimes also referred to 'drought tolerance'. Desiccation tolerance can be defined by the ability of a bacterium to resist dying. During the desiccation process, until the level of equilibrium is achieved, the bacterium stops all the metabolic activities but returns to its normal functions after rehydration (Alpert, 2005).

2.5.2 The desiccation mechanism

To investigate the mechanism of desiccation many studies have been performed on both Gram-positive and Gram-negative bacteria but the exact effects and explanation of the post-desiccation behavior are still unknown (Potts *et al.*, 2005). It was found that Gram-positive bacteria (all lactobacilli and some other efficacious probiotics) in general are more tolerant to desiccation and the hypothesis behind this has been postulated as the accumulation of Mn^{2+} inside the cells and having high Mn^{2+}/Fe^{2+} ratio. These bacteria have also been found to be resistant to ionizing radiation (Daly *et al.*, 2004). Intracellular accumulation of Mn^{2+} is now being proposed as the most probable factor causing such resistance, which in effect protects the cell proteins from oxidation during and after the

desiccation (Fredrickson *et al.*, 2008; Poddar *et al*., 2014). Figure 2.4 shows a schematic

diagram of the desiccation process and the consequences of it.

Figure 2.4: *The mechanisms of damage, reduction in the size of the cell and the loss of water due to desiccation (Garcia, 2011)*

2.5.3 Processing techniques used for the stabilization of probiotics

Freeze-drying is the most popular stabilizing technique for probiotics because of the

milder heat processing it involves. However, the detrimental effects of freeze-drying

have also been reported, including undesirable changes in the physical state of cell

membrane lipids and the structures of sensitive cell proteins (Leslie *et al.,* 1995). Most

of the cellular inactivation occurs in the freezing step by the formation of extracellular

ice crystals, leading to very high extracellular osmolality and consequent dehydration of

the cells (Tsvetkov & Brankova, 1983; To & Etzel, 1997). In addition, the freeze-drying

process is expensive, and therefore inaccessible to the underprivileged population of the

world. An inexpensive alternative process is the spray drying of the probiotic cells suspended in a suitable protectant matrix. However, the spray drying process has the disadvantages of heat stress, oxygen exposure, osmotic stress (Teixeira *et al.,* 1997) and increased permeability of the cell walls leading to the leakage of the intracellular components (Teixeira *et al.,* 1995). Vacuum drying is also an important alternative process which is carried out at reduced temperature and the water evaporation rate is controlled by the degree of vacuum maintained in the drying chamber. The use of fluidized bed drying in probiotic stabilization has not been extensively explored, and only a handful of studies could be found where the fluidized bed has been used as a spray coater (Kumar *et al.,* 2010; Haris *et al.,* 2012; Stummer *et al.,* 2012). Fluidized bed drying process is comparatively economic, involves low energy consumption, and imparts moderate heat stress to the bacterial cells; however, it has the limitation of being a batch process (Kudra & Mujumdar, 1988; George *et al.,* 2004). Figure 2.5 shows a phase diagram of the water removal process in relation to these four drying mechanisms.
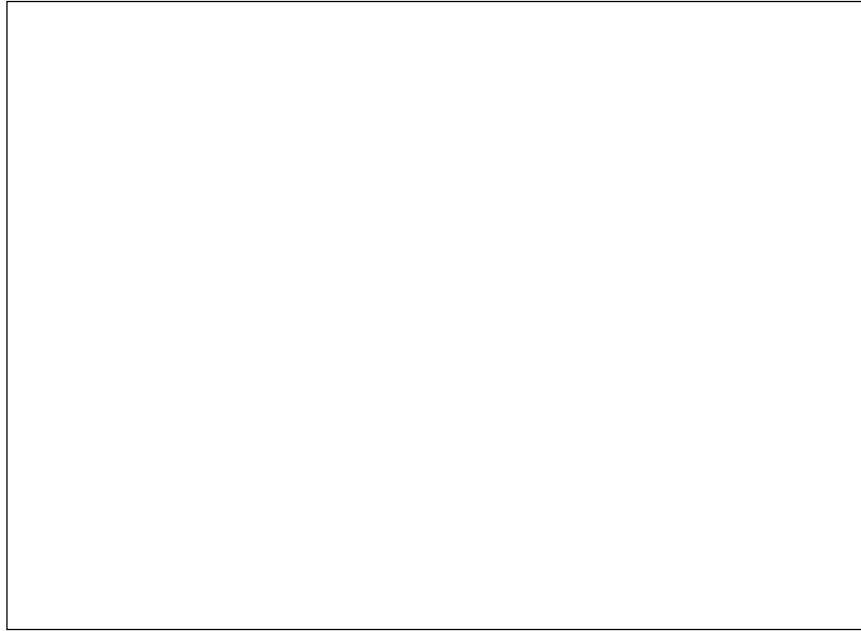
Figure 2.5: *Common drying processes and the phase diagram in relation to water evaporation (Santivarangkna et al., 2007)*

In a fluidized bed dryer, a stream of hot air is blown upwards through the particle bed at a velocity high enough to set them in motion and form a floating bed of powder particles suspended in air. The airflow creates turbulence and aids rapid mixing of the particles. The dehydration process is carried out by rapid exchange of heat and mass between the particles and air. Fluidized bed drying time (varies between 5 to 30 min) is longer than the spray drying process which means bacterial cells are exposed to enhanced oxidative stress but the heat inactivation can be minimized by using relatively lower drying temperatures. This process has also certain limitations too. The irregular particle sizes and stickiness of the granulated particles creates a heterogeneous bed and the agglomeration process reduces the drying rate.

2.6 Problems associated with the loss in cell viability in different stabilization techniques

2.6.1 Spray drying

Heat inactivation and consequently the cell wall injury is the main cause of the loss of probiotic viability during the spray drying process. The heat inactivation process is not solely dependent on the inlet drying temperature but the residence time inside the drying chamber and this time-temperature combination are also major factors in deciding the inactivation coefficient (Santivarangkna *et al.,* 2007). Many researchers have found the outlet temperature of spray drying is more important and residual cell viability can be positively correlated with lower outlet temperatures (Kim & Bhowmik, 1990; Roelans & Taeymans, 1990; Bielecka & Majkowska, 2000; Desmond *et al.,* 2002 and Ananta *et al.,* 2005) as shown in Fig 2.6. Low outlet temperature drying has also been found to yield better storage stability by Desmond *et al.* (2002) and Gardiner *et al.* (2000).
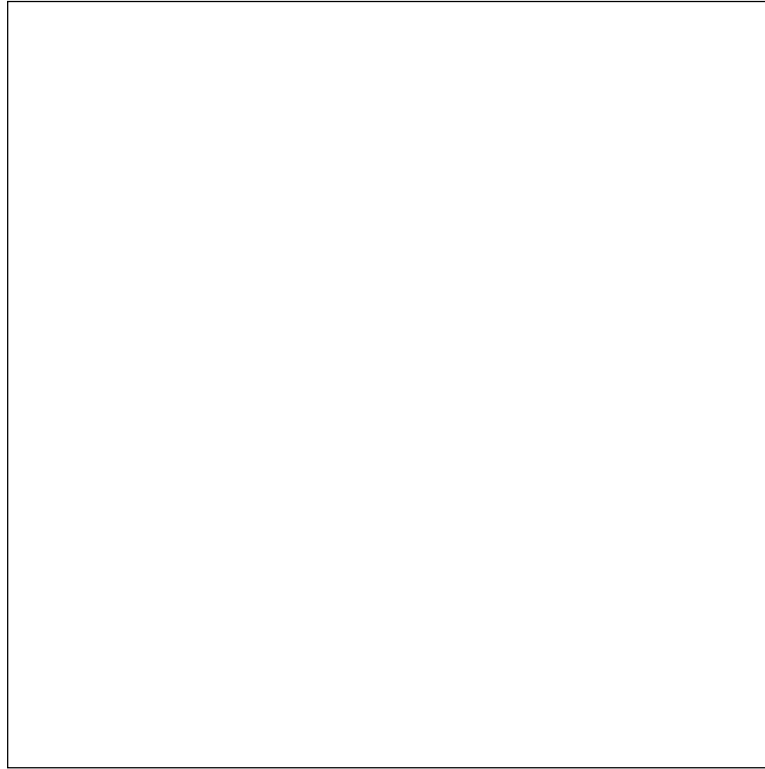
Figure 2.6: (A) Survival of L. paracasei NFBC 338 during spray drying in 20% (wt/vol) RSM supplemented with 0.5% (wt/vol) yeast extract at different air outlet temperatures (bar graph). The line shows the moisture contents of the resulting powders. The air inlet temperature was maintained at 170°C. (B and C) CSLM micrographs of NFBC 338-containing powders produced at air outlet temperatures of 70 to 75°C (B) and 120°C (C). The powders were stained with the LIVE/DEAD BacLight viability stain; live cells are green, and dead cells are red. Bars = 10 μm. (Gardiner et al., 2000)

The survival of different bacteria against desiccation stress depends a lot on the individual intrinsic tolerance levels of the cells. When three bacteriocin-producing strains of a lactic acid bacteria were spray dried under similar process conditions, only one of them was viable after 3 months of storage (Silva *et al.,* 2002). Gardiner *et al.* (2000) observed significant reduction (about 1 log) in viability between two strains even when both were stored under refrigeration for 2 months. This indicates that optimizing the process parameters of spray drying is just one aspect of the challenge of successful

stabilization but understanding the individual bacterial strain for its desiccation tolerance and other intrinsic properties such as cell membrane composition, morphology or Mn^{2+} intake capability etc. are equally important. The genomic study may also offer useful guidance for manipulating the growth conditions in order to induce stress tolerance in the subjected bacteria by the production of stress proteins (Potts *et al.*, 2005).

2.6.2 Freeze drying

Generally, the freeze-drying process used in the stabilization of probiotics yields higher survival rates because of the milder heat treatment involved (Wang *et al.,* 2004). The freezing step of this drying process causes more cellular inactivation than the desiccation step and depends on the choice of slower or faster freezing rate (Tsvetkov & Brankova, 1983; To & Etzel, 1997; Fowler & Toner, 2005). Cell morphology also plays an important role here. Bacterial cells with the higher surface area are subjected to a higher degree of membrane damage by the extracellular ice crystals formation. This was supported by the findings of Fonseca *et al*. (2000) when small round shaped enterococci cells were found more resistant to freeze-drying stresses than the larger sized rod shaped lactobacilli. The other causes of cell deaths during freeze-drying have been proposed as removal of bound water from the cell bodies, membrane lipid peroxidation (Brennan *et al.,* 1986) and destabilization of the RNA and DNA structures which causes poor replication, transcription and translation of the DNA during rehydration and future growth (van de Guchte *et al.,* 2002).

2.6.3 Fluidized bed drying

Fluidized bed drying is an established method for drying granulated solids. This is a good potential technique to stabilize heat sensitive probiotics because a moderate and controlled drying temperature range between 40 °C to 70 °C can be used. The heat inactivation and freezing related problems mentioned earlier can be avoided in this drying method.

Stabilization of yeasts using fluidized bed drying has been studied extensively (Bayrock *et al.,* 1997; Bayrock *et al*., 1997a) as well as tested for commercial level productions (Beker & Rapoport, 1987; Caron, 1995). The use of this technique in probiotic stabilization has not been extensively explored and only a few studies could be found where the fluidized bed has been used as a spray coater (Kumar *et al.,* 2010; Haris *et al.,* 2012; Stummer *et al.,* 2012; Poddar *et al*., 2014). The major reason for the reduction in cell viability in a fluidized bed dryer is the osmotic shock caused to the cells when low water activity carrier agents such as milk powders, maltodextrin, starch etc. come in contact with the wet cells (Santivarangkna *et al.,* 2007). Mille *et al.* (2004) found the recovery of *L. plantarum* cells to be extremely low (<2.5%) when they were mixed directly with desiccated casein powder having a water activity (a_w) of <0.1. But the advantages of this process has been reported as the granulation achieved by the interaction between the cell bodies and the carrier medium which creates a protective

environment against oxidative stress during drying and storage (Santivarangkna *et al.,* 2007).

2.7 Problems associated with the storage stability of desiccated probiotics and the responsible factors

The major factors affecting the stability of desiccated probiotics during storage are temperature, moisture content and a_w of the matrix, relative humidity of the storage environment and exposure to light and oxygen (Meng *et al.,* 2008).

There is a consensus among the researchers that in the dried form of storage, probiotic viability is inversely related with the storage temperature (Gardiner *et al.,* 2000, Mary *et al.,* 1993, Silva *et al.,* 2002 and Teixeira *et al.,* 1995). Bruno and Shah (2003) found the optimum storage temperature to be −18 °C to maintain high level of viability for bifidobacteria and observed significant reduction at 20 °C which is considered lower than 'ambient' temperature. A number of bifidobacteria species showed a significant decline in viability when spray dried with skim milk and stored at 15 °C and 25 °C (Simpson *et al.,* 2005). Costa *et al.* (2000) found the viability of *Pantoea agglomerans* (having anti-pathogenic properties against *Penicillium digitatum*) cells declined by only 0.5 log when stored at 4 °C for 90 days but by more than 3 logs within 28 days at 25°C.

The moisture content and water activity of the stabilized probiotic powders have an inverse relationship with the residual bacterial viability during storage. Water activity is

influenced by the relative vapor pressure (RVP) of the storage environment/container and it was found in one study that 11.4% RVP ($\approx a_w$ 0.11) yielded maximum viability for freeze-dried probiotics (Meng *et al.,* 2008). Zayed & Roos (2004) found the most optimum moisture content to be 2.8% to 5.6% for ensuring maximum viability of freeze-dried *L. salivarius* subsp. *salivarius cells.*

Exposure to light and oxygen during storage is related to the packaging materials used for stabilized probiotics but very few research papers are available on this topic. Storage of dried probiotics under N_2 was found to cause no loss in viability of *Lactobacillus oenos* (Clementi, 1984) and slightly improve the same for *L. acidophilus* cells (Espina & Packard, 1979). High barrier plastic bags allowing lesser penetration of oxygen and moisture were found to be more effective for *Pantoea agglomerans* cells by Costa *et al.* (2000). A similar observation was noted by Bozoglu *et al.* (1987) when yogurt starters were packed in the presence or absence of oxygen. It was concluded that oxygen diffusion takes place through the cell interfacial area which is considered permeable. In another study, Wang *et al.* (2004) spray-dried *S. thermophillus* and *B. longum* cells and found laminated pouches with better oxygen and light barrier properties protected the cells better during storage than glass bottles and PET bottles. A few studies have found a higher degree of oxidative damage during storage in case of freeze-dried probiotics compared to the spray dried ones when both were stored under similar packaging and storage conditions (Korobkina *et al*., 1982; Wang *et al*., 2004; Ferreira *et al*., 2005).

The oxidative damage is mainly caused by the products of lipid peroxidation which have been demonstrated by showing a decrease in the unsaturated/saturated fatty acid ratio for *L.delbrueckii* ssp. *Bulgaricus* (Teixeira *et al.,* 1996) and irreversible damage to the cell

DNA of *Salmonella tester* TA104 (Marnett *et al.,* 1985). A higher level of unsaturation of the lipids is believed to passively affect the cell permeability and subsequent oxidative damage (In't Veld *et al*., 1992). The viability decreases at a faster rate during the early months but the change in fatty acid ratio continues at a slower rate with passing time (Santivarangkna *et al.,* 2007). This trend analysis is very important for developing standard curves and modeling the rate of decay but authors mostly use first-order, i.e linear models to present their findings. Each bacterial species shows very different characteristics during storage and their inactivation patterns can be very dissimilar with different level of concavities in the stability graph (Garcia, 2011). Hernandez *et al.* (2009) have introduced a new modeling technique by defining the 'anhydrobiosis quotient' to characterize the viability of stabilized cells. A non-linear model was used in their study to predict the future population of viable cells stored in a desiccated state.

2.8 Approaches to improving the viability of probiotics upon desiccation and during storage

A few more critical and complex factors thought to be responsible for the loss in cell viability during storage are the cell physiology and expression of specific stress proteins, the role of various protectants, glass transition temperature (T_g) of the stabilization mix, membrane integrity and the molecular mobility of free water within the matrix. Also, manipulating the growth media and determining the optimum cell harvesting phase have been found beneficial in many studies (Meng *et al.,* 2008).

Successful drying and storage stability of probiotic cells also very much depend on cell physiology, which is influenced by a variety of factors including pre-adaptation to mild stress, the phase of harvesting, growth media composition and genetic modification (Meng *et al.,* 2008). Before stabilizing probiotic cells, an application of a sub-lethal stress to enhance the stress responses has been found to be highly effective by many researchers in improving viability and retention of physiological activities after dehydration (Kim *et al.,* 1988; Teixeira *et al.,* 1995; deUrraza & DeAntoni, 1997; Desmond *et al.,* 2002). It has been shown that probiotic cells positively respond to the changes in the immediate surroundings by reprogramming their metabolic activities and that this causes enhanced resistance to loss of viability during prolonged storage in an inactive state (Pichereau *et al.,* 2000). Prasad *et al.* (2003) found that *L. rhamnosus* HN019 cells that were heat stressed at the stationary phase or osmotic stressed at the growth phase showed superior storage stability at 30 °C compared to the unstressed cells. The authors reported that multiple-fold upregulation of heat shock proteins and synthesis of certain glycolytic enzymes were responsible for improving cell stability during desiccated storage.

It seems that the harvesting phase also plays a role in the stress adaptability of the bacterial cells since carbon exhaustion at the early stationary phase leads to starvation which aids better stress tolerance during the desiccation process (Morgan *et al.,* 2006). It was reported that fermentation with uncontrolled pH yields more robust probiotic cells, probably because of the acid stress adaptation and consequent synthesis of heat shock proteins (Ferreira *et al.,* 2005). In several experiments performed in the Riddet Institute

(unpublished), this behavior was observed in a range of probiotic cultures where significant differences in storage stability were recorded between pH controlled and uncontrolled fermentation batches.

Protectants or cryoprotectants (in case of freeze drying) can be a component of or even form the whole drying matrix for probiotic cells. Among the most commonly used compounds are non-fat milk solids, whey proteins, trehalose, glycerol, betaine, adonitol, sucrose, glucose, lactose, dextran, and polyethylene glycol. (Hubalek, 2003; Morgan *et al.,* 2006). Each of these compounds has specific functional properties and protective mechanisms, but the maximum effectiveness of these protectants has been found to be reducing the loss of cell viability during the drying process rather than in the storage period. Protectants which are carbohydrate in nature and can transform into a glassy state upon dehydration have been reported to offer better storage stability to the dried bacterial cells (Vega & Roos, 2006). Therefore, the moisture content of the drying matrix which is a key variable for the T_g of the mix becomes important once again. Buitink *et al*. (2000) explained the role of T_g in stabilizing biological systems and identified the role of reduced molecular mobility behind this phenomenon when the high viscous sugar glasses are stored at a lower temperature than their T_g. Keeping other parameters constant, the inclusion of certain compounds in the drying mix, which raised the T_g of the mix has been reported to offer better storage stability of biological materials (Aguilera, 1997). Anhydrous trehalose, for example, is one such compound having very high T_g of 110 °C and has been found to offer very high survival rates compared with a base-sugar such as sucrose with T_g of 65 °C (Leslie *et al*., 1995; Crowe *et al*., 1998; Gómez Zavaglia *et al*., 2003; Streeter, 2003). Fowler & Toner (2005)

explained the role of carbohydrates as protectants in raising the glass-phase transition temperature so that the viable cells can remain in the glassy phase without forming intracellular ice crystals. In light of the water replacement theory, Ananta *et al.* (2005) proposed that in the spray-drying of *Lactobacillus rhamnosus* GG with reconstituted skim milk (RSM) and mixtures of RSM and prebiotic oligosaccharides, the RSM was more effective at interacting with the polar headgroups of membrane phospholipids than the oligosaccharides. Using flow cell cytometry, damage to cell membranes during spray drying was identified as the primary cause of cell injury and it was also confirmed that these cells did not grow subsequently on MRS agar (Ananta *et al.,* 2005). The study of Swabb *et al.* (2007) investigated the influence of sucrose, fructooligosaccharides (FOS), inulin and skim milk on the viability and membrane integrity of *Lactobacillus reuteri* TMW1.106 cells during freezing, freeze-drying, and storage (14 days at ambient temperature). Increased membrane integrity of stationary phase *L. reuteri* TMW1.106 cells was attributed to direct interactions between FOS and the membrane which led to increased membrane fluidity and thereby improved the stability of the membranes during storage and rehydration.

Apart from the carbohydrates, the polypeptides also have been found to enhance desiccation tolerance by altering the vitreous properties of sugars. In a mix of proteins and sugars, the proteins were found to be more stable above the T_g and thereby to contribute to better glass formation (Buitink *et al.,* 2000). This can probably explain the very good protectant properties of milk serum or reconstituted skim milk (RSM) as reported in many studies (Ananta *et al.,* 2005, Corcoran *et al.,* 2004 and Desmond *et al.,*

2002). The underlying mechanisms were described as the prevention of cell injuries by cell membrane stabilization (Castro, Teixeira & Kirby, 1995) and by forming protective coatings over the cell walls (King & Su, 1993). RSM alone or in combination with betaine or glycerol (Selmer-Olsen *et al.,* 1999), gum acacia (Desmond *et al.,* 2002), soluble starch, gelatine or gum acacia (Lian *et al.,* 2002) were found to be good protectants for probiotics of lactobacilli and bifidobacteria origin, when no compound from the carbohydrate group mentioned previously was used.

2.9 Use of sugar alcohols in the stabilization of probiotics

In an extensive study, Linders *et al.* (1997) investigated the effects of various sugars (sucrose, maltose, lactose, trehalose, glucose, and fructose) and one polyol compound (sorbitol) on the inactivation of *L. plantarum* cells upon desiccation and observed the role of water activity as well as the moisture distribution within the dried matrix. Fluidized bed drying technique was used in this study and it was found that maltose and sorbitol were the most effective desiccation protectant among the additives used. Table 2.1 shows that maximum desiccation tolerance was exhibited by *L. plantarum* cells when dried in presence of sorbitol in a FBD.

Table 2.3: *Phase transition temperature and residual activity (post drying survival) of L. plantarum cells stabilized in a convective drying process (Linders et al., 1997)*

However, in that study, the role of maltose or sorbitol was not found to be connected with the moisture distribution, water activity or the lowering of the membrane phase transition temperature. Instead, the authors attributed the improved results to the free radical scavenging activity of these compounds. The free radical scavenging activity of another sugar alcohol, mannitol, was found to be responsible for the protection of *L. lactis* cells in the study by Efiuvwevwere *et al.* (1999). The role of sorbitol in improving the survival rate of *L. helveticus* upon vacuum drying was confirmed by Santivarangkna *et al*. (2006). As described earlier, trehalose is probably the mostly used additive in stabilizing probiotics but the focus of almost all the studies was centered on understanding the protective effect of trehalose during the desiccation process but not during the high-temperature storage period. In an interesting study in 2012, the roles of sorbitol and trehalose were compared during desiccation as well as during non-refrigerated storage (Foerst *et al.,* 2012). It was found that both trehalose and sorbitol

yielded better post drying survival of *L. paracasei* F19 cells by 70% and 54% respectively, compared to the control cells with 29% survival. However, the high stability during storage was only observed in presence of sorbitol. Trehalose was not found to have any significant contribution to it. The responsible factor was found to be the increase in the T_g of dry cell-sorbitol mix from -32 °C to 12 °C at the storage temperature of 37 °C (Foerst *et al.,* 2012). Damage to the cell membrane was also studied by Santivarangkna *et al.* (2010) in the presence and absence of sorbitol by measuring the membrane phase transition temperature (T_m) using FTIR technique. Sorbitol was found to lower the T_m by 6 °C and 10.5 °C. They concluded that sorbitol protects the cells during drying by depressing the T_m via interactions with the phosphate groups (P = O band) of the membranes.

2.10 Role of water mobility in successful stabilization of probiotics

The fundamental approach to understanding the effects of moisture on the viability of microorganisms, including probiotics, is to assume that the absence of water molecules has no intrinsically adverse effect on the viability of cells. Rather it is the increasing concentration of water molecules, in which solutes become hydrated, that results in the osmotic effects that have an adverse effect on the cells. Mugnier and Jung (1985) using bacteria, (rhizobium, agrobacterium, and arthrobacter spp.), fungal spores (penicillium sp.), and yeasts (saccharomyces sp.) entrapped in dehydrated polysaccharide (xanthan

and carob gums) gels at 28 °C, observed that microbial cells died at $a_w > 0.069$ and survived during prolonged storage (>3 years) at $a_w \leq 0.069$. They proposed that water molecules present in dried preparations of cells and biopolymers, at $a_w \leq 0.069$, are tightly bound to the biopolymer, speculatively as a monolayer, and are insufficient in amount to allow the biopolymer to become mobile. At $a_w > 0.069$, the water molecules that are not tightly bound as a monolayer to the biopolymer solute are able to make other solutes sufficiently mobile facilitating migration to the cell surface and causing damages associated with osmotic stress. However, the presence of 14% mannitol in the dehydrated gel increased the survival of cells at $a_w > 0.3$ (>17% moisture) compared with controls not containing mannitol. This observation has a high significance in developing probiotic ingredients designed to be applied on dry food products (eg. infant formula powders) where the prevailing a_w is expected to be in the range of 0.20 to 0.30. Practically it is hard to find a food formulation with extreme low a_w of < 0.10 where the probiotics can be made stable over ambient storage period solely relying on minimal a_w. It was suggested that mannitol influenced the water hydrating processes involved with the mobility of solutes.

In a further development of the investigations of Mugnier and Jung (1985), Vittadini *et al.* (2002 and 2005) evaluated the water mobility in xanthan and locust bean gums using a number of analytical techniques. Macromolecular mobility was evaluated by measurement of glass to rubbery transition using thermal analyses of Differential Scanning Calorimetry (DSC) and Dynamic Mechanical Analysis (DMA) and the molecular mobility was determined by spin relaxation times of protons and deuterium using solid-state Nuclear Magnetic Resonance (NMR) procedures (Vittadini *et al.,*

2002). These researchers concluded that changes in structural mobility as determined by glassy to rubbery transition were not observed in the dehydrated gel test system (between a_w of 0.07 - 0.97) and this was therefore of little value in explaining the effects on microbial survival. However, the presence of mannitol lowered water mobility at less than 25% moisture content as determined by proton and deuterium signals of NMR measurements. At moisture contents below 5%, 90% of protons and 80% of deuterons were highly immobile and as moisture content increased up to 25-30%, all protons and deuterons were highly mobile and became liquid-like. As moisture content increased from 6 to 25%, cell viability decreased greatly and at a greater rate in control samples compared with the mannitol-containing samples (Vittadini *et al.,* 2002). It was, therefore, suggested that the reduced water mobility may allow initiation but not the completion of metabolic reactions, resulting in cell death. Fig. 2.7 shows how different additive compounds were responsible for different levels of cell survival at some controlled a_w levels, indicating water activity is not the sole control parameter responsible for ensuring storage stability of microorganisms.

Figure 2.7: *Effect of a_w value and solute type on the survival of the Gram-negative bacterium R. japonicum USDA 138 and the Gram-positive bacterium Arthrobacter sp. entrapped in xanthan-carob inocula after 30 days of storage at 28 °C. (Mugnier and Jung, 1985)*

2.11 Quality assurance aspects of the stabilized probiotic bacteria

The regulatory framework of food products containing probiotics focusses mainly on the presence of sufficient numbers of viable cells at the end of the claimed shelf life of the products. Accordingly, the quality control approach for such products has traditionally relied upon the tests which ensure the viable cell counts throughout the product lives.

Cell viability is nonetheless the most important factor but should not be the only criterion to evaluate such products. In order to ensure the consumption of efficacious probiotics by the end users, a manufacturer must ensure that the functional properties of individual probiotic strains are not lost or changed during the processing and storage of such products. Therefore, it is necessary to ensure that probiotic strains retain the health benefit characteristics for which they were originally chosen (Tuomola *et al.,* 2001). This becomes more important in case of stabilized probiotics where harsher processing steps and relatively long storage period are involved compared to the short lived fermentation products such as, probiotic yogurts or probiotic drinks.

The efficacy of every probiotic strain is associated with some form of desired health benefits but the most common criteria for their selection are strong resistance to stomach acid, intestinal bile salts and their adhesion properties to the intestinal epithelium or mucosa, all three of which supposedly ensure (*in vitro*) their passage and subsequent colonization. The other strain specific criteria for selection are high plasmid stability, carbohydrate and protein utilization patterns, resistance against antibiotics, synthesis of antimicrobial compounds, inhibition ability against gut pathogens and immunogenicity (Tuomola *et al*., 2001). However, there are pieces of evidences in the literature that probiotics lose a part of these properties during the processing and storage of different food formats in which they are applied (Tuomola *et al*., 2001).

Acid tolerance is considered as the intrinsic property of a strain and hence least affected by the environmental factors. However, the growth conditions and any stress experienced during the growth phase may lead to the production of shock proteins and

thereby alter this property (Lee & Wong, 1998). Tuomola *et al.* (2001) shown that the acid tolerance properties of three bifidobacteria strains and observed high correlations between the survival rates when incubated in simulated gastric juice and during the storage within low pH yogurt. They concluded that if the intrinsic acid tolerance level of a particular strain is low, it is expected to lose viability at a faster rate if applied in a low-pH food format. Adhesion property is very important for probiotic strains but the results may vary widely depending on the method by which it is tested. For example, adhesion percentages of six commercial probiotic strains were found to vary significantly when tested in two different *in vitro* models. Two strains showed superior adhesion properties in the intestinal mucin model and four other strains showed multiple times higher adhesion percentages in the differentiated Caco-2 cell monolayer (Letho *et al.*, 1996 & 1997). The effect of long term storage of a culture (under controlled, refrigerated conditions) on adhesion properties was found to be significant when *L. acidophilus* cells were isolated from the yogurt production line between a span of 1 year. Cells, isolated after 1 year, had significantly poorer adhesion properties in both Caco-2 and mucin models (Tuomola *et al.*, 1998). These studies have reinforced the necessity of maintaining a constant vigil over the major functional properties of the processed and stored probiotic strains without solely relying on their viable counts.

2.12 Summary and key findings from the literature review

The rationale behind conceiving the project was to tap into the commercial opportunity in successful stabilization of probiotic bacteria which should ensure the minimum required live population of viable cells at the end of the stipulated storage period,

preferably under ambient storage conditions and should retain the specific probiotic qualities at any point between manufacturing and consumption. Numerous published studies have supported this idea by pointing out the continuous efforts by researchers around the world using a variety of approaches.

The literature review explored the mechanics of the probiotic cell dehydration during the stabilization process and identified the common industrial technologies available for this purpose and did a comparison among them. The popular techniques were identified as spray drying, freeze drying and fluidized bed drying. The processing stress associated with each of these techniques were identified as heat inactivation and cell membrane injury for spray drying, osmotic stress and freezing injury for freeze drying, osmotic and oxidative stress for fluidized bed drying. Comparatively, fluidized bed drying technique was found to be the least explored one and having another big advantage of imparting only moderate heat stress to the probiotic cells.

By searching a reasonably large database of published scientific literature, the review has compiled a comprehensive list of the major factors responsible for the probiotics viability during enhanced temperature storage. The factors were identified as storage temperature, moisture content and a_w of the matrix, relative humidity of the storage environment and exposure to light and oxygen. The review also described each of these factors in detail and mentioned the probable explanations as suggested by the previous researchers.

The review also looked into various approaches taken by the researchers to address the associated problems and categorized them as,

1) Adaptation to sub-lethal stress conditions in order to induce enhanced expression of certain stress proteins within the cells.

2) Maintaining the optimum glass transition temperature of the stabilization matrix at a level well above the targeted storage temperature.

3) Ensuring maximum cell membrane integrity and minimum oxidative damage by incorporation of most appropriate protectant compounds into the matrix.

4) Restricting the mobility of free water molecules within the stabilized matrix to the minimum possible level but at the same time not reducing the water activity to an extremely low level which may prove unrealistic for the common food applications.

5) By facilitating the accumulation of certain compatible solutes within the probiotic cells by manipulation of the cell growth media.

It was realised that a multi-directional approach targeting all the crucial factors was necessary to offer a complete solution to the identified problem but this project had to limit the periphery of its scope hence it was initially planned to identify the most promising protectant compounds and investigate their actions during the drying and storage period for a better understanding of the underlying mechanism. A subjective analysis of the research trends indicated that the use of sugar alcohols such as, sorbitol or xylitol in combination with non-fat milk solids may prove to be a promising combination of effective protectants.

The review also kept in mind the available resources in the university premise to carry out this project and helped to identify the use of the food pilot plant, common microbiological enumeration techniques, high resolution microscopy, DSC and FTIR instruments for testing of the hypothesis, which is believed to have some merits as supported by the published literatures as well as from the in-house experiments conducted previously.

Chapter 3.0. Materials and methods

3.1 Materials and methods for Chapter 4.0

3.1.1 Preparation of bacterial cell suspension

In this study, altogether 3 lactobacilli and 2 bifidobacterium strains were used. The lactobacilli strains were *Lactobacillus casei* CRL 431 (ATCC Accession no. 55544), *L. acidophilus* ATCC 4356, *L. rhamnosus* ATCC 53103 sourced through Cryosite Ltd. (Lane Cove, NSW, Australia), the bifidobacterium strains were *B. lactis* BB12 from Chr. Hansen (Horsholm, Denmark) and *B. lactis* HN019 from Danisco (Braband, Denmark). The freeze dried cultures were rehydrated in MRS broth from Difco Lab (Franklin Lakes, NJ, USA) at 37 ºC for 24 hours followed by 2 consecutive growth cycles up to their early stationary phase. The growth phases of individual strains, especially the early log, mid-log and early stationary phases were identified by the standard optical density measurement at 610 nm. Cells were harvested by centrifugation at 4600 x g for 10 min and washed 2 times with 0.25% peptone water (Nag *et al*., 2011).

3.1.2 The stress adaptation process

The initial set of experiments were conducted using only *L. casei* CRL431 cells by applying heat, osmotic, a combination of heat and osmotic stresses and compared with

unstressed cells as a control. The aim was to develop the methods and techniques with one species first and then look at other strains of the same species to validate the findings; hence the choice of *L. casei* CRL 431 was random. A slightly modified experimental design based on the work of Pichereau *et al*. (2000) and Prasad *et al*. (2003) was planned. According to the growth curve obtained for this strain, 14th and 20th hours were identified as the mid-log and early stationary phases, respectively. For the heat stress adaptation, after 20 hours of growth, the cells suspended in the growth media were subjected to gradual heating in a water bath maintained at 50 ºC and held for 30 min. Thereafter, the suspension was immediately cooled down to room temperature (~22 ºC) and cells were harvested as mentioned in the section 3.1.1. The osmotic stress to the cells was induced at the mid-log phase (14th hour) by addition of the required quantity of NaCl to give a final osmolality of the media as 0.6M NaCl. Incubation at 37 ºC was continued till the early stationary phase and the same harvesting procedure as mentioned above was followed. For combined heat and osmotic stress adaptation, cells were subjected to the osmotic stress first as described above and then were harvested by centrifugation. The pellet was re-suspended into fresh MRS broth and the same heat stress (50 ºC/30 min) was applied thereafter.

3.1.3 The stabilization process

For freeze-drying process, washed cell pellets were added to 20% (w/w) pre-sterilized reconstituted whole milk. The mix was homogenized in an Ultra-Turax mixer from Ika Works (Guangzhou, China) at 9000 rpm and freeze-dried in a dryer (Model 0610) from

W.G.G. Cuddon Ltd. (Blenheim, New Zealand). The steps involved in the process were freezing at -18 °C, followed by sublimation at 20 °C under a vacuum of 0.4 Torr for a 48 hour period. The dried flakes were manually ground with a mortar and repacked in double layered LDPE sachets. In the case of fluidized bed drying (FBD), a modified protocol suggested by Haris *et al*. (2012) and Stummer *et al*. (2012) was followed. Washed cell pellets were first mixed homogeneously with whole milk powder obtained from Fonterra Co-operative Ltd. (Palmerston North, New Zealand) containing approximately 26% fat. The ratio of cell pellet to milk powder was determined based on a final desired water activity of 0.45 to 0.50, which have been found easy to handle and dry in the FBD.

In one of the experiments, the effects of vitamin E as an antioxidant and inulin as a prebiotic compound were evaluated by incorporating them into the stabilization matrix. Vitamin E (50% dl-alpha-tocopheryl acetate) powder from DSM Nutritional Products Ltd. (Wurmisweg, Switzerland) at 0.5% (w/w) and inulin from Sensus Operations CV (Roosendal, The Netherlands) at 5.0% (w/w) of the final product, were mixed directly with the centrifuged cell pellet. The cell suspension and whole milk powder mix were then transferred to a pilot plant scale FBD from Glatt GmbH (Binzen, Germany), maintained at an inlet temperature of 50-55 ˚C and 35-40˚C outlet temperature. Drying was continued for 15-20 min till the final water activity of 0.24-0.28. The dried product in the free-flowing powder form was packed in double layered LDPE pouches and stored at different temperatures as per different experimental designs. Apart from precise temperature control, no other parameters, such as humidity and oxygen content of the

storage area were controlled. The aim was to simulate the real-life storage environment of a commercial marketplace.

3.1.4 Storage stability of *L. casei* CRL 431 cells and periodical enumeration

For the initial set of experiments, only *L. casei* CRL 431 cells were stabilized with freeze and fluidized bed drying techniques and samples were stored at 25 °C for 52 weeks. In the second phase, cells subjected to various stress adaptation processes were stabilized in FBD only and their storage stability at 25 °C for 52 weeks was compared with unstressed cells as control.

All samples prepared with fortified vitamin E or inulin as well as the samples in the validation experiment with other 4 probiotic strains were stored at 25 °C and periodical viability was analyzed for 12 to 24 weeks, depending upon the residual viable cell population. Each sample was tested for viable cell counts every 7 days and allowed to be rehydrated for 120 min at 37 °C followed by homogenizing in a laboratory stomacher (Model BA6021) from Seward Ltd. (Sussex, UK) and pour plating on MRS agar. The enumeration protocol with particular emphasis on rehydration conditions was followed from the suggestions of Morgan *et al*. (2006) and Garcia (2011).

3.1.5 Particle size distribution of probiotic powder

The Scirocco-2000 unit of the Malvern Mastersizer 2000 Ver. 5.54 (Malvern Instruments Ltd., Malvern, U.K) was used to determine the mean particle size and surface-based diameter range. The principle behind this technology is based on the diffraction of a laser beam on a stream of air containing suspended particles. The diffracted laser beam is collected at different scattering angles by numerous semi-circular photoelectric diodes. The surface-based particle diameter distribution is calculated by measuring the diffracted intensities for each angle using the Lorenz-Mie theory. The refractive index of whole milk powder, i.e; 1.370 was used as a reference because the exact value of the same for the complex encapsulation mix and bacterial cells used in the probiotic powder was unknown. For comparison purposes, two common dairy ingredients viz., whole milk powder (WMP) and skim milk powder (SMP) obtained from Fonterra Co-operative Ltd. (Palmerston North, New Zealand) were also tested for particle size distribution and all other physical properties. Duplicate samples were analyzed and the average result is presented here.

3.1.6 Measurement of water activity

The water activities (a_w) of the probiotic powder and the control samples mentioned above were determined in a Decagon CX-2 (Formula Foods, Christchurch, NZ) instrument utilizing the chilled mirror dew point technique, which measures the relative humidity based on fundamental thermodynamic principles.

3.1.7 Measurement of solubility index

The solubility index was measured as per the guidelines described in the reference manual for U.S milk powders issued by American Dairy Products Institute. In a mixer, 14 g of each sample was mixed with 100 ml of water at 24 ºC at high speed for 90 sec. The mix was then left for 15 min, after which it was stirred with a spatula. 50 ml of the mixture was filled into a graduated 50 ml centrifuge glass with a conically graduated bottom and centrifuged for 5 min at 4600 x g. The sediment-free liquid was drained off, the tube was filled up again with water (to make the reading easier), and the contents were stirred. Then the tube was put into the centrifuge and spun for 5 min at 4600 x g. after which the sediment was read from the graduated cone bottom.

3.1.8 Measurement of bulk density and flowability

The bulk density and flowability of the samples were measured in a Powder Flow Tester from Brookfield Eng. Lab. (Middleboro, MA, USA). The principle of operation of the instrument is to drive a compression lid vertically downward into the powder sample contained in an annular shear cell. The powder sample has a defined volume and the weight of the sample is measured before the start of the test, which helps to calculate the bulk density. A calibrated beam load cell is used to control the compaction stress applied to the powder. The annular shear cell is then rotated at a defined speed and the torque resistance of the sample is measured by a calibrated reaction torque sensor. The

geometries of the lid, shear cell, rotational speed and compressive loads applied to contribute to the calculations, which determine the flow-ability of the powder sample.

3.1.9 Acid and bile salts resistance in a simulated gastro-intestinal environment

Pepsin (P7000) and porcine bile extract powder (B8631) were supplied by Sigma Aldrich (St. Louis, MO, USA). All other chemicals used in this experiment were procured from VVR International (Paris, France). Simulated gastric fluid (SGF) was prepared as per US Pharmacopeia (USP31-NF26, 2008) with 0.2% (w/v) NaCl and the pH was adjusted to 2.0 with hydrochloric acid. Pepsin (800-2500 units/mg of protein) in powder form, obtained from porcine gastric mucosa was added into this preparation at 0.32% (w/v). One g of the powdered sample was added to each of the 5 test tubes containing 9 ml of pre-warmed (37 °C) SGF preparations, the pH was immediately further adjusted to 2.0 with HCl (addition of the powder raised the SGF pH) and incubated in a water bath maintained at 37°C under orbital agitation at 100 rpm (Guerin *et al*., 2003). The pH measurements after the final incubation of 120 min did not show any significant change in value. After every 30 min, one sample tube was taken out and immediately the pH of the medium was raised to 7.0 with 0.1N NaOH to stop the enzymatic reaction on the encapsulation matrix and to prevent from the detrimental acidic effect of acid on the remaining viable cells. To verify and compare the protective ability of the encapsulating matrix, free *L. casei* CRL431 cells were also subjected to the

SGF incubation test and 1 ml of cell suspension was added into every 9 ml of SGF. Neutralization followed the incubation in this case.

The Simulated environment containing bile salts was prepared as per the method described by Muthukumarasamy *et al*. (2006). Monobasic potassium phosphate (KH_2PO_4) at 0.68% (w/v) and 1.0% (w/v) porcine bile extract were dissolved into deionized milliQ water (Millipore, Molsheim, France) and adjusted the pH to 6.8 with 0.2N NaOH solution. The incubation protocol for both the probiotic powder and free cells was the same as in SGF with the only exception of a prolonged incubation period of 8 hours.

3.2 Materials and methods for Chapter 5.0

3.2.1. Determination of the early stationary phase of *L. reuteri* LR6 cells
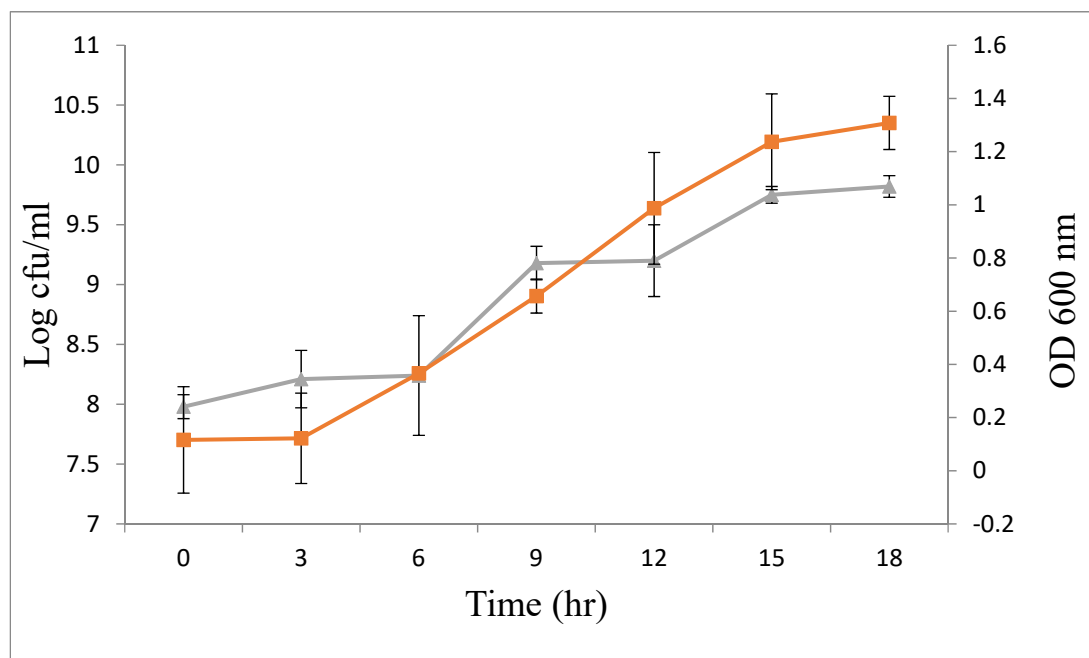
Figure 3.1: *Growth curve of L. reuteri LR6 cells grown in MRS broth. Pour plate counts of the (▲) plotted along with the optical density (■) of the growth media*

To identify the early stationary phase during the growth cycle, LR6 cells were inoculated into MRS broth (Oxoid chemicals) at 1.0% (v/v) and incubated at 37 ˚C for 18 hours. At 3 hour intervals, the media was enumerated for viable cell counts on MRS agar plates and the optical density (600 nm) was measured in a spectrophotometer using proper dilutions. From Fig. 3.1 it was estimated that the cells reached the early stationary phase after 16 hours and henceforth harvesting of the cells was done after 16 hours from inoculation for all the subsequent experiments.

3.2.2 Vacuum oven drying of the cells without any carrier agent

Before stabilizing the cells in the fluid bed dryer (FBD), which requires a carrier excipient or supporting material to hold the cells in a granular form, it was planned to desiccate the unsupported cells in a vacuum drier maintained at 55 ˚C or 75 ˚C. Figure 3.2 shows the process flow diagram designed for stabilizing the LR6 strain in the unsupported form.

Cultures stored in frozen beads at -80°C

↓

Rehydrated in 10 ml MRS Broth at 37°C

↓

After 24 hours inoculated into 50 ml MRSB at 37.C

↓

After 16 hours inculated at 37°C into 1000 ml MRSB

↓

After 16 hours cells harvested with centrifugation at 11750 x g

↓

Washing the cells with 0.25% peptone water

↓

Final harvesting with centrifugation at 11750 x g

↓

Addition of Sorbitol/Xylitol powder directly to the pellet

↓

Mixing and dissolving of additives into the cell pellet

↓

Vacuum oven drying at 55°C or 75°C for 2 hours

Figure 3.2: Process flow diagram for the stabilization of LR6 cells in the unsupported form

After two growth cycles (up to stationary phase) the cells were inoculated at 1.0% level into MRS broth (Oxoid Ltd., England) and harvested at the early stationary phase with centrifugation (11,750 x g for 10 min). After discarding the spent liquor the cells were washed once with 0.25% (w/v) peptone water and pellets were collected. Average pellet weight or wet biomass obtained from 500 ml of growth media was 4.0 ± 0.11 gm. Since the fluidized bed drying of the cells cannot be undertaken without a supporting material, unsupported drying of the cells was performed with a different technique that tried to mimic the conditions in a FBD. The freshly harvested cell pellet was spread evenly on a sterile Petri dish and placed inside the tray dryer maintained at 55 ˚C or 75 ˚C under vacuum. The same quantity of cell pellet was mixed with 5 gm (which represents 5% w/w of the stabilized samples when prepared in FBD with the carrier agents) of D-sorbitol (Sigma Aldrich, USA) or xylitol (Danisco, Finland) powder until dissolved completely. These blends were then subjected to vacuum drying as described above.

3.2.3 Stabilization using wet granulation and fluidized bed drying

Figure 3.3 shows the process flow diagram designed for stabilizing the LR6 strain. The protocol for cell harvesting and concentrating was same as described in section 3.1.1. In one set of samples the cell pellet (obtained from 1000 ml growth media) was slowly mixed under agitation to a bed of 200 gm skim milk powder (Fonterra co-op., NZ) and blended in a dough mixer (Make: Kenwood, Model: Chef) for the wet granulation purpose. In other two sets, sorbitol or xylitol powder (5.0% by weight of the total sample weight, i.e, 10 gm of sorbitol/xylitol added into the quantity of cell mass that goes into

200 gm of SMP) was directly added to the cell pellets and allowed to dissolve fully before adding to the SMP. The granulation process was carried out for 15 min followed by drying of the mix in the fluidized bed dryer (Glatt GMBH, Germany).

Cultures stored in frozen beads at -80°C

↓

Rehydrated in 10 ml MRS Broth at 37°C

↓

After 24 hours inoculated into 50 ml MRSB at 37°C

↓

After 16 hours inculated at 37°C into 1000 ml MRSB

↓

After 16 hours cells harvested with centrifugation at 11750 x g

↓

Washing the cells with 0.25% peptone water

↓

Final harvesting with centrifugation at 11750 x g

↓

Addition of Sorbitol/Xylitol powder directly to the pellet

↓

Mixing and dissolving of additives into the cell pellet

↓

Slow and intermittent addition of the pellet+additive mix into 200 gm SMP

↓

Slow mixing in a Kenwood dough blender

↓

Rapid granulation in the dough blender for 15 min

↓

Fluidized bed drying of wet granules

Figure 3.3: Process flow diagram for the stabilization of LR6 cells using fluidized bed drying

It was also important to identify the most optimum drying temperature in order to yield the desired level of moisture content/a_w but without compromising the viable cell counts in the dried samples. The combination of four different inlet temperatures (55 ˚C, 65 ˚C, 75 ˚C & 85 ˚C) for a fixed drying time of 20 min for each batch was chosen for this purpose. In the fluidized bed dryer (FBD) there is no provision to control the outlet temperature but a consistent temperature difference of 20-25 ˚C was observed between the inlet and outlet temperatures.

3.2.4 Stabilization using excipients other than skim milk

The chosen excipients in the second set of experiments reported in section 4.3 were microcrystalline cellulose (Sigma Aldrich, USA), corn starch (National Starch, USA) and milk protein concentrate or MPC-80 (Fonterra co-op, NZ). Microcrystalline cellulose (MCC) is refined wood pulp and it is a white, free-flowing powder. Chemically, it is an inert substance, insoluble in water and resistant to reagents, not degraded during digestion and has no appreciable absorption. It is a commonly used excipient in the pharmaceutical industry but also found in many processed food products, and may be used as an anti-caking agent, stabilizer, texture modifier, or suspending agent among other uses. Corn starch (CS) is used as a thickening agent in liquid-based foods. It is comprised of long chains of starch molecules, insoluble in water at low temperatures and contains no protein. MPC-80 contains 80% protein in the same ratio as found in the milk and very low level of lactose (approximately 5%). It is prepared by the membrane filtration of milk. The rationale behind choosing these carrier

excipients was the selection of a relatively inert but food grade substance (MCC), pure carbohydrate but with longer chain lengths of polysaccharides (CS) than lactose and high concentration of milk proteins in dried form (MPC). The cell harvesting process, granulation and drying parameters were exactly the same as maintained previously for the set of experiments with SMP/sorbitol/xylitol as detailed in Fig. 3.3.

3.3 Materials and methods for Chapter 6.0

3.3.1 Preparation of samples for FTIR spectroscopy

The advantage of studying bacterial cell components using FTIR spectroscopy is its ability to investigate *in situ* without extracting or modifying the structure of the components (Oldenhof *et al*., 2005). *Lactobacillus reuteri* LR6 cells dried in the vacuum oven were rehydrated in Ringer's solution (Sigma Aldrich, USA) with the composition of 6.5g NaCl, 0.42g KCl, 0.25g $CaCl_2$ and 0.2g of sodium bicarbonate dissolved in one litre of distilled water. Cells coated with sorbitol or xylitol were washed twice by vortexing while suspended in Ringer's solution to remove the polyol coating the cells were finally harvested by centrifugation at 4400 x g for 5 min. The cell suspension (approximate volume 10 µl) was then spread as a thin film onto the 1 mm thick CaF_2 window (Crystran Ltd., Poole, UK) and the moisture was dried off by holding the plate at 42 ˚C for 60 min as per the method suggested by Santivarangkna *et al*. (2007). Triplicate samples from each batch were spread onto three such plates and taken for the direct IR measurement. The whole experiment was replicated twice.

The cells which were dried in the FBD alongwith the supporting excipients had to be isolated first from the dried powder matrix. The individual powder samples containing the embedded cells on the particle surfaces or in an agglomerated form were mixed thoroughly with the same Ringer's solution (approximately 10 gms powder made upto the volume of 50 ml) in 50 ml sterile centrifuge tubes. To breakdown the granules, the samples were vortexed and the tubes were then kept stationary for 1 hour to settle insoluble solids. MCC and starch powders, both of which were insoluble in cold water settled at the bottom of the tube and the cell suspension at the top was decanted carefully into another tube. This process was repeated once again to remove any heavier powder particles from the decanted cell suspension and finally the cells were harvested by centrifugation. The insolubles in the MPC and SMP based samples, principally thought to be comprised of aggregated proteins, were allowed to settle. The dissolved portions at the top of the tubes were decanted out into fresh tubes and the same process was repeated for the final cell harvesting but with extra two washing cycles for these samples. The pellets at the bottom cone of the tubes were resuspended into fresh Ringer's solution. The film making process as described in the case of vacuum dried cells was the same for all these FB dried samples.

3.3.2 Determination of the state of cell envelopes and the secondary protein structures using FTIR spectroscopy

Wavenumbers (cm^{-1}) of molecular vibrations and the corresponding infrared absorbances of the specific functional groups of the cell envelope and the secondary

protein structures were recorded using the FTIR spectrometer (Thermofisher Scientific, USA) fitted with a KBr beam splitter. The parameters for spectral measurement were: resolution 4 cm^{-1}, encoding interval 1 cm^{-1}, scanning speed 0.2 cm^{-1}/s. Mid IR range of 4000-500 cm^{-1} was recorded for each sample after averaging 256 scans. A background spectrum of air was measured and subtracted by the instrument itself before each operation. Mean spectra were the average of three samples from two independent and replicated experiments. The optical chamber was purged continuously with nitrogen gas to avoid interference of water vapor and CO_2.

The obtained spectra were analyzed in the Omnic software (Thermofisher Scientific, version 7.1), saved in the CSV format compatible with Microsoft Excel 2010 and R environment (64 bit, version 3.2.2). The resolution of the complex bands was increased by calculating the 1st derivative using Savitzky – Golay algorithm with 9 point smoothing followed by the deconvoluted 2nd derivative spectra calculation using the same algorithm and smoothing technique (Dziuba *et al*., 2007). In 1964 Savitzky and Golay (Savitzky and Golay, 1964) published a paper on calculating the smoothing and differentiation of data series by employing a new type of least-squares method. This work became very popular afterwards because this method simplified the computation by replacing the previously practiced lengthy least-squares calculations with an equivalent convolution (Gorry, 1990). After obtaining the convoluted 2nd derivative spectra using this method, the peak values at specific areas of interests were recorded.

3.3.3 Using R environment for the principal component analysis and computation of the hierarchical clustering

For a quantitative analysis to detect the variations among individual sample spectrum, the data in CSV format were subjected to a multivariate statistical analysis called principal component analysis. "Principal component analysis (PCA) is a statistical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components. The number of principal components is less than or equal to the number of original variables. This transformation is defined in such a way that the first principal component has the largest possible variance (that is, accounts for as much of the variability in the data as possible), and each succeeding component in turn has the highest variance possible under the constraint that it is orthogonal to the preceding components." (Wikipedia, Principal Component Analysis). PCA is a dimension reduction techniques applied for simplifying the data and for visualizing the most important information in the data set. Individuals are considered in a high dimensional Euclidean space and studying the similarities between individuals means studying the shape of the cloud of points. Principal component methods then approximate this cloud of points into a Euclidean subspace of lower dimensions while preserving as much as possible the distances between individuals. Another way to study the similarities between individuals with respect to all the variables is to perform hierarchical clustering. Hierarchical clustering requires defining a distance and an agglomeration criterion. Many distances (Manhattan, Euclidean, etc.) as well as several

agglomeration methods (Ward, single, centroid, etc.) are available. The indexed hierarchy is represented by a tree named a dendrogram (Husson *et al*., 2010).

Clustering is applied for identifying groups (i.e clusters) among the observations. Clustering can be subdivided into five general strategies:

- Partitioning methods

- Hierarchical clustering

- Fuzzy clustering

- Density-based clustering

- Model-based clustering

The Ward criterion is preferred in the hierarchical clustering because it is based on the multidimensional variance (i.e.inertia) as well as principal component methods (Husson *et al*., 2010).

"R is an integrated suite of software facilities for data manipulation, calculation and graphical display. Among other things it has

- an effective data handling and storage facility,

- a suite of operators for calculations on arrays, in particular matrices,

- a large, coherent, integrated collection of intermediate tools for data analysis,

- graphical facilities for data analysis and display either directly at the computer or on hardcopy, and

- a well developed, simple and effective programming language (called 'S') which includes conditionals, loops, user defined recursive functions and input and output facilities. (Indeed most of the system supplied functions are themselves written in the S language.)

The term "environment" is intended to characterize it as a fully planned and coherent system, rather than an incremental accretion of very specific and inflexible tools, as is frequently the case with other data analysis software (Venables *et al.*, 2004).

R is a vehicle for newly developing methods of interactive data analysis. It has developed rapidly, and has been extended by a large collection of packages. Two such packages namely, Factoextra and FactoMineR have been used in developing the programming code with consultation from the Dept. of Statistics, Institute of Fundamental Sciences, Massey University. The principal component analysis of all spectra was performed using the developed code mentioned below.

```
library("factoextra")
compiled_dendrogram<-read.csv("file_name.csv",row.names=1)
head(compiled_dendrogram)

library("FactoMineR")
res.pca <- PCA(compiled_dendrogram, graph = FALSE)
print(res.pca)

eigenvalues <- res.pca$eig
```

```r
head(eigenvalues[, 1:2])

library("factoextra")
fviz_screeplot(res.pca, ncp=10)

# Coordinates of variables
head(res.pca$var$coord)

fviz_pca_var(res.pca)
head(res.pca$var$cos2)
fviz_pca_var(res.pca, col.var="cos2") +
scale_color_gradient2(low="white", mid="blue",
            high="red", midpoint=0.5) + theme_minimal()

head(res.pca$var$contrib)
# Contributions of variables on PC1
fviz_contrib(res.pca, choice = "var", axes = 1)
# Contributions of variables on PC2
fviz_contrib(res.pca, choice = "var", axes = 2)
# Total contribution on PC1 and PC2
fviz_contrib(res.pca, choice = "var", axes = 1:2)

# Control variable colors using their contributions
fviz_pca_var(res.pca, col.var="contrib")

# Change the gradient color
fviz_pca_var(res.pca, col.var="contrib") +
scale_color_gradient2(low="white", mid="blue",
            high="red", midpoint=50) + theme_minimal()

dimdesc(res, axes = 1:3, proba = 0.05)
res.desc <- dimdesc(res.pca, axes = c(1,2))
# Description of dimension 1
res.desc$Dim.1
# Description of dimension 2
res.desc$Dim.2
head(res.pca$ind$coord)
fviz_pca_ind(res.pca)
fviz_pca_ind(res.pca, col.ind="cos2") +
scale_color_gradient2(low="white", mid="blue",
  high="red", midpoint=0.50) + theme_minimal()
```

```
head(res.pca$ind$contrib)

# Contributions of individuals to PC1
fviz_contrib(res.pca, choice = "ind", axes = 1)
# Contributions of the individuals to PC2
fviz_contrib(res.pca, choice = "ind", axes = 2)
# Total contribution on PC1 and PC2
fviz_contrib(res.pca, choice = "ind", axes = 1:2)

# Contributions of the top 10 individuals to PC1
fviz_contrib(res.pca, choice = "ind", axes = 1, top = 10)

# Change the gradient color
fviz_pca_ind(res.pca, col.ind="contrib") +
scale_color_gradient2(low="white", mid="blue",
            high="red", midpoint=50) + theme_minimal()

res.hcpc <- HCPC(res.pca, nb.clust=0, conso=0, min=3, max=30)
```

For computing the hierarchical clustering using the Ward algorithm, another programme
compatible to R was written as mentioned below.

```
# Compute distances and hierarchical clustering
dd <- dist(scale(head), method = "euclidean")
hc <- hclust(dd, method = "ward.D2")
plot(x, labels = NULL, hang = 0.1,
    main = "Cluster dendrogram", sub = NULL,
    xlab = NULL, ylab = "Height", ...)
# Default plot
plot(hc)

# Put the labels at the same height: hang = -1
plot(hc, hang = -1, cex = 0.6)
plot(x, type = c("rectangle", "triangle"), horiz = FALSE)

# Convert hclust into a dendrogram and plot
hcd <- as.dendrogram(hc)
# Default plot
```

```
plot(hcd, type = "rectangle", ylab = "Height")

# Triangle plot
plot(hcd, type = "triangle", ylab = "Height")

# Zoom in to the first dendrogram
plot(hcd, xlim = c(1, 20), ylim = c(1,8))

# Define nodePar
nodePar <- list(lab.cex = 0.6, pch = c(NA, 19),
         cex = 0.7, col = "blue")
# Customized plot; remove labels
plot(hcd, ylab = "Dissimilarity %", nodePar = nodePar, leaflab = "none")

# Horizontal plot
plot(hcd,  xlab = "Heterogeinity",
    nodePar = nodePar, horiz = TRUE)
```

3.3.4 Storage stability of the stabilized LR6 cells

After the fluidized bed drying the stabilized LR6 cells supported by the selected carrier agents were adjusted for a_w equilibrium. A supersaturated solution of lithium chloride (LiCl) kept inside a desiccator was used to bring down the a_w to 0.10-0.11 level. For another set of samples supersaturated solution of magnesium chloride ($MgCl_2$) was used to adjust the a_w to 0.30-0.32. The desiccators were placed in a room maintained constantly at 37 ˚C and periodic examination and necessary adjustment to the concentration of the salt solutions were performed. After the equilibrium was reached within 7-14 days, the samples were packed in double layered polypropylene sachets each containing ~5 gm of the powders and were placed inside heat-sealed alluminum foil pouches. The packages were stored at 37 ˚C for 6 months. Samples were analyzed every 30 days for viable lactobacillus counts on MRS agar plates using pour plating technique.

For FTIR spectroscopic examination of the isolated cells, the same protocol was followed as described in sections 3.3.1 and 3.3.2. For principal components and hierarchical clustering analysis of the obtained FTIR spectra, the methodology described in section 3.3.3 was used.

3.3.5 Segregation by sieving

To investigate the role of granule sizes i.e, the effect on agglomeration efficiency on the LR6 cells viability following stabilization and storage, the samples in triplicate were segregated into four fractions based on particle diameters. Three laboratory test sieves made out of stainless steel (Lab Technics, Kilkenny, South Australia) with apertures 1000 µm, 500 µm, and 250 µm were placed in a series from to bottom in the same order. A measured quantity of the samples was placed on the topmost sieve (1000 µm). The setup was manually shaken until the samples stopped passing out of the bottom sieve (250 µm). The segregated fractions were collected, accurately weighed and packed into double layered LDPE pouches followed by heat sealed alluminum foils. LR6 cells stabilized with only SMP was used in this experiment and no adjustment of a_w was done. Samples were stored at 37°C for 180 days and enumeration of viable cells was performed at day 0 and day 180.

3.3.6 Scanning electron microscopic examination

Freshly harvested *L. reuteri* LR6 cells and the isolated cells from the stabilization matrix were examined with the help of scanning electron microscopy (SEM). The samples were observed under a FEI Quanta 200 scanning electron microscope (Eindhoven, The Netherlands). The samples were first mounted on a standard SEM sample stud made of aluminum, with the help of double sided sticky tape. The sample was then sputter coated with gold with the help of a BAL-TEC SCD 050 sputter coater under a vacuum of 5 x 10^{-2} millibar. The images were recorded in TIF format using 20 kV accelerated voltage and 250 to 20,000 times magnification.

3.3.7 Transmission electron microscopic examination

The microscope used for this purpose was from Philips Export Dvn., Eindhoven, The Netherlands (Model CM10). The samples were first observed under light microscopy, which is a preliminary step towards TEM observation. The sample preparation steps included primary fixation with 3% glutaraldehyde in 0.1M phosphate buffer, holding for two hours at room temperature followed by centrifugation at 4000 rpm. The pellet was then mixed with 20% bovine serum albumin (BSA), coagulated the BSA with 25% glutaraldehyde and the resultant pellet was sliced into thin pieces. After 3 washes with buffer, the thin pellets were dehydrated with a gradually increasing concentration of acetone. One micron cross sections were cut from the thin blocks and placed on

microscopic slides after staining with Toluidine Blue. The samples were then ready for light microscopic observations.

Further processing of these resin blocks was done to prepare for TEM observation. The blocks were trimmed to approximately half by half a millimeter square sizes which included the area of interests and then made into small 100 nm sections using a diamond knife and Ultra-microtome. The cross-sections were then stretched using chloroform vapor and double stained first with saturated uranyl acetate in 50% ethanol for 4 min and again with lead citrate for next 4 min. The equipment used for cutting the resin blocks was Leica Ultracut R from Microsystem GmBH, Austria.

3.3.8 Measurement of glass transition temperature (T_g) using differential scanning calorimetry

Differential scanning calorimetry (DSC, TA Instruments, New Castle, USA) was used to determine the glass transition temperatures of the stabilized LR6 cells carried by various excipient materials. The samples were weighed (about 10 mg) in the stainless steel DSC pans and hermetically sealed thereafter by putting on the lids and using pressure. The first run of heating was designed to heat the samples up to 70 °C at a constant heating rate of 10 °C/min followed by cooling down to 0 °C at the same rate of cooling. The second heating round was comprised of heating the samples from 0 °C to 120 °C at the uniform increment of 10 °C/min. The mid-point glass transition temperature (T_g) was

determined by spotting the change in the heat flow curve using the Universal Analysis 2000 software (TA Instruments, New Castle, USA).

3.3.9 Measurement of surface hydrophobicity

The cell surface hydrophobicity of the LR6 cells towards three hydrocarbons viz. n-hexadecane, xylene or n-octane (Sigma Aldrich, USA) was measured following the protocol suggested by Singh *et al.* (2014). The freeze-dried cultures were rehydrated in MRS broth from Difco Lab (Franklin Lakes, NJ, USA) at 37 ºC for 24 hours followed by 2 consecutive growth cycles up to their early stationary phase (16 hours). Cells were harvested by centrifugation at 4600 x g for 10 min, washed 2 times with Ringer's solution and resuspended into the same. The cell suspension OD at 610 nm was adjusted to approximately 0.8 – 1.0 using a spectrophotometer. This suspension was then mixed with each of the hydrocarbons at 3:10 (v/v) ratio in Eppendorf tubes and incubated at 37 ºC for 10 min followed by thorough vortexing for 2 min. After this mixing process, the tubes were incubated at 37 ºC for 60 min to allow the cells to bind to the hydrocarbons. During this time, due to phase separation, the hydrocarbon parts formed a layer on the top. The aqueous parts at the bottom of the tubes were collected carefully with a Pasteur pipette and the OD was measured. The hydrophobicity (%) was calculated by the following formula.

Surface Hydrophobicity (%) = $(ODi - ODf) \div ODf * 100$

ODi = **Initial optical density**

ODf = **Final optical density**

The retention of surface hydrophobicity over the storage period was calculated by the following formula.

Retention of surface hydrophobicity = $(Hyd0 - Hyd12) \div Hyd0 * 100$

Hyd0 = **Hydrophobicity at week 0**

Hyd12 = **Hydrophobicity at week 12**

3.4 Statistical analysis

Statistical analysis of the obtained results was done using Minitab 15.1.0 software from Minitab Inc. (State College, PA, USA). Analysis of Variance (ANOVA) between two series of data was performed and a difference is considered to be of significant importance only at more than 95% confidence level ($p \leq 0.05$). The results were expressed as mean values of the viable cell population per g (converted into logarithmic scale) of the sample from 2 replicated experiments and the standard deviations were shown as error bars.

Chapter 4.0. Improving ambient temperature stability of probiotics with stress adaptation and fluidized bed drying

The contents of this chapter have been published in a peer-reviewed journal article and reproduced with permission from:

Nag, A, & Das, S. (2013) Improving ambient temperature stability of probiotics with stress adaptation and fluidized bed drying, *Journal of Functional Foods*, **5**(1), 170-177,

Based on the work reported in this chapter, an international patent has been secured for the developed technology:

Nag, A., Das, S., and Singh, H. (2013) Process of producing shelf stable probiotic food, US 2013/0224303 A1.

4.1 Introduction

The survival of probiotics during the product shelf-life is of high importance considering their commercial and regulatory aspects but a technological gap exists to successfully deliver live probiotics in this category of ambient-stable foods. Although several microencapsulation techniques have been developed and published which can improve the survival of probiotic cells in simulated physiological conditions (Ainsley-Reid *et al*., 2005; Nag *et al*., 2011; Rajam *et al*., 2012), improvement in storage stability, especially under ambient conditions, has been rarely reported. Many attempts can be found to successfully stabilize the popular probiotic strains of lactobacilli, bifidobacterium or enterococcus origin using various drying techniques (Gardiner *et al*., 2000; Goderska & Czarnecki, 2008; Stummer *et al*., 2012). In general, freeze drying of probiotic cultures is associated with many detrimental effects such as damage of membrane lipids and change in cell protein structures (Leslie *et al*., 1995), physical and/or chemical degradation during dehydration and storage (Santivarangkna *et al*., 2008). This results in poor cell recovery and loss of metabolic and probiotic characteristics during unfavorable storage conditions at ambient temperature. Several recent studies have highlighted the challenge of maintaining the viability of probiotics when stabilized with either spray or freeze-drying techniques and stored for long duration at ambient temperature (Prasad *et al.,* 2003; Suharja *et al.,* 2014; Dianawati *et al.,* 2017; Zhao *et al.,* 2018).

This chapter discusses the stabilization of a probiotic strain *Lactobacillus casei* CRL 431 with both freeze drying and fluidized bed drying and compares the residual viability

over a period of 52 weeks when stored at 25 ºC. Then the effects of heat, osmotic and combined stress adaptations on long term ambient stability of this strain were investigated. The best combination of drying technique and stress adaptation which showed promising results during storage at 25 ºC was selected and further experiments were conducted to enhance the stability by the incorporation of an antioxidant and a prebiotic compound in the stabilization matrix. Finally, the findings were validated over a range of other popular probiotic strains and therefore the best-known technique was applied to another 2 lactobacilli and 2 bifidobacterium strains. Their ambient stability with the freeze-dried samples of the same strains when stored at 25 ºC were compared. To characterize the stabilized ingredient for the physical properties, such as water activity, particle size distribution, flowability, solubility, and bulk density, were measured and reported here.

4.2 Results and discussion

4.2.1 Comparison between freeze drying and fluid bed drying

Figure 4.1 shows a gradual decline in the viable population of *L. casei* CRL431 cells over a long-term storage period at 25 °C. For freeze-dried cells, the viable population was reduced to 0.5 log cfu/g within first 4 weeks and thereafter the decline
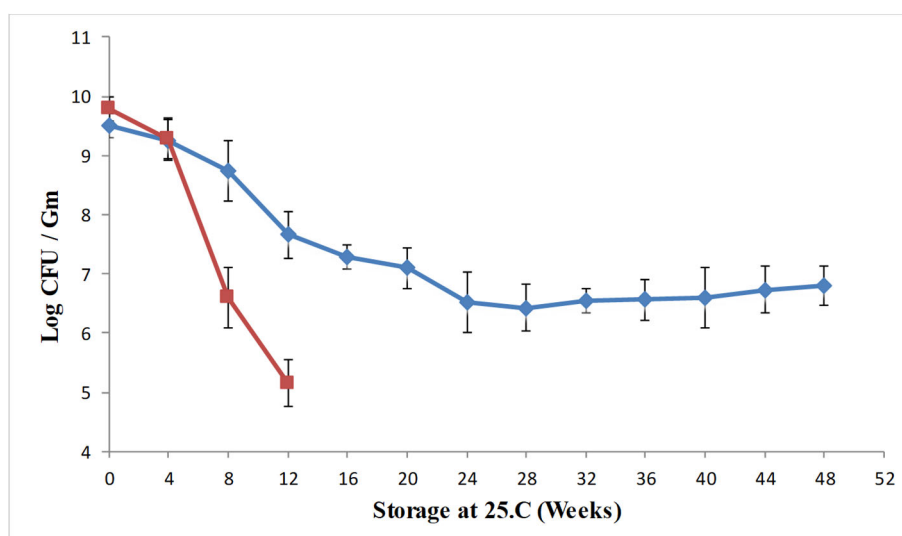
Figure 4.1: *Storage stability of fluidized bed dried L. casei CRL 431 cells stabilized within WMP matrix at 25 °C (♦) compared with freeze-dried cells as a control (■)*

was sharper and within 12 weeks the viable cell count was reduced to 5.2 log cfu/g. This level was considered to be very low and of little significance, therefore further enumeration was discontinued. During this first 12 weeks, cells dried in FBD (within a matrix comprised of whole milk powder) lost the viable population from 9.5 log to 7.7 log cfu/g only, which has been found significantly ($p \leq 0.05$) better than the freeze-dried samples. Up to 24 weeks the loss in viability was gradual and thereafter the cell population was comparatively stable till 52 weeks. The total loss in viability after 52 weeks was recorded as 2.7 log cfu/g.

In this study, freeze-dried *L. casei* CRL 431 cells suffered from a high loss in recovery during ambient storage (Fig. 4.1). The same trend was observed for other 2 lactobacilli and 2 bifidobacterium strains (Fig. 4.3). This is not an uncommon phenomenon and several studies have reported that freeze-dried probiotic cells lose their viability

completely within 3 months of storage at temperatures between 20 ºC and 30 ºC (Tsvetkov & Brankova, 1983; Viernstein *et al*., 2005).

On the other hand, all the 5 strains stabilized with FBD showed significantly better survival when stored at 25 °C. The probable explanation could be the enhanced protection obtained by the cells in the form of a coating of fat, protein and carbohydrate particles during the fluid bed drying but avoided the freezing stress associated with freeze-drying (Gouin, 2004; Haris *et al*., 2012). It has been shown earlier by several authors that by ensuring proper control over drying temperature (Ringer & Mujumdar, 1984; Bayrock & Ingledew, 1997) and the kinetics of osmotic pressure variations (Kosanke *et al*., 1992; Beney *et al*., 2000), it is possible to obtain a very high level of microbial survival rate and their storage stability is also improved due to less damage to the membrane integrity (Cardona *et al*., 2002). Stabilization of probiotics with FBD is not only cost effective (Joshi & Thorat, 2011) but offers very good control over residual moisture content, water activity, powder flowability, and particle sizing.

The superiority of the fluid bed drying technique over freeze drying in terms of storage stability of probiotics was validated by Poddar *et al*. (2014). According to their study, even if the dried powders from two drying methods are brought to equilibrium towards a_w 0.11 or a_w 0.33, the differences in their absolute moisture contents still remain significant. In both, the a_w levels fluid bed dried powders contained lower moisture than the freeze-dried powders. Storage stability of the fluid bed dried powders particularly at a_w 0.33 was much superior and was directly correlated with the moisture content (less moisture = better stability). Since most of the targeted dry or intermediate moisture food

applications will have an equilibrium a_w around 0.30, it was recommended to develop probiotic ingredient using fluid bed drying technique. Poddar *et al*. (2014) also explained this difference in absolute moisture content at the same a_w levels by examining the porosity and surface morphology of the dried particles. They concluded that the lower moisture contents of the fluid bed dried powders (at a_w 0.33) were due to the lower internal surface area of the dried particles resulting into lesser exposure to the environment from where the water gets absorbed into the particles during the equilibrium process.

4.2.2 Effect of stress adaptation and fluidized bed drying on long term storage stability

L. casei CRL431 cells subjected to mild heat stress at 50 °C for 30 min showed a very similar pattern of storage stability as the control cells mentioned in section 4.2.1 (Fig. 4.2). Up to 20 weeks, the viability was reduced at a faster rate (from 9.6 to 7.1 log cfu/g)
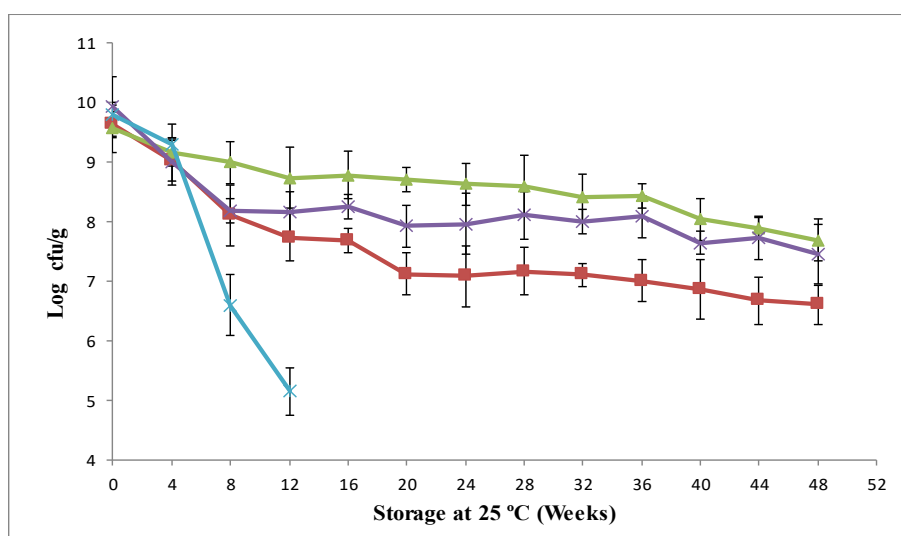
Figure 4.2: *Storage stability of fluidized bed dried L. casei CRL 431 cells at 25 °C pre-adapted with heat stress (■), osmotic stress (▲), combined heat and osmotic stresses (X), when compared freeze-dried cells as a control (X)*

and thereafter it remained steady till 36 weeks, before reaching to 6.6 log cfu/g at the end of 52 weeks. The net reduction in viable cell count was 3.0 log, very similar to the unstressed, control cells. The effect of applying a combined heat and osmotic stress was found to be significantly (p≤0.05) greater than the heat-stressed cells in terms of storage stability. After an initial sharp reduction within the first 8 weeks, the viable cell population was very steady during rest of the storage period. From 8 to 52 weeks, the reduction was only 0.7 log and the net reduction during the entire period was 2.47 log cfu/g. However, the best result was obtained in case of osmotic stress adaptation where only 1.87 log cfu/g reductions in the viable count were observed at the end of 52 weeks of storage test. The loss in storage stability was found to be significantly lower (p≤0.05) than the other two stressed and the control samples.

Before stabilizing probiotic cells, an application of a sub-lethal stress to enhance the stress responses has been found highly effective and has been attempted by many researchers to ensure high viability and retention of physiological activities after dehydration (Kim *et al*., 1988; Teixeira *et al*., 1995; deUrraza & DeAntoni, 1997; Desmond *et al*., 2002).

It was shown that probiotic cells positively respond to the changes in the immediate surroundings by reprogramming their metabolic activities and that causes enhanced resistance during prolonged storage in an inactive state (Pichereau *et al*., 2000). Prasad *et al*. (2003) found that heat-stressed *L. rhamnosus* HN019 cells at stationary phase and osmotic stressed cells at growth phase showed superior storage stability than the unstressed cells and reported that multiple fold upregulation of heat shock proteins; the synthesis of some glycolytic enzymes was possibly responsible for improving the cell stability during a desiccated storage. The results shown in Figure 4.2 confirmed that adaptation to the osmotic stress significantly improved the storage stability of desiccated probiotic cells. However, the application of heat stress did not have any impact on the storage stability of *L. casei* CRL 431 cells, in contrast to the findings of Prasad *et al*. (2003), probably because a different bacterial species was used in this study.

4.2.3 Validation of the findings with other probiotic strains of lactobacilli and bifidobacterium origin

After establishing the effect of a combination of osmotic stress adaptation and FBD, other two lactobacilli and bifidobacterium strains were treated under the same conditions and their storage stability evaluated. However, the freeze-dried cells used as controls were not adapted to any stressed environment. The viabilities of the freeze-dried cultures of *L. acidophilus* and *L. rhamnosus* decreased below significant levels (< 6.0 log/g) within 8 and 16 weeks respectively (Fig. 4.3).
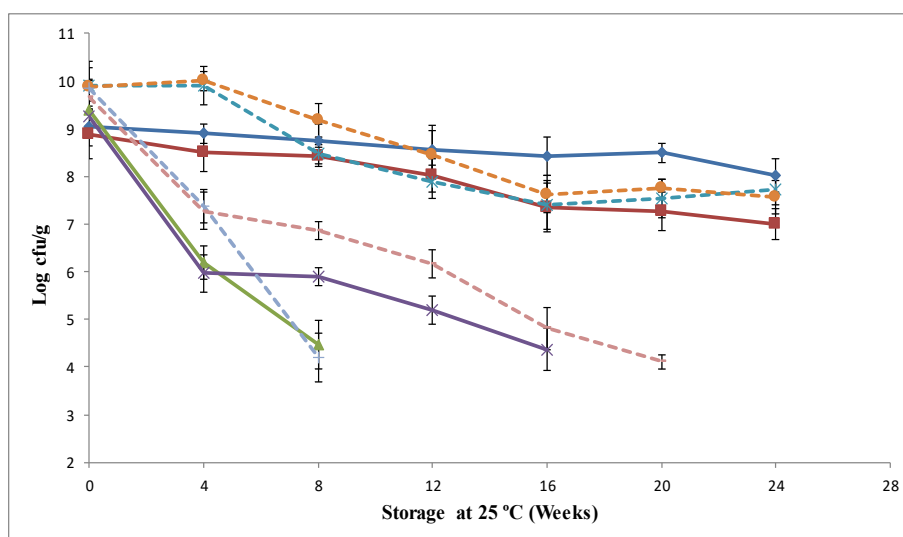
Figure 4.3: *Storage stability at 25 °C of osmotically stressed and FB dried vs. freeze dried control samples for L. acidophilus (FBD ♦, FD ▲), L. rhamnosus (FBD ■, FD X), B. lactis BB12 (FBD *, FD +) and B. lactis HN019 (FBD ●, FD -) cells. Continuous lines are shown for lactobacillus and dashed lines are for bifidobacterium strains*

The same trend was observed for bifidobacterium strains where it took just 8 and 16 weeks respectively for the *B. lactis* BB12 and *B. lactis* HN019 strains to be reduced to

the below significant level of cell population. As evident from Fig. 4.3, significant improvements in storage stability were observed irrespective of the probiotic strain chosen when osmotically stressed cells were stabilized using FBD, although there were some variations in the final viable cell population among different strains which is a common phenomenon (Morgan *et al*., 2006). Comparison between the 2 genera of probiotic cells showed bifidobacterium strains were significantly more susceptible to lose in storage viability than the lactobacilli cells, irrespective of the drying technique chosen.

Freshly grown freeze-dried cells of *L. acidophilus* ATCC 4356 and *L. rhamnosus* ATCC 53103 were harvested at stationary phase and freeze dried after blending with 20% (w/w) reconstituted whole milk. The loss in viability for *L. acidophilus* cells was more pronounced with 4.9 log/g reduction by 8 weeks. *L. rhamnosus* cells were also reduced by 4.9 log cfu/g but after 16 weeks (Fig. 4.3). Below these levels, further enumeration was not continued. The same freeze-drying protocol was followed for *B. lactis* BB12 and *B. lactis* HN019 cells. After 8 weeks the loss in viability for BB12 cells was 5.6 log cfu/g and HN019 strain showed 5.5 log cfu/g reductions after 20 weeks.

As expected, osmotically stressed and fluidized bed dried cells in all the 4 samples showed significantly better (p≤0.05) stability. After 24 weeks of storage at 25 °C, *L. rhamnosus*, BB12 and HN019 cells lost the net viability of 1.9, 2.2 and 2.3 log cfu/g, respectively and the best result was obtained for *L. acidophilus* cells where only 1.0 log reduction during the same period was recorded (Fig. 4.2).

4.2.4 Effects of an antioxidant and a prebiotic compound on ambient storage stability

Dehydration of bacterial cells results into serious oxidative stress and consequent damage to the cell membranes by the formation of reactive oxygen species (ROS), which in turn causes lipid peroxidation and de-esterification, protein denaturation and damage in cell nucleic acids (Hansen *et al*., 2006; Garcia, 2011). In a metabolically active state, these ROS are trapped by the antioxidant defense system in a normal way but during prolonged dry storage the cell membranes are more susceptible to ROS attack (Franca *et al*., 2007). Hence it was hypothesized that during the stabilization process if the cells are adequately coated with vitamin E powder then its proven antioxidative property could offer better protection against the oxidative damages during the dry storage.

Samples fortified with vitamin E or inulin (0.5% and 5.0% w/w of the final products) were stored at 25 °C for 20 weeks. The average net reductions in viable cell count during this period were 0.72 and 1.05 log cfu/g for vitamin E and inulin, respectively. Control samples with only whole milk powder as the stabilization matrix showed 0.9 log reduction in cell population during this period (Fig. 4.4). Test of significance showed that the antioxidative properties of vitamin E significantly improved ($p \leq 0.05$) the storage stability of the *L. casei* CRL 431 cells but any synergistic effect of inulin as a prebiotic was not observed.

The results suggest (Fig. 4.4) that a preliminary conclusion can be drawn about the efficacy of using such antioxidative compounds in the bacterial cell stabilization matrix but a decisive result needs more experimentation. It was reported earlier that inulin helped improving the stability of spray dried bifidobacterium cells during refrigerated
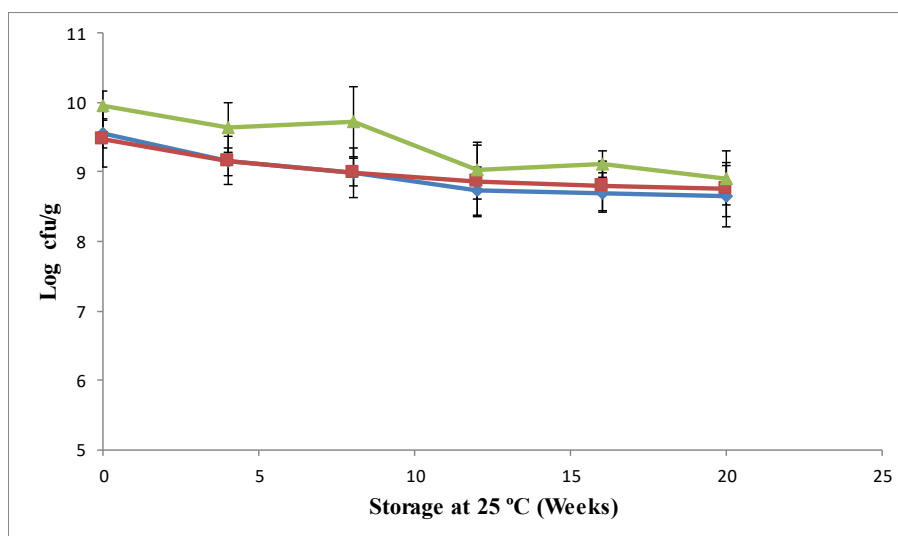
Figure 4.4: *Effects of inulin (▲) and Vit. E (■) fortifications into the drying matrix on storage stability of osmotically stressed and FBD stabilized L. casei CRL 431 cells at 25 °C. Control samples (♦) without any fortificant were analyzed for comparison*

storage (Fritzen-Freire *et al*., 2012). However, at 25 ºC storage temperature, up to 7 log reduction in viability in 120 days was reported even in presence of inulin in the freeze-drying matrix (Fatemeh *et al*., 2011). No improvement in storage viability was found when inulin was in direct contact with the cells during desiccation. A symbiotic effect of prebiotic fibers and probiotic bacteria which aids easy colonization of probiotics in the intestinal wall has been reported (Foye *et al*., 2012; Maathuis *et al*., 2012) but the results presented here suggest that at ambient temperature storage no such synbiotic effect of the prebiotic and probiotic combination is evident.

4.2.5 Physical characteristics of the probiotic powder

Different physical properties, e.g. particle size distribution, flowability and solubility of the experimental powder containing *L. casei* CRL431 cells were measured and compared with the two most common powders in the food industry, SMP and WMP.
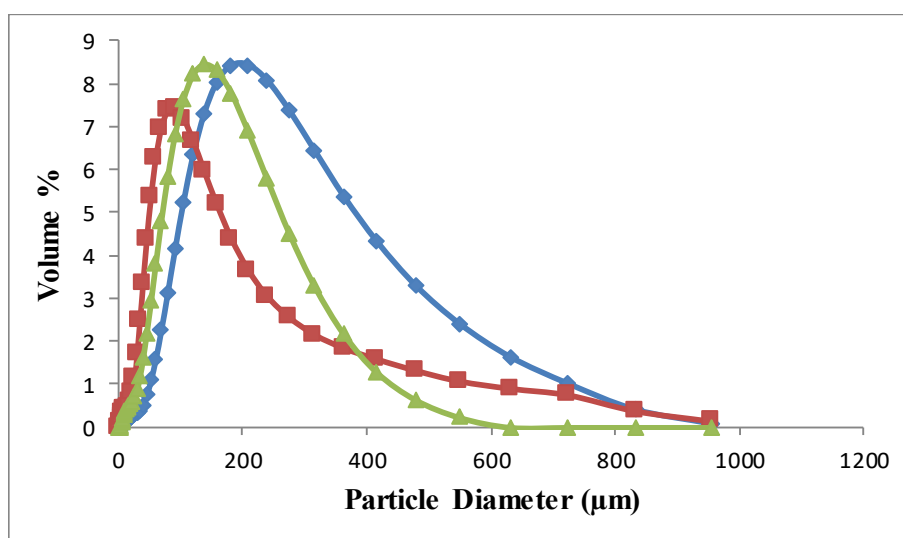
Figure 4.5: *Particle size distributions of the developed probiotic powder (♦) and comparison with whole milk (▲) and skim milk (■) powders*

The particle size distribution of the probiotic powder was uniform (Fig. 4.5) with a surface-based mean diameter (D_{32}) of 125.6 µm and ranged from 7 to 832 µm. D_{32} for WMP and SMP were found to be 76.1 and 66.3 µm respectively. The diameter range for WMP was slightly shorter (6 to 550 µm) and the same for SMP was very similar (5 to 954 µm). The notably higher mean diameter clearly indicates the probiotic powder was highly agglomerated, which is a desirable property for easy handling and better dispersibility.

The flow functional properties of the experimental probiotic powder, WMP and SMP are presented in Fig. 4.6. According to the principle described in section 3.2.8, the flowability of powder is defined by the slope of the curve. The highest flowability is
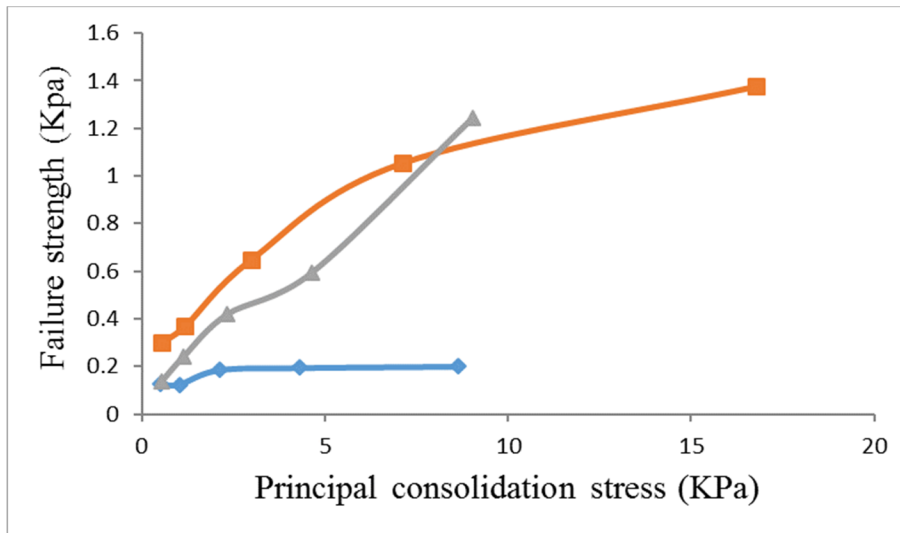
Figure 4.6: *Flow properties of probiotic powder (■) in comparison with whole milk (▲) and skim milk (♦) powders*

recorded for SMP and the flow properties of WMP and probiotic powder were very close, with probiotic powder being slightly better. The higher particle sizes due to agglomeration in the probiotic powder as reported above may be a probable reason for better flow properties.

The average bulk densities of the samples were found to be 469.72 Kg/m^3, 540.52 Kg/m^3 and 583.25 Kg/m^3 for probiotic powder, WMP and SMP respectively. The comparatively lower density for the probiotic powder is also because of the presence of highly agglomerated bigger sized particles as reported earlier (Szulc & Lenart, 2010).

The solubility index for probiotic powder, WMP and SMP were found to be 1.5, 1.0 and 1.0 ml respectively. As per CODEX standard 207-1999, the maximum allowed solubility index for SMP and WMP is 1.0 ml. The experimental probiotic powder had a slightly poorer solubility probably due to the presence of a very high population of bacterial cells in the precipitate portion.

The water activity of the probiotic powder was found to be 0.27 which is lower compared to both WMP (0.33) and SMP (0.30). A lower water activity means less amount of free water available to the bacteria which restricts any possibility of biological activity and hence considered to be favorable for the product shelf stability.

4.2.6 Acid and bile tolerance properties of *L. casei* CRL431 cells

One key criterion for a particular bacterial strain to be called as probiotic is its adherence in the human gastro-intestinal tract (Saxelin *et al.,* 2010), which is only possible when the strain is capable of surviving the harsh environmental conditions present therein. One of the objectives in this study was to compare the degree of survival of *L. casei* CRL431 cells in free and stabilized forms (in the experimental powder) in a simulated gastro-intestinal environment.

Fig. 4.7 describes the loss in viability suffered by the *L. casei* CRL431 cells when incubated in SGF. The extreme harsh effect of the acidic environment was very

pronounced in case of cells in free form, where a reduction of 7.0 log cfu/ml was recorded within 60 min but thereafter no viable count was recorded.
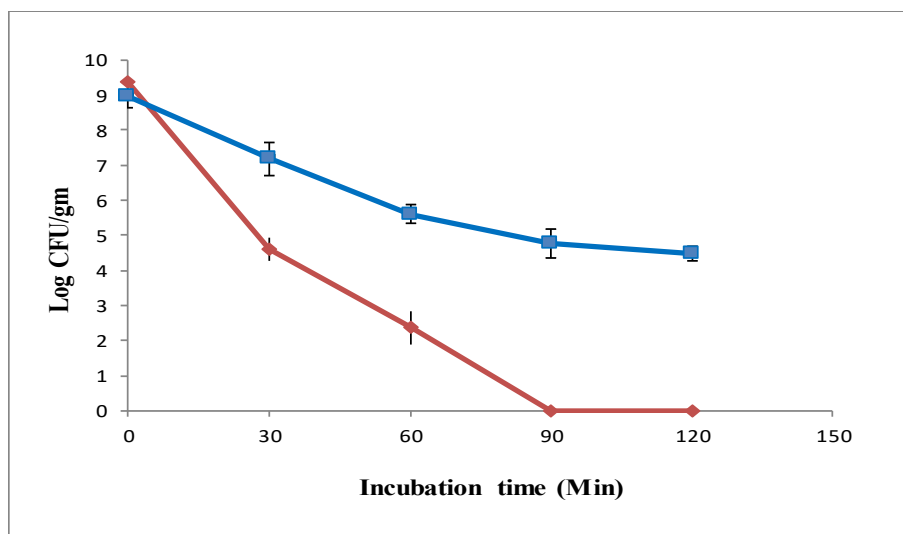
Figure 4.7: *Viability of free (♦) and stabilized (■) L. casei 431 cells during simulated gastric transit. The error bars indicate standard deviations from the mean values of 3 replicated experiments*

The stabilized cells also a suffered loss in viability but to a milder degree. During the 2 hours incubation period the cell population was reduced by 4.4 log. Test of statistical significance showed that the bacterial cells were significantly better ($p \leq 0.05$) protected in the stabilized environment than the control sample. This result is comparable to the previous similar work of Ding & Shah (2009) and Heidebach *et al.* (2009). Encapsulation (entrapment inside a protective food particle) or embedding (adherence to a protective food-grade matrix) techniques have been used by many researchers as a shield to protect probiotic bacteria against the gastro-intestinal environment. The degree of success depends on many factors, such as choice of wall material, the robustness of the chosen strain or the technology used in the process. The presence of milk proteins in

the protective matrix has been found to offer very good protection due to their buffering nature (Kos *et al*., 2000; Guerin *et al*., 2003; Ainsley-Reid *et al*., 2005; Nag *et al*., 2011). The presence of milk proteins in high concentrations in this developed encapsulation matrix is thought to be a key beneficial factor towards the better protection of *L. casei* CRL431 cells.
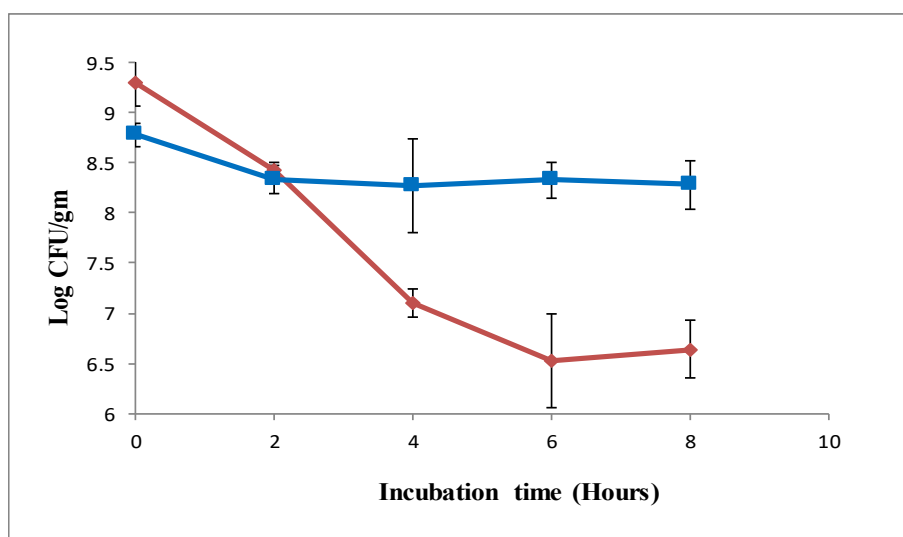
Figure 4.8: *Loss in cell viability for free (♦) and stabilized (■) L. casei 431 cells when incubated in bile salts solution. The error bars indicate standard deviations from the mean values of 3 replicated experiments*

In case of tolerance against bile salts, the encapsulation offered significantly better protection. Compared to 2.7 log cycle reduction of free cells during the 8 hours incubation period, protected cells showed only 0.6 log reduction in viability (Fig. 4.8). Test of statistical significance showed that similar to the acid tolerance test, the bacterial cells were significantly better ($p \leq 0.05$) protected in the protected samples than the control one. Bile salts present in the human intestine are generally considered to be

detrimental to probiotic cells due to their lipid emulsifying action on the bacterial cell membrane (Song *et al*., 2003; Ding and Shah, 2009).

The bile salts present in the small intestine are growth inhibitors to the probiotics because of their biological detergent nature (Chadwick *et al*., 2003). Membrane damage, protein misfolding, lowering intracellular pH and causing DNA injury by oxidative stress are considered as the reasons for such growth inhibition (Mills *et al*., 2011). Therefore, resistance to the bile salts either coming from the intrinsic properties of a particular probiotic strain or through any external protection is an important factor to be considered while designing probiotic containing foods. Microencapsulation of probiotics with polymers, such as chitosan, alginate, carrageenan, starch, whey proteins, dextrans, inulin is aimed for offering such external protection against intestinal bile salts (Nazzaro *et al*., 2012; Tripathi and Giri 2014). It was also found that apart from providing physical protection, microencapsulation also preserves the functionality of the probiotics by protecting the bioactive and effector molecules of the bacterial cell envelops, which are important for probiotic effects on the host (de Vos *et al*., 2010). However, the stabilizing technique used in the current study did not use any established encapsulation technique; therefore, the enhanced protection observed above (Fig. 4.8) can not be explained by non-release of the cells from protective capsules. It can be assumed that agglomerated milk particles were dissolved easily into the simulated intestinal fluid thereby releasing the adhered cells immediately. Therefore the protection must have come from some other means. The most probable reason could be the buffering nature of the milk proteins present there. It was reported that food carriers containing milk (most commonly yoghurt) improved the survival of probiotics during gastro-intestinal transit

because of mutual interactions (Ranadheera *et al*., 2010). Also, lactic acid bacteria which were grown in fermented milk were found to be more robust in the in vitro gastric digestion test than the cells grown in synthetic culture media (Faye *et al*., 2012; Burns *et al*., 2014). It was also shown that the APF gene (aggregation-promoting factor) were highly expressed during the growth of a *Lactobacillus acidophilus* strain in milk media and was responsible for enhanced bile tolerance, gastrointestinal survival, and interactions with epithelial cells (Baugher and Klaenhammer 2011). Therefore, it can be assumed that dairy carrier foods have important role to play in probiotics survival and maintaining functionalities.

4.3 Conclusions

While formulating a food product fortified with functional ingredients such as live probiotic bacteria, the formulator needs to make sure that the concentration of the functional ingredient is adequate at the point of consumption to deliver the expected health benefits. This becomes very important if the fortified foods are meant for ambient storage, therefore improving the ambient stability of dehydrated probiotic bacteria is a big challenge to the food industry. In case of probiotic fortified foods, so far there is no strict regulatory requirement in terms of the viable probiotic cell population in the EU or U.S.A (Hicky, 2005) but the clinical efficacy must be established if a particular health benefit is claimed (Venugopalan *et al*., 2010). A general guideline issued by FAO/WHO states that any good effect of probiotic bacteria on human health can be obtained only if consumed at a level of 10^7 to 10^8 viable cells per day (FAO/WHO, 2003). This study

indicates that a combination of stress adaptation, embedding in a dried milk matrix and fluidized bed drying technique can be effectively applied to improve such stability compared to the conventional way of stabilizing probiotics with freeze drying.

Chapter 5.0. Improving the viable cell recovery of *Lactobacillus reuteri* LR6 after fluidized bed drying

The contents of this chapter have been submitted in October 2018, in the form of a manuscript, for publication in the peer-reviewed journal "Journal of Food Science":

5.1. Introduction

In the previous chapter, the basic technology development for improving the ambient temperature storage stability for a range of probiotic strains was described. As a part of the process development, the produced powder was also characterized for its physical properties and protection ability against the harsh gastro-intestinal environment. However, when the stabilized strains were stored at a higher temperature of 37 ˚C, the stability was found to be very poor. As the project progressed further, it was decided to stabilize a new probiotic strain of Indian origin, keeping the Indian consumer markets in mind, it was important to ensure a satisfactory level of stability is achieved upon storage at 37 ˚C, which is not uncommon in most parts of India during the summer season. Therefore, improvement in the technology was an imminent need. For this part of the project, a validated probiotic strain (more details on the validation process in Chapter 8.0) *Lactobacillus reuteri* LR6 was selected and received from National Dairy Research Institute (NDRI), Karnal, India. The characterization and validation of this strain was done in NDRI and the details of that work are given below.

Lactobacillus reuteri LR6 was originally isolated from the feces of the breast-fed human infants (less than 3 months). This particular strain survived in acid, bile, and simulated stomach–duodenum passage conditions, indicating its high tolerance to gastric juice, duodenal juice and bile environments. The strain LR6 did not show strong hydrophobic properties because the percentages of adhesion to the apolar solvent, n-hexadecane, did not exceed 40%, showing that its surface was predominantly hydrophilic. The functionality of this probiotic isolate was supported by its ability to adhere to Caco-2 cells, its antagonistic activity and its ability to deconjugate bile salts. The safety of this indigenous *L. reuteri* isolate was supported by the absence of transferable antibiotic resistance determinants, DNase activity, gelatinase activity, and hemolysis. This strain has shown the ability to competitively exclude pathogens from adhesion to Caco-2 cells *in vitro*. Thus, the results suggest that the LR6 is resistant to low pH, bile salts and duodenum juice, so they could survive when passing through the upper part of the gastrointestinal tract and fulfill their potential probiotic action in the host organism (Singh *et al.,* 2012).

An *in vivo* study focusing on the cholesterol-lowering action of *L. reuteri* LR6 was also conducted in NDRI. Thirty-two Male Albino rats were divided into four groups of eight each. For 60 days, group A was fed normal synthetic diet, group B was fed cholesterol-enriched diet only, group C was fed cholesterol-enriched diet supplemented with skim milk and group D was fed cholesterol-enriched diet supplemented with *L. reuteri* LR6 fermented skim milk (10^8 cfu/ml). Blood samples were withdrawn to study the lipid profile on 0th, 15th, 30th and 60th day. The values for total cholesterol, triglyceride and LDL were reduced significantly in the group fed with *L. reuteri* LR6 but for HDL this

difference was not significant. According to these results, the strain LR6 was found to possess interesting probiotic properties that make them potentially good candidates for probiotics and could be utilized as an additive for health assistance foods (Singh *et al.,* 2015).

The main focus of the previous work was result-oriented where achieving superior stability was the objective whereas the scientific understanding of the underlying mechanisms responsible for such stability did not develop strongly. It was realized that without such understanding the stumbling block towards further improvement will not be possible. The loss in probiotic viability was found to be taking place in two stages, viz., during the desiccation and related processing steps and thereafter during the storage period. This chapter describes the approach taken towards minimizing viable cell losses during the first stage only. The process parameters were optimized and the effect of different carrier excipients was studied. The most promising combinations were further analyzed with advanced techniques for a better understanding of the factors responsible for such losses.

5.2. Results and discussion

5.2.1 Desiccation of LR6 cells in the unsupported form

In section 2.9 of the literature review, it was described how researchers have successfully stabilized probiotic bacteria by preventing the heat inactivation through protection available from the polyol or sugar alcohol compounds, utilizing their free radical scavenging property (mannitol) or the depression of the membrane phase transition temperature property (sorbitol) (Linders *et al*., 1997; Efiuvwevwere *et al*., 1999; Santivarangkna *et al*., 2006). It was also shown that trehalose, often considered as the best protectant for probiotics during stabilization, performed poorly than sugar alcohols in this regard in some studies. However, the use of such polyols in these studies was mostly restricted to using either freeze or spray dying for the stabilization purpose. In this project, a coating of polyol compounds (sorbitol or xylitol) over the experimental bacterial cells and its effect during fluidized bed drying was investigated, which was thought to be a novel approach.

The initial a_w of the cell pellet was recorded as 0.96 and after mixing with sorbitol or xylitol, it decreased to 0.84 and 0.85 respectively. After 2 hours of drying at 55 ˚C, the final a_w values were recorded as 0.24 for pure cells and 0.26 and 0.28 for cells mixed with sorbitol or xylitol respectively and the same for the samples dried at 75 ˚C were 0.17, 0.14 and 0.15 respectively (Fig. 5.1). The drying was discontinued at this point and the samples were enumerated for total lactobacilli counts by pour plating on MRS agar

(Oxoid Ltd., England). Fig. 5.1 shows the reduction in cell viability per gram of wet biomass. The viability of pure cells was reduced from 10.58 to 9.79 log cfu/g by the desiccation process applied at 55°C. Cells mixed with sorbitol were slightly more affected, with the cell viability on average reducing from 10.28 to 8.98 log cfu/g. Cells mixed with xylitol were better protected against desiccation, with the viable cell count on average being reduced from 10.37 to 10.10 log cfu/g. Drying at 75 °C caused a significant loss in cell viability for all samples, with sorbitol and xylitol having minor protective effects compared with samples with low-temperature drying (55 °C). The viable cell populations were reduced drastically to 2.35, 2.92 and 3.04 log cfu/g of wet biomass for the pure, sorbitol-coated and xylitol-coated cells respectively.
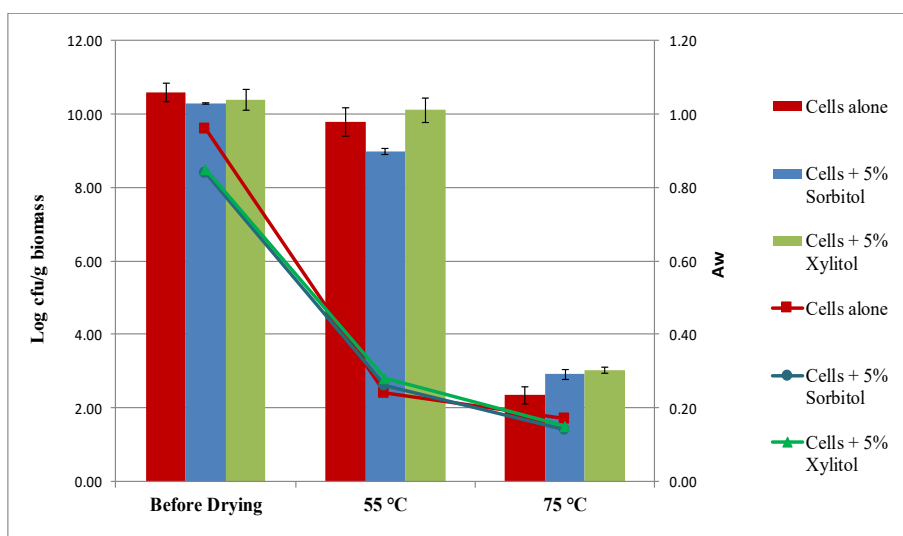
Figure 5.1: *Effect of low-temperature air desiccation on unsupported LR6 cell pellets. Columns showing the decline in viable counts and the lines showing a_w values before and after drying*

When subjected to moderate levels of desiccation stress, the unsupported cells did not change substantially. However, exposure to a high drying temperature caused severe

irreversible damage to the cells, which failed to propagate upon rehydration. This experiment was designed to determine the loss in viable cell population when the cells were desiccated without using any supporting agent. As it is not feasible to store probiotic cells in such a format (a thin and almost invisible film) and then to fortify them into a product, it was necessary to stabilize them using a food-grade ingredient and a conventional and industrially scalable drying technique. SMP was chosen for this purpose because of its cheap cost, easy availability and nutritional qualities. Based on this, all subsequent stabilization experiments were carried out using a pilot-plant-scale fluidized bed dryer (FBD). However, the principle of forced-air drying was almost similar in both drying systems. A vacuum chamber was used only to facilitate a faster rate of moisture removal.

5.2.2 Desiccation of LR6 cells in a fluidized bed dryer with skim milk powder as carrier excipient

In the following experiments, an important objective was to optimize the drying temperature in the FBD to identify the most effective time-temperature combination of the stabilization process without significant compromise on the post-drying viability.

Figure 5.2 shows the changes in a_w as drying progressed at different inlet air temperatures. As expected, a higher temperature of drying resulted in lower a_w during the process and at the end of it. At each sample collection point, the a_w data correlated well with the drying temperature in an almost linear fashion. An important observation

was the sharp reduction in a_w for the 'SMP only' samples within the first 5 min of drying. The rate of further reduction during the next 15 min was much much less (Fig. 5.2a).
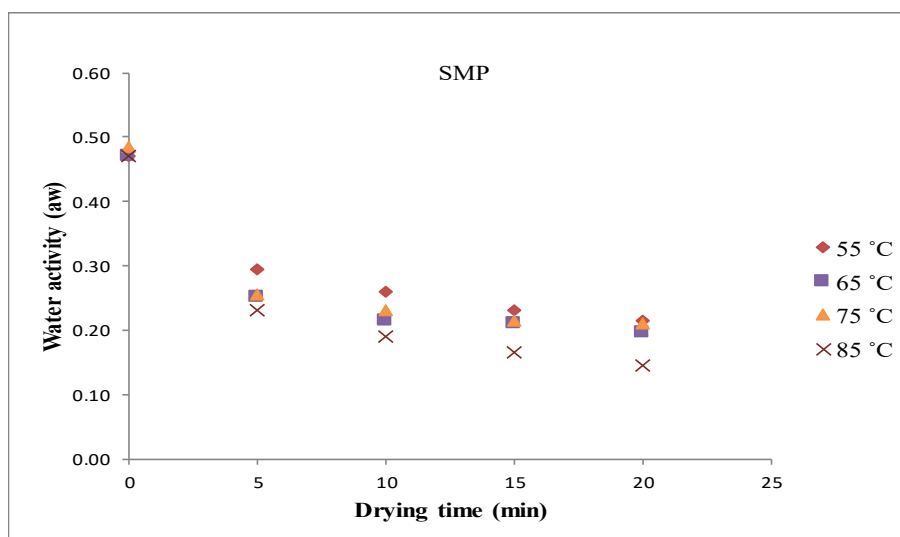
Fig. 5.2 (a)
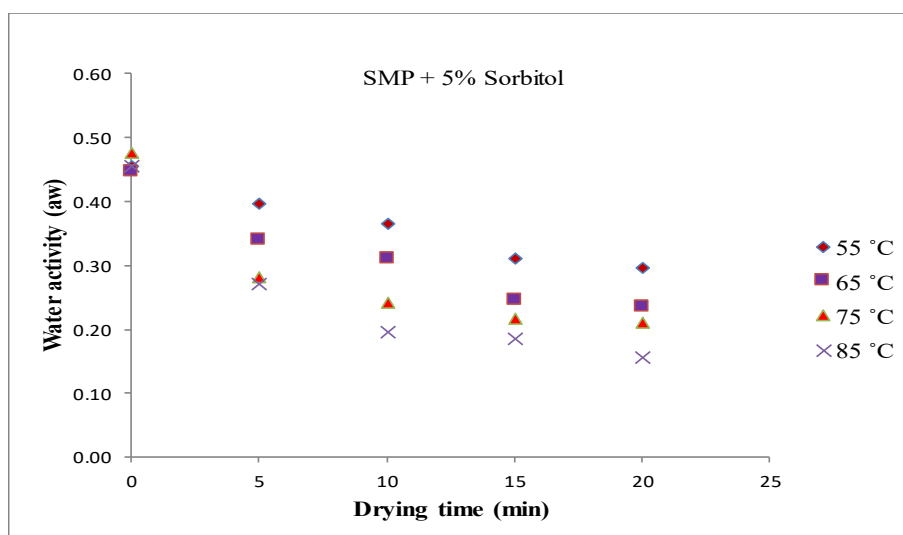
Fig. 5.2 (b)
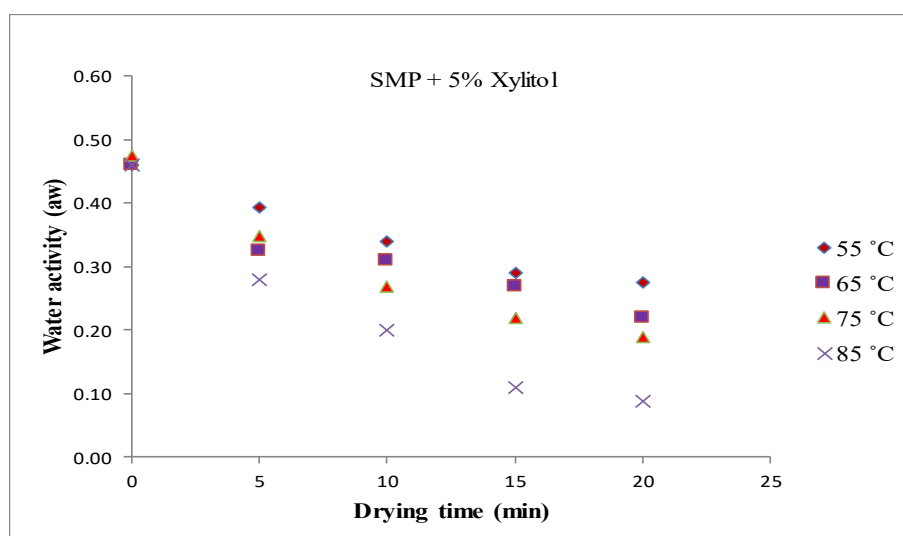
Fig. 5.2 (c)

Figure 5.2: *Effect of fluid bed drying temperatures (inlet) on water activity of the stabilized LR6 cells carried by 'SMP only' (a) and coated with 5% sorbitol (b) or xylitol (c) and carried by SMP*

In case of the sorbitol and xylitol containing samples, the rate of decline was more uniform, for example at 55 ˚C the curves were almost linear. This indicates a probable interaction between the polyol and the free water during the mixing and the wet granulation processes which might have prevented quick evaporation. This also explains the higher values of a_w recorded for the polyol samples at the end of drying, with the exception of the xylitol sample with 85 ˚C inlet temperature where a slightly lower value was obtained. This phenomenon could have also influenced the type of glassy matrix formation around the bacterial cells, which could be confirmed by measuring the T_g of the samples. Different types of glass formation may also be partially or fully responsible for the higher viable cell population recorded in the polyol samples (Fig. 5.2 b & c). If molecular interactions between the bacterial cell envelop and the carrier agent are important factors in reducing the desiccation stress to the cells, the other important

factor is the physical state of the carrier agent in the dried condition. The dehydrated state results in extremely high viscosity which helps in decreasing molecular mobility and related adverse chemical reactions (Dianawati *et al.,* 2017). However, this physical state will strongly depend upon the glass transition temperature of the matrix which needs to be examined.

The effect of inlet drying temperatures on the reduction of cell viability can be viewed in Fig. 5.3. For the SMP samples, minimum loss in viability during drying was recorded for 55˚C inlet temperature (0.21 log cfu/g). No loss in viable cell counts was recorded for sorbitol and xylitol samples at this inlet temperature drying. At 65 ˚C inlet, losses for SMP and sorbitol samples were nearly the same (0.40 and 0.35 log cfu/g) but the xylitol samples showed a loss of only 0.07 log cfu/g. Inlet temperatures of 75 ˚C and 85 ˚C did not show much difference for SMP samples (loss of 0.36 and 0.44 log CFU/g) but were found to be more lethal for sorbitol and xylitol samples.

Fig. 5.3 (a)
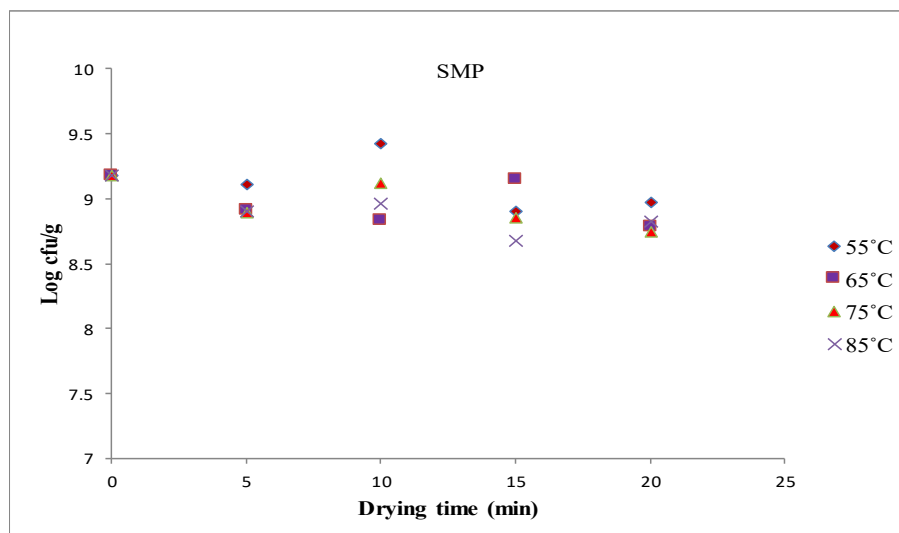
Fig. 5.3 (b)
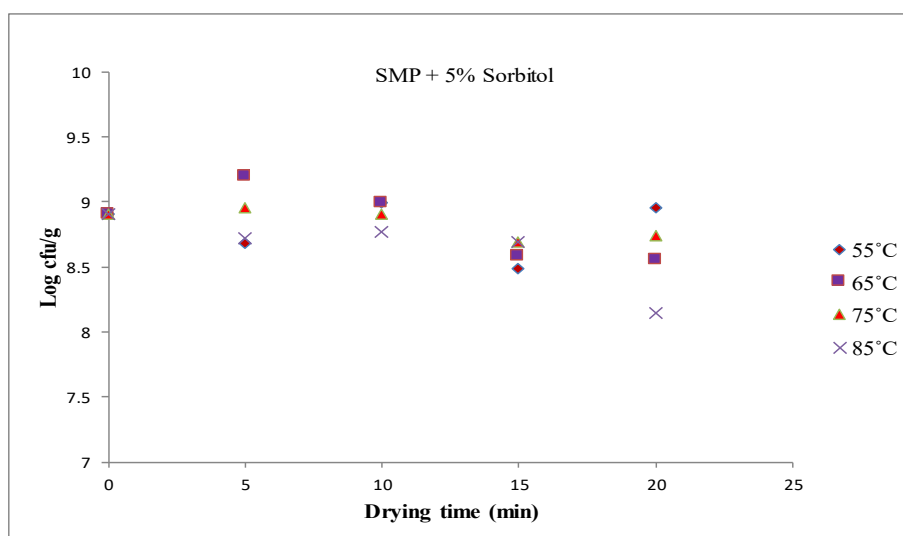
Fig. 5.3 (c)
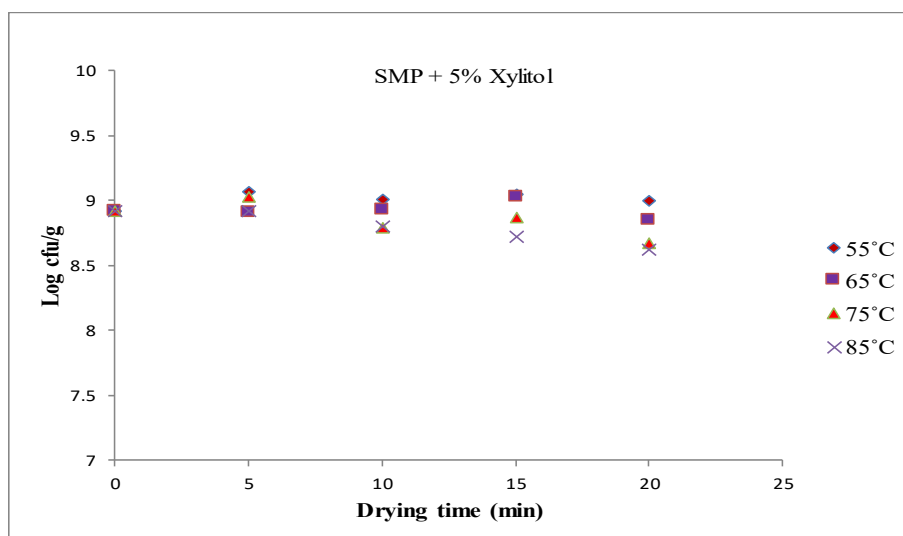
Figure 5.3: *Effect of fluid bed drying temperatures (inlet) on residual viability of the LR6 cells stabilized only with 'SMP only' (a) and coated with 5% sorbitol (b) or xylitol (c)*

Sorbitol samples showed 0.76 log reduction at 85 ˚C and the same for xylitol samples was 0.29 log. Overall, cells coated with xylitol proved to be better protected during the desiccation process, which was similar to the results obtained for unsupported drying

(Fig. 4.4). All subsequent experiments were carried out at 75 ˚C inlet temperature only, which corresponds to ~55 ˚C outlet temperature so that the results could be comparable to the unsupported samples dried at 55 ˚C.

Figure 5.4(a) shows the average results from 3 replicated drying experiments using 75 ˚C inlet temperature. After granulation but before drying, the differences in viable cell counts in all three types of samples were very close within the range of 0.06 log cfu/g and with a standard deviation of 0.24. After 20 min of drying, almost the same level of viable counts was recorded for SMP and with sorbitol samples (9.08 and 9.10 log respectively) but the population of xylitol-containing samples was slightly higher with

Fig 5.4(a)
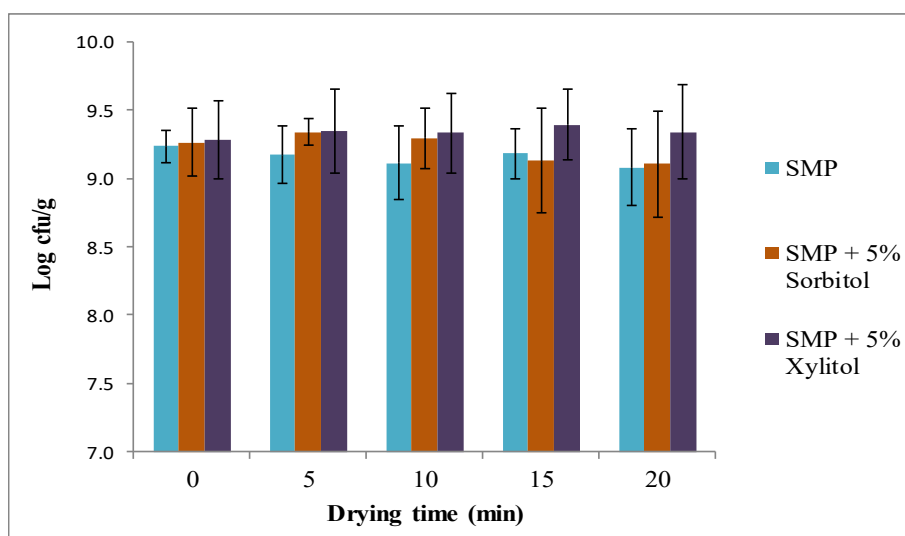
Fig 5.4(b)
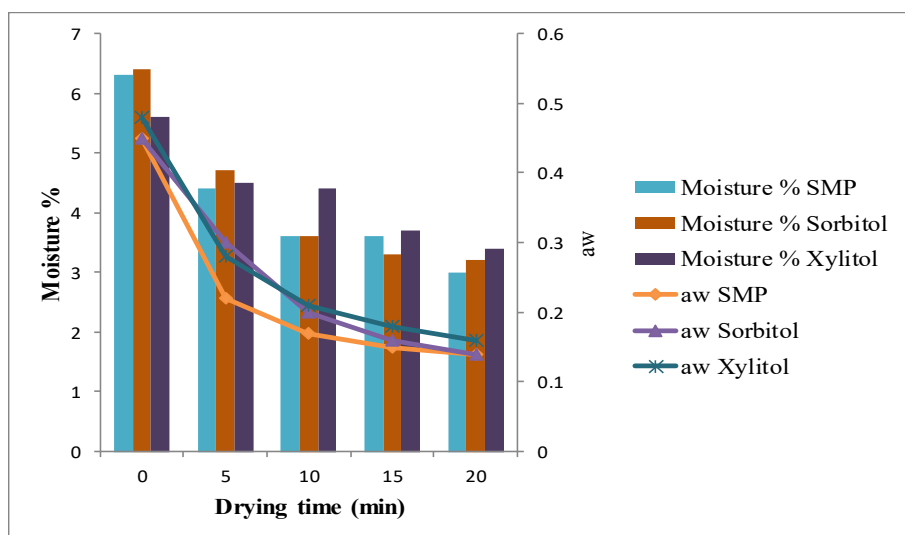
Figure 5.4: *Viable cell counts during the fluid bed drying process with 'SMP only' and coated with 5% sorbitol or xylitol (a). Line graphs showing the reduction in water activity and columns showing residual moisture content during the drying process (b). Three replicate trials at 75 ˚C inlet / 55 ˚C outlet temperatures*

9.34 log cfu/g with a standard deviation of 0.34 log among the samples. The gradient of water evaporation (residual moisture content) during the drying process is shown in Fig. 5.4(b).

Table 5.1: *Summary of the results shown in Fig. 5.4*

		Before Drying	5 min	10 min	15 min	20 min
SMP	aw	0.45	0.22	0.19	0.17	0.17
	Moisture %	6.30	4.40	3.60	3.60	3.00
	Log cfu/g	9.23	9.17	9.11	9.18	9.08
SMP + 5% Sorbitol	aw	0.45	0.32	0.23	0.19	0.18
	Moisture %	6.40	4.70	3.60	3.30	3.20
	Log cfu/g	9.26	9.34	9.29	9.13	9.10
SMP + 5% Xylitol	aw	0.46	0.29	0.24	0.21	0.19
	Moisture %	5.60	4.50	4.40	3.70	3.40
	Log cfu/g	9.28	9.34	9.33	9.39	9.34

*Pure SMP aw = 0.35

The results shown in Figs. 5.1, 5.3 and 5.4 indicated no substantial advantage of coating the cells with sorbitol over the uncoated ones but in all the experiments it appeared that xylitol seemed to work as a better protectant over the uncoated cells. However, statistical analysis of the data did not confirm this view. A test of two-factor ANOVA with replication was performed using Microsoft Excel 2010 and the results are summarized in Table 5.2.

Table 5.2: *ANOVA result for the samples described in Figure 5.4*

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Process parameters	0.06	4.00	0.02	0.14	0.97	2.69
Sample types	0.25	2.00	0.13	1.15	0.33	3.32
Interaction (matrix)	0.13	8.00	0.02	0.15	1.00	2.27
Within	3.30	30.00	0.11			
Total	3.75	44.00				

According to this analysis of variances, no significant difference was present either among the replicate trials ($p = 0.97$) or the types of samples ($p = 0.33$). This indicates that the hypothesis of considering sorbitol and xylitol as superior protectants during the desiccation process cannot be supported with confidence, therefore the hypothesis can be statistically rejected. However, it should be kept in mind that the principal focus of this project is to minimize the loss in viable cell counts during long term ambient temperature storage. Investigation for any beneficial role of such polyols during the storage period is equally important similar to their role as effective desiccation protectants. Therefore this hypothesis should be fully rejected only if no significant difference with the pure SMP samples are observed at the end of the storage trial.

In the granulation process, sorbitol or xylitol powder was directly mixed with the cell pellets before the pellets came in contact with the SMP. It is not yet known how the cell surface interacts with SMP particles when there is direct contact and when a layer of sorbitol or xylitol is present around the cell bodies before coming in contact with the SMP particles. Hence it is likely that the positioning of the cells within the stabilized matrix would be quite different between the samples with or without the polyol compound. Whether such different positioning has any significant effect on the storage stability would be an important investigation in the subsequent stages of this work.

5.2.3 Desiccation of *L. reuteri* LR6 cells with corn starch, microcrystalline cellulose, and milk protein concentrate as carrier agents

For all samples prepared with approximately 95% SMP, the polyols demonstrated insignificant roles as desiccation protectants. The loss in viable cell population was relatively small, which could be attributed to the presence of sufficiently high proportions of a combination of lactose (49.5–52%) and milk protein (34–37%) in SMP. However, the individual contributions of these two different types of compounds could not be determined. Although the buffering action of milk solids against desiccation stress has been confirmed in many studies (Hubalek, 2003; Vega & Roos, 2006), it is not clear whether pure carbohydrate or pure protein is a more effective protectant than their combination, as is found in SMP. Therefore, a set of experiments was designed to collect more information on how other food excipients behave as protectants to bacterial cells when subjected to the same stabilization process.

In the following set of experiments, two different drying temperatures were used for separate batches. For this purpose, the drying inlet temperatures were chosen as 75 ˚C and 95 ˚C which approximately corresponded to 55 ˚C and 75 ˚C outlet temperatures respectively and hence directly comparable to the results from the unsupported drying experiment reported earlier (section 5.2.1). The rationale was to understand the effect of drying temperature on the conformational changes and damages to the bacterial cell envelope and to identify any correlation with the loss in cell viability during the

desiccation process. This analysis was performed by developing a novel cell isolation protocol (section 3.3.1) and using the infrared absorbance technique (section 3.3.2), the results of which will be presented in the next chapter. In this chapter, only the loss in cell viability during the desiccation process at two different drying temperatures and carried by different excipient materials is presented (section 3.2.4).

Figure 5.5 shows the average results from three replicated experiments with the three new excipients as introduced above and plotted along with the results obtained in the previous set of experiments with SMP, SMP+sorbitol, and SMP+xylitol (from Fig. 5.4a).
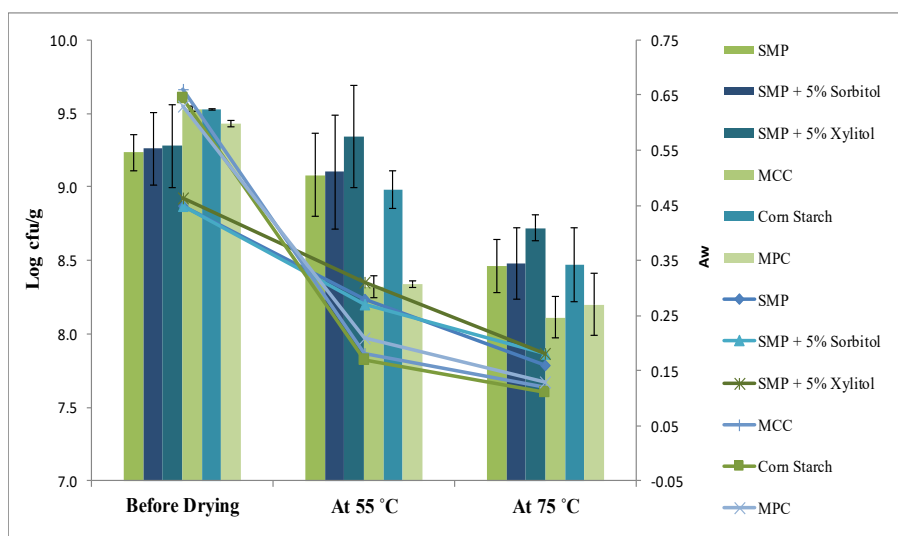
Figure 5.5: *Changes in viable cells counts (columns) and water activities (lines) during fluid bed drying of LR6 cells at 55˚C and 75 ˚C outlet temperatures*

As reported earlier, at 55 ˚C outlet temperature, the loss in viable cell counts due to drying was marginal for SMP, SMP + sorbitol and SMP + xylitol samples. Samples with SMP and sorbitol showed reductions of 0.15 and 0.16 log cfu/g and no change was

recorded for the xylitol sample. However, the other three sets of samples showed considerable reductions in viability. Among them, MCC appeared to be the best protectant with 0.30 log reduction followed by CS (0.55 log) and MPC (1.09 log). At 75 ˚C outlet drying temperature, all the samples showed a higher loss in viability which was not unexpected (Table 5.3). However, the viable population in this form of drying was higher for all the samples compared to the unsupported drying results (at 75 ˚C) as reported earlier in Fig. 5.1, which confirms the protective effect of the excipients irrespective of their nature and composition.

Table 5.3: Summary data for the results shown in Fig. 5.5

		Before Drying	At 55 ˚C	At 75 ˚C
SMP	aw	0.45	0.28	0.16
	Log cfu/g	9.23	9.08	8.46
SMP + 5% Sorbitol	aw	0.45	0.27	0.18
	Log cfu/g	9.26	9.10	8.48
SMP + 5% Xylitol	aw	0.46	0.31	0.18
	Log cfu/g	9.28	9.34	8.72
MCC	aw	0.66	0.18	0.12
	Log cfu/g	9.53	8.32	8.11
Corn Starch	aw	0.65	0.17	0.11
	Log cfu/g	9.53	8.98	8.47
MPC	aw	0.63	0.21	0.13
	Log cfu/g	9.43	8.34	8.20

*Raw material aw: SMP = 0.35, MCC = 0.42, Corn Starch = 0.48, MPC = 0.45

Interestingly, the initial a_ws of the non-SMP samples (MCC, CS, and MPC) were much higher than those of the SMP, SMP + sorbitol, and SMP + xylitol samples. After the wet granulation process, the a_ws for the MCC, CS, and MPC samples were 0.66, 0.65, and 0.63 respectively, whereas those for the SMP, SMP + sorbitol, and SMP + xylitol samples were 0.45, 0.45, and 0.46 respectively (Table 5.3). This indicates the enhanced

water-binding capability of SMP, which is due to the presence of the protein–lactose combination, but no incremental effect of sorbitol or xylitol was observed. Furthermore, the viable cell counts before drying were higher for the samples dried with MCC, CS, and MPC than for the SMP group (Table 5.3), which could possibly be correlated with the higher initial a_w values in these samples.

Table 5.4: *ANOVA result for the samples described in Figure 5.5*

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Process parameters	1.38	1.00	1.38	75.39	0.000001	4.60
Sample types	1.33	6.00	0.22	12.13	0.000075	2.85
Interaction (matrix)	1.69	6.00	0.28	15.43	0.000019	2.85
Within	0.26	14.00	0.02			
Total	4.65	27.00				

The cells in the SMP group samples were probably exposed to a higher osmotic stress environment, which could have had an adverse effect on their viability. The final a_w of the non-SMP group was 0.09 on average, which was much less than that of the SMP group, i.e. 0.18 on average. It is possible that the low water-binding property of the pure carbohydrate and pure protein group was responsible for not retaining as much moisture during the drying process as for the SMP group. Statistical test of significance using two-factor ANOVA (Table 5.4) shows significant differences among the types of samples ($p<0.05$) as well as between the results before and after the drying process ($p<0.05$).

5.3 Conclusions

Stabilization of the LR6 cells in presence of a supporting agent has been found responsible for substantial improvements in residual viability. The improvement is more pronounced at high-temperature drying. Marginal protection against desiccation stress was available when the cells were coated with xylitol. However, it was found that the major differentiating factor was the physicochemical nature of the supporting agents used to carry the cells either through agglomeration of the powder particles or the adherence of the cells to them. The superior agglomeration and adherence properties of SMP because of the lactose-protein combination present therein was found to be a better supporting agent compared to other ingredients tested. It is now important to investigate the effect of this physical form of coating on the structural integrity of the LR6 cells, particularly on the cell membrane, which becomes directly exposed to the desiccation related stresses discussed in Chapter 2.0. A better understanding of the process should help in designing a more optimized supporting matrix to yield more viable cells post desiccation. In the following set of experiments, the desiccated LR6 cells will be isolated from the stabilization matrix and inspected for any structural deformations. The results will be correlated with the degree of cell death observed in this chapter.

Chapter 6.0. Effect of drying methods and the role of different carrier excipients on the viability of *Lactobacillus reuteri* LR6 cells; Fourier transform infrared spectroscopy studies

The contents of this chapter have been submitted in October 2018, in the form of a manuscript, for publication in the peer-reviewed journal "Journal of Food Science":

6.1 Introduction

Drying or stabilization of probiotic bacteria for long time preservation aids in easy handling, flexibility in product formulations and reduces the cost of transportation. As discussed previously, the most popular industrial techniques adapted for this purpose are spray and freeze drying, and fluidized bed or vacuum oven drying in some cases. The alternative drying methods have not become commercially popular because of the high loss in the viable cell population during drying in these methods (Santivarangkna *et al*., 2006). To tackle this problem, it is important to understand the physiological response of the probiotic cells to the applied drying process. It has been reported that drying causes some adverse effects on the cell envelopes and the secondary protein structures therein (Leslie *et al*., 1995). The inactivation kinetics during drying were studied by many researchers and the cytoplasmic membrane was found to be the most susceptible area (Gardiner *et al*., 2000). The damage to the cytoplasmic membrane leads to the leakage of

cell constituents, as evident from the increase in cellular enzymes and proteins in the surrounding medium of the cells upon rehydration and also from the increased sensitivity of the cells towards NaCl, oxgal or lysozyme (Brennan *et al*., 1986; Lievense *et al*., 1994; Teixeira *et al*., 1995; Selmer-Olsen *et al*., 1999a).

The water replacement theory proposed by Crowe *et al*. (1987) is probably the most recognized one in explaining the mechanism of dehydration and bacterial protection by certain sugars. This theory suggests that removal of the hydrogen-bonded water from the phospholipid bilayer causes an increase in the melting temperature (T_m) of membrane lipids, resulting in lipids changing from a liquid crystalline to a gel phase. Upon rehydration, when these lipids return to the liquid crystalline phase again, the process results into cell membrane leakage which is the principal cause for inactivation or loss in cell viability. When the cell dehydration is carried out in presence of sugars or sugar-type compounds such as sorbitol or mannitol, the hydrogen bonds are retained and a membrane stabilization effect is seen. Similarly, damage to the cell wall is also considered as a probable factor for dehydration inactivation but mostly predominant in Gram-negative bacteria. Lactobacilli, being Gram-positive bacteria, possess about 10 times stronger cell wall; hence the cell wall damage is considered to be a less significant contributor to the inactivation mechanism (Delcour *et al*., 1999; Santivarangkna *et al*., 2006).

The Fourier transform infrared technique (FTIR) has been used extensively to investigate the conformational changes in the secondary protein structures (Leslie *et al*., 1995, Oldenhof *et al*., 2005; Santivarangkna *et al*., 2010). The FTIR technique is able to

assign infrared absorbance bands in the mid-IR region (wavenumber 4000-500 cm^{-1}) to the cell components, out of which the proteins and polypeptides comprised of the amide 1 and amide 2 bands. The polypeptide and protein repeat units represent nine infra-red absorption bands which are named as amide A, B, and 1-7 (Kong and Yu, 2007). The most prominent vibrational bands among these representing the protein backbone are amide 1 and 2. Approximately 80% of the C=O stretching vibrations of the peptide linkages are assigned to the amide 1 band (1700 – 1600 cm^{-1}) of the protein secondary structures. The frequencies of the amide 1 band have been found to be highly correlated to each of the secondary structural elements of the proteins. The amide 2 band is relatively less correlated to the protein conformation compared to the amide 1 band and is derived from the in-pane NH bending (40-60%) and the CN stretching vibration (18-40%) (Krimm and Bandekar, 1986). Due to the highly complex nature of the side chains and hydrogen bonding, the other amide vibrational bands are of less practical use in the study of protein conformational changes (Banker, 1992). Apart from the most important polypeptide and the protein repeat units, the FTIR spectra of a bacterial strain is also able to show the characteristics of other cell components such as fatty acids, membrane and intracellular proteins, polysaccharides and nucleic acids (Dziuba *et al*., 2007).

The advantages of the FTIR technique are minimal handling of the powdered samples without destruction of their original structure, the ability to inspect the whole bacterial cells without causing lysis by any chemical or mechanical treatment and the ability to inspect the samples in either H_2O-based, D_2O-based media or in the dried form. This is a well-established technique for investigating the secondary structures of proteins and polypeptides and has been used in many areas other than cell biology (Elliot &

Ambrose, 1950; Krimm & bandekar, 1986; Smith, 1996; Susi & Byler, 1986). FTIR spectroscopy measures the wavelength and the absorption intensity of the infrared radiation of a sample. The obtained spectral data is interpreted by the vibrations of a structural repeat unit (Kong & Yu, 2007).

Fourier Transform Infra-Red (FTIR) spectroscopy is getting popular to study the entire molecular composition of microbial cells and can detect minimal cell composition changes by fingerprinting the entire cell (membrane and cytoplasm). The advantage of using the FTIR technique is the ability to inspect the stabilized cells in the dried format as well as in the reconstituted or wet format. The dried samples can be inspected for the matrix molecular structure and the interactions of individual matrix constituents with the cell membrane. The stabilized cells can also be concentrated and isolated from the powder by reconstitution and centrifugation and be checked for changes in cell compositions that would help to understand the type of membrane injuries taking place due to desiccation. Not only is this technique rapid and non-invasive to the cells but the sample preparation steps are also minimal.

A recent study by Booyens and Thantsha (2014) has looked into the injuries caused to various bifidobacterium strains by an antimicrobial compound and used FTIR technique for this purpose. They have shown the unique distinctions in the spectra between the control and injured cells and by doing the principal component analysis of the spectra, the probable and most significant sources of differences were also shown. Another relevant work using FTIR spectroscopy to detect changes in the phase transition temperature (T_m), in the presence and absence of sorbitol in a stabilization matrix, was

carried out by Santivarangkna *et al.* 2010. It was shown that sorbitol interacts with the membrane lipids and helps in reducing the T_m thereby offering protection against desiccation stress.

In Chapter 5.0 it was shown that drying of cells in the absence of any carrier agent, particularly at elevated temperature causes severe loss in cell viability. When dried along with a carrier excipient with granules formation, the degree of survival improves overall but the extent of protection depends upon the physical and chemical nature of the excipient. In this chapter, the desiccated bacterial cells in unsupported form and after isolation from the powdered matrix were investigated for any conformational changes in the secondary protein structures using FTIR spectroscopy in order to understand the protective mechanisms of the individual carrier excipients.

6.2 Results and discussion

6.2.1 FTIR spectra of fresh and desiccated LR6 cells

The overall primary spectra of the cells vacuum dried at 55 ˚C and in presence of sorbitol or xylitol show very similar patterns with no major peak alterations in the selected area of interests representing important cell components (Fig 6.1).

A peak flattening in the amide region (1700-1500 cm^{-1}) was noticeable only for the xylitol coated cells (circled). Further investigations into these specific regions were carried out by analyzing the secondary derivatives of these spectra and by comparing the detected peaks. The findings are reported in the following sections.

(a)   (b)

(c)   (d)
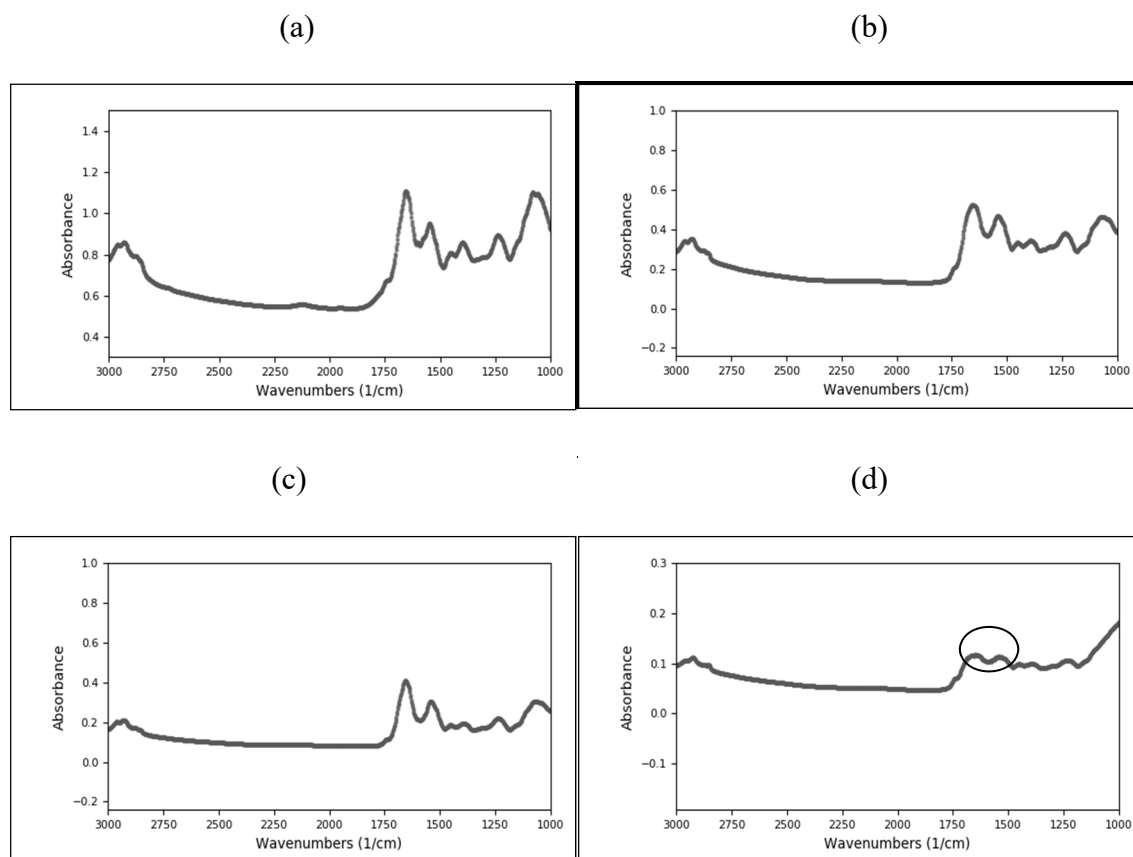
Figure 6.1: *Total primary spectra of the raw LR6 cells (a) and cells dried in vacuum oven without any supporting agent and with no coating (b), coated with sorbitol (c) or xylitol (d). The circled region in the xylitol coated sample spectra indicating peak flattening in the amide regions.*

Figure 6.2 shows the total primary spectra of the cells isolated from the six types of powdered samples. The SMP group samples (Fig. 6.2 – a, b, c) showed similar spectra

with the noticeable difference (flattened) in the amide region of the sorbitol coated cells (circled). Cells isolated from the corn starch, MCC and MPC showed differently

(a)   (b)

(c)   (d)

(e)   (f)
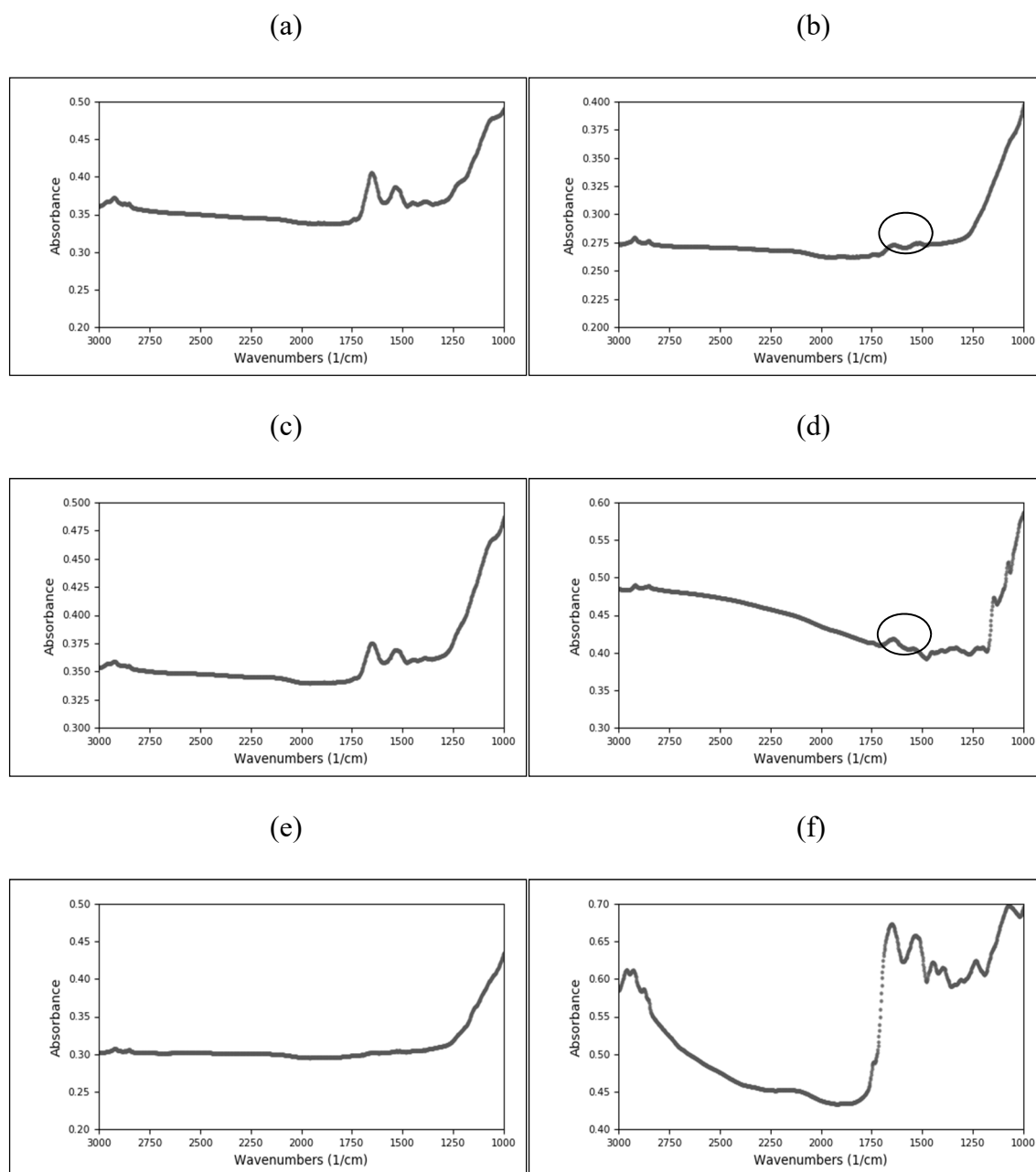
Figure 6.2: *Total primary spectra of the cells dried in FBD with supporting agents – SMP (a), SMP + sorbitol coating (b), SMP + xylitol coating (c), corn starch (d), MCC (e) and MPC (f). The circled regions showing distincttively different peak patterns.*

patterned spectra than the SMP group. No clear amide peak was visible in the MCC samples (Fig. 6.2 - e) and was very subtle for the corn starch samples (circled in Fig. 6.2 - d).

6.2.2 Stretching vibrations of the fatty acids functional groups of the cell envelope

Second derivative spectroscopy is a commonly used spectral analysis technique that has been used for decades (Rieppo *et al.*, 2012). According to Beer—Lambcrt law, absorbance can be expressed as follows:

$$A(\tilde{\nu}) = \alpha(\tilde{\nu})lc,$$

where A is the wavenumber (v)-dependent absorbance, α is the wavenumber-dependent absorption coefficient. l is the optical path length (mainly determined by the section thickness) and c is the concentration. When this equation is differentiated twice, the output is the second derivative of the first equation as described below.

$$\frac{d^2 A(\tilde{\nu})}{d\tilde{\nu}^2} = \frac{d^2 \alpha(\tilde{\nu})}{d\tilde{\nu}^2} lc.$$

To reduce the noise from the second derivative data and to increase the resolution, a smoothing filter was applied as per the protocol described in Dziuba *et al.* (2007). The Savitzky–Golay filter is a low-pass filter well adapted to smoothing noisy data. After the baseline correction, first derivatives and second derivatives were calculated (Savitzky–Golay algorithm with 9 point smoothing) using the Omnic version 7.1 software.

The second derivative spectral peaks of the desiccated cells in unsupported drying format are shown in Table 6.1. As reported earlier by Cacela and Hincha (2006) and Dianawati *et al*. (2013), and the $N^+(CH_3)_3$ asymmetric stretching of choline of the freshly harvested LR6 cells was indicated at wavenumber 980.8 cm^{-1}. The C-H asymmetric and symmetric stretching vibrations of the cell envelope containing fatty acids (FA) were detected at 2947.4 and 2862.8 cm^{-1} respectively. The peak at 1770.1 cm^{-1} was identified as C=O bond located at the interface between polar headgroup and apolar tailgroup of the phospholipid bilayers. The vibration of P=O symmetric stretching was found at 1068 cm^{-1}. The peaks located in this experiment were close but slightly different than the figures published in Dianawati *et al*. (2013) who looked into two strains of *Lactobacillus acidophilus* and *Lactococcus cremoris* when subjected to freeze and spray drying processes. These differences can be justified by the suggestion from Dziuba *et al*. (2007) who found FTIR spectra of bacteria are very specific to a given strain and therefore may vary to some extent depending on the type of organism.

Table 6.1 *Symmetric and asymmetric streching vibration peaks of the fatty acids, choline groups, phosphate group of the phospholipids and carboxylic esters. Samples representing freshly harvested cells compared with the desiccated cells in vacuum drying without any carrier agent*

	Unsupported drying				
	Band assignment for Functional groups				
	CH3 asym	CH3 sym	N-(CH3)3 asym	P=O sym	C=O
Fresh cells	2967.2	2878.8	952.3	1062.0	1756.0
Uncoated 55°c	2966.9	2879.4	950.8	1063.7	1750.1
Uncoated 75°c	2967.0	2879.3	958.2	1063.0	1752.0
Sorbitol 55°c	2966.6	2879.4	944.6	1059.8	1750.0
Sorbitol 75°c	2967.6	2878.8	958.0	1059.0	1750.1
Xylitol 55°c	2966.5	2878.6	940.0	1058.0	1750.2
Xylitol 75°c	2966.8	2879.2	955.7	1057.3	1751.9

The following spectral peaks (Table 6.2) represent the stretching vibrations from specific cell components from the samples which were dried in the FBD. These were measured from the second derivative spectra of the fresh and desiccated cells, as guided by the literature mentioned earlier. The peak measurement tool inbuilt in the Omnic software was used for this purpose.

Table 6.2 *Symmetric and asymmetric stretching vibration peaks of the fatty acids, choline groups, phosphate group of the phospholipids and carboxylic esters. Samples representing freshly harvested cells compared with the desiccated cells in fluid bed drying with carrier agents*

		Drying with carrier agents				
		Band assignment for Functional groups				
		CH3 asym	**CH3 sym**	**N-(CH3)3 asym**	**P=O sym**	**C=O**
Fresh cells		2967.2	2878.8	952.3	1062.0	1756.0
MCC	55°c	2962.0	2876.7	950.0	1060.8	1771.5
	75°c	2967.7	2878.6	955.0	1063.9	1770.9
MPC	55°c	2965.8	2878.8	948.3	1061.4	1771.7
	75°c	2966.0	2878.6	957.1	1062.7	1770.6
Starch	55°c	2964.6	2876.8	952.8	1061.1	1771.3
	75°c	2966.2	2877.6	958.0	1060.7	1770.9
SMP	55°c	2965.2	2877.5	944.8	1050.1	1771.2
	75°c	2965.9	2878.3	959.6	1055.6	1771.1
SMP(sorb)	55°c	2963.2	2876.5	949.1	1056.6	1771.3
	75°c	2965.2	2877.6	960.2	1058.8	1771.2
SMP(xyl)	55°c	2964.9	2877.2	944.8	1052.3	1771.1
	75°c	2666.1	2878.4	959.9	1054.7	1771.5

For the unsupported samples, no significant up or downshift in the CH_3 asymmetric and symmetric stretching of the FA of the cell envelopes was noticed. The maximum range of deviation was 0.5 cm^{-1} and 0.8 cm^{-1} for CH_3 asymmetric and symmetric frequencies respectively, which can be considered negligible. In case of supported drying at both 55 ˚C and 75 ˚C, the CH_3 asymmetric frequency peaks for all the samples were slightly shifted down in the range of 2962 to 2967.7 cm^{-1} compared to the fresh cells (2967.2 cm^{-1}). The CH_3 symmetric frequency peaks did not alter for any of these samples similar to the unsupported drying experiment. An upshift of the peaks in these two regions indicates conformational changes in the FA which were converted from lyotropic gel to the liquid crystalline phase (Goodrich *et al*., 1991). According to Gauger *et al*. (2002 and 2007), at higher moisture content a phenomenon called chain melting is triggered which causes conformational disorders of the acyl chains and subsequent increase in the

C-H frequencies. However, it is not known if this phenomenon is connected to any significant damage to the cells leading to the loss in viability.

Some major alterations were noted in the rest of the principal regions within the total spectrum of the desiccated samples. The P=O symmetrical frequency peak for the fresh cells was at 1062 cm^{-1} and the same for the unsupported samples in the absence of polyols were slightly increased to 1063.7 cm^{-1} and 1063 cm^{-1} for 55 ˚C and 75 ˚C drying temperatures, respectively. Minor peak reductions were recorded in case of the polyol coated cells. The range was 1057.3 to 1059.8 cm^{-1} and any effect of drying temperature was not prominent. In case of supported drying at 55 ˚C, reduced peak frequencies were observed for SMP and SMP with sorbitol or xylitol samples and maximum downshift to 1050.1 cm^{-1} was observed for the SMP carried cells. For 75 ˚C drying, 4 out of 6 samples showed a considerable reduction in their peaks. Except for MCC and MPC, which are not considered as sugars or sugar derivatives, the P=O symmetrical frequencies of all others were affected by drying.

It was apparent that drying of the cells in presence of sugars and/or polyols clearly yielded different conformational changes to the P=O symmetrical frequencies of the fatty acids compared to other excipients which are mostly protein (MPC) or carbohydrate in nature but not necessarily be regarded as sugars (MCC and starch). The decrease in P=O wavenumbers during desiccation of bacterial cells in the presence of sugars and polyol compounds have been previously reported by Leslie *et al*. (1995), Oldenhof *et al*. (2005), Santivarangkna *et al*. (2010) and Dianawati *et al*. (2013). The phenomenon was explained by them as a result of the interaction between the sugar

molecules and the polar site of lipids, causing a decrease in lateral lipid movement. Sugar and sugar alcohols are known to replace water during desiccation and thereby protecting the cell envelopes (Goodrich *et al*., 1991). It was also reported earlier that the phospholipid bilayers of the bacterial cells are protected when dried in presence of mannitol through an interaction between the polar surface of phospholipid and mannitol and in such case a reduction in the P=O vibration frequencies can be expected (Grdadolnik and Hadzi, 1998). Such interaction took place through a strong hydrogen bond formation between sugar hydroxyl–lipid headgroups where mannitol acted as a proton donor (Grdadolnik and Hadzi, 1998; Ricker *et al*., 2003; Dianawati *et al*., 2013). However, as per the residual cell viability observed in Figs. 5.1 and 5.5, such protection of the FA headgroups was not found to be correlated with the actual protection of the cells from decay in viability during vacuum or fluid bed drying. The depression in P=O vibration frequencies was common for both group of samples when the drying was carried out at 55 ˚C and 75 ˚C but a considerable loss in viability was recorded at 75 ˚C even in presence of milk compounds, sorbitol or xylitol. This is probably because exposure to a higher temperature is expected to cause more conformational changes to the cell proteins along with the lipid constituents and consequently might be expected to be more correlated to the decay in cell viability.

The frequencies for the $N^+(CH_3)_3$ asymmetric stretching vibrations of the unsupported cells were mostly reduced at low-temperature drying and increased when the drying temperature was high. Cells dried at 55 ˚C showed more depression in peaks when dried in the presence of polyols. At the higher temperature of drying, all three samples showed broadening of peaks in the range of 955.7 to 958.2 cm^{-1} compared to the fresh cells at

952.3 cm^{-1}. In case of supported drying at 55 ˚C, the lowering of peaks was very small for MCC, MPC, and starch but became more pronounced for SMP and polyol mixed samples; the trend was similar to unsupported drying (Table 6.1 and 6.2). The temperature of drying was a major factor in the peak alterations of the choline functional group. Drying at 75 ˚C caused a considerable increase in frequency peaks for all the samples irrespective of the carrier agent. This probably indicates a greater degree of conformational changes in this cell component and might be linked with the higher loss in cell viability recorded for these samples (Fig. 5.1 and 5.5). According to Cacela and Hincha (2006), the presence of sugars and polyols aids in the depression of choline peaks because of the interactions of these compounds with the polar site of phosphatidylcholine. The probable mechanism was explained by Grdadolink and Hadzi (1998) with the proposed torsional angles theory according to which increase in H-bonds in presence of sugars and polyols result from the alteration in rotamer population and changes in the *ap* and *sc* torsion angles.

The C=O double bonds of all the unsupported samples (Table 6.1) were found to be considerably lower (range 1750.1 – 1752 cm^{-1}) than the fresh cells (1756 cm^{-1}). However, an opposite trend was observed in case of supported drying (Table 6.2) where a major upshift to the range of 1770.6 – 1771.7 cm^{-1} was noted and the variation due to the temperature of drying could not be noted. The pattern of the peak alterations in opposite directions can be explained from the findings of Santivarangkna *et al*. (2010). They reported that the C=O stretching vibration of the ester carbonyl groups of the bacterial phospholipids can be identified at 1716 – 1750 cm^{-1} wavenumber. An increase in this number may be attributed to the formation of non-hydrogen bonds and a decrease

could be due to weak or strong hydrogen bond formation (Lewis and McElhaney, 1998; Santivarangkna *et al*., 2010). In the unsupported drying experiment, the hydrogen bond formation could possibly have taken place when water was replaced by the polyols during evaporation. However, the same phenomenon recorded in the case of uncoated samples and the samples containing non-sugar compounds cannot be explained in this way. When drying was carried out in presence of a complex matrix material, the frequencies were found to be increased probably as a result of non-hydrogen bonds of more complex in nature which are beyond explanation from this study only.

The study of the peak alterations in the five regions of the FTIR spectra as described above could not provide a clear picture of the underlying factors responsible for a significant amount of loss in viable cells during drying when compared between unsupported and supported formats containing various carrying agents. It is possible that peak shifts in the regions mentioned in the previous section were very subtle and the actual impact could be seen from the alterations in the entire spectra rather than just the individual peaks.

6.2.3 The amide region consisting of amide 1 and 2 bands of proteins and peptides

From Figs. 5.1 and 5.5 it can be seen that the higher temperature of drying is the principal factor for the cell death irrespective of any protection received in the form of a liquid coating (vacuum drying) or from the interactions with an excipient (fluid bed

drying). However, between these two forms of drying, the presence of an excipient powder during fluid bed drying, even at 75 ˚C, offers superior protection. Many researchers have stated that complete protein denaturation or even minor conformational changes in the secondary protein structures (α-helix, ß-sheet, ß-turn) are responsible to bacterial cell death during desiccation and that the stabilization of the membrane proteins improves bacterial viability. Therefore, the amide 1 and 2 bands have been of maximum interest to many previous researchers (Brennan *et al*., 1986; Carpenter and Crowe, 1989; Santivarangkna *et al*., 2006; Dianawati *et al*., 2013).

Using the same protocol to identify the spectral peaks as used in the previous section, the secondary derivatives of the amide 1 band were identified and assigned to the particular components of the secondary protein structures as described by Kong and Yu (2007) (Table 6.3 and 6.4).

Table 6.3: *Assignment of band components of the secondary protein structures of LR6 cells dried without any supporting agent*

		Unsupported drying		
		Band assignment		
	ß-Sheet	**α-Helix**	**ß-Turn**	**Random**
Fresh cells	1695.1 1643.6	1658.8	1680.7	
Uncoated 55°c	1697.2 1646.6	1659.4	1681.8	
Uncoated 75°c	1635.7	1660.1		
Sorbitol 55°c	1695.8 1643.8	1659.3	1682.3	
Sorbitol 75°c	1696.3 1643.4	1658.5		
Xylitol 55°c	1695.1 1636.8 1628.3	1658.8	1682.4 1670.9	1650.1
Xylitol 75°c	1697.0 1642.5	1658.5		

Table 6.4: *Assignment of band components of the secondary protein structures of LR6 cells dried with various supporting agents*

		Drying with carrier agents											
	Fresh cells	MCC		MPC		Starch		SMP		SMP(sorb)		SMP(xyl)	
Band Assignment		55°c	75°c	55°c	75°c	55°c	75°c	55°c	75°c	55°c	75°c	55°c	75°c
ß-Sheet	1643.6 1695.1	1627.7 1635.8 1693.0	1643.5 1696.0	1630.1	1630.8	1627.7 1636.0 1693.3	1644.6 1694.9	1628.4 1694.6	1632.0 1697.6	1627.6 1635.3 1692.8	1627.9 1695.1	1628.1 1693.7	1631.9 1697.2
α-Helix	1658.8	1657.9	1658.8	1658.7	1658.6	1657.8	1657.7	1657.1	1657.8	1657.6	1658.0	1657.8	1658.4
ß-Turn	1680.7	1681.2 1669.9	1681.6	1680.7	1679.9	1681.2	1680.1	1681.3	1679.4	1680.9	1681.1	1680.8	1680.1
Random		1649.1		1648.8	1646.0	1648.7		1646.7	1646.1	1648.5		1647.0	1646.7

The amide 1 band of the fresh cultures of LR6 cells showed a peak at 1658.8 which was assigned to the α-helix component (Table 6.3). In some proteins, such as hemoglobin, myoglobin, lysozyme, the α-helix component constitutes of up to 80% of the total secondary structures (Dong *et al*., 1992) and the IR spectra of this band is very well correlated (98%) with the X-ray crystallography estimates (Kong and Yu, 2007). The α-helix band was present in all the desiccated samples in both methods of drying with a very minor alteration in the range 1657.1 to 1660.1 cm^{-1} (Table 6.3 and 6.4). Both ß-sheet and ß-turns are very important components of the protein secondary structures and their IR spectra correlate up to 90-99% with the X-ray crystallography results (Kong and Yu, 2007).

A peak in the range 1627.6 to 1643.8 cm^{-1} (in some cases 2 peaks within this range) was found in all the samples with minor variations and was assigned to the ß-sheet component. Another peak near the vicinity of 1695 cm^{-1} was seen in some of the samples and was also assigned to the ß-sheet component. Interestingly, this peak near 1695 cm^{-1} was absent in the samples dried at 75 ˚C in uncoated form and with MPC at both the temperatures. Particularly these samples yielded very low viable cell counts after drying. The ß-turn bands were identified near 1681 and 1670 cm^{-1}. However, the most interesting observation was the absence of any ß-turn band in the spectra of unsupported cells dried at 75 ˚C whether they were coated or not coated with polyols (Table 6.3 and 6.4). This indicates the disruption of this area because of excessive direct heating. It was previously observed that drying at any temperature with carrier agents caused less inactivation to the cells compared to unsupported drying. Spectral analysis of all such samples with carrier excipients indicated the presence of the ß-turn bands. A

band near 1650 cm^{-1} was observed in some of the sample spectra which were assigned to the 'Random' component of the protein secondary structures (Kong and Yu, 2007). This band was absent in the fresh cells spectrum and hence probably indicates an important contributory factor towards the total conformational changes within the cellular proteins due to desiccation.

Similar deformations in the secondary protein structures of the cell envelope were observed previously by Dianawati *et al*. (2013). They found that desiccation by freeze drying or spray drying and more importantly the level of residual moistures in the dried samples were responsible for different types of damages to the cell envelopes and accordingly the pattern of change in the band components varied. In their study, it was shown that storage of the desiccated *L. acidophilus* and *L. cremoris* cells in presence of silica gel with reduced moisture level caused the α-helix structures to convert into β-sheet and β-turn structures compared to storage in presence of NaOH or LiCl. They also found that any conformational change depends on the type of organism being desiccated. For example, *L. cremoris* was found to be more sensitive to freeze-drying process as indicated by the conversion of α-helix structures to 'no order' and β-sheet whereas the structures of *L. acidophilus* were comparatively less affected.

Better retention of secondary structures during freeze-drying of proteins in the presence of sugars, such as sucrose, maltodextrin, or disaccharides in combination with starch was previously reported by Oldenhof *et al*. (2005) and Garzon-Rodriguez *et al*. (2004). Mannitol, a sugar alcohol similar to sorbitol and xylitol, was found to be protecting the structures of antibodies when spray dried at a moderate outlet temperature of 50 ˚C

(Schuele *et al.*, 2007). It was first postulated by Crowe *et al.* (1988) and later confirmed by Murray *et al.* (2005) that when water is evaporated in the presence of sugars, the unfolding of proteins and consequent preservation of the structures takes place by hydrogen bond formation. As shown in Table 6.1 and 6.2, in case of bacterial cell desiccation, the sugars or sugar like compounds also interact with the polar side of the membrane phospholipid bilayers, the actual factors responsible for protection against desiccation stress are not limited to retention of protein structures only.

By comparing the spectral peaks it was found that the changes observed in the secondary protein structures, compared to the fatty acid region, are more useful in explaining the role of drying temperature and certain protectants against the loss of cell viability. In the following sections, the entire spectra for this important region representing the general state of proteins as well as the secondary structures of the polypeptides will be analyzed using advanced statistical techniques. The objective would be to see if the differences seen among the samples in that way are more correlated with the post-drying viability levels.

6.2.4 Principal component analysis and hierarchical clustering of the FTIR spectra of the desiccated *Lactobacillus reuteri* LR6 cells

Fig. 6.3 shows the second derivative spectra of the amide zones (~1500 to 1700 cm^{-1}) for the LR6 cells desiccated in both the drying systems at 55 ˚C and compared with the same for freshly harvested cells. Fig. 6.4 shows the same for the samples dried at 75 ˚C. The amide zones in the unprocessed total spectra were found as two smooth peaks (Fig. 6.1) whereas the second derivative spectra show the overlapping minor bands representing the secondary structure, such as α-helix, ß-sheet and ß-turn of peptide components (Dianawati *et al*., 2013).
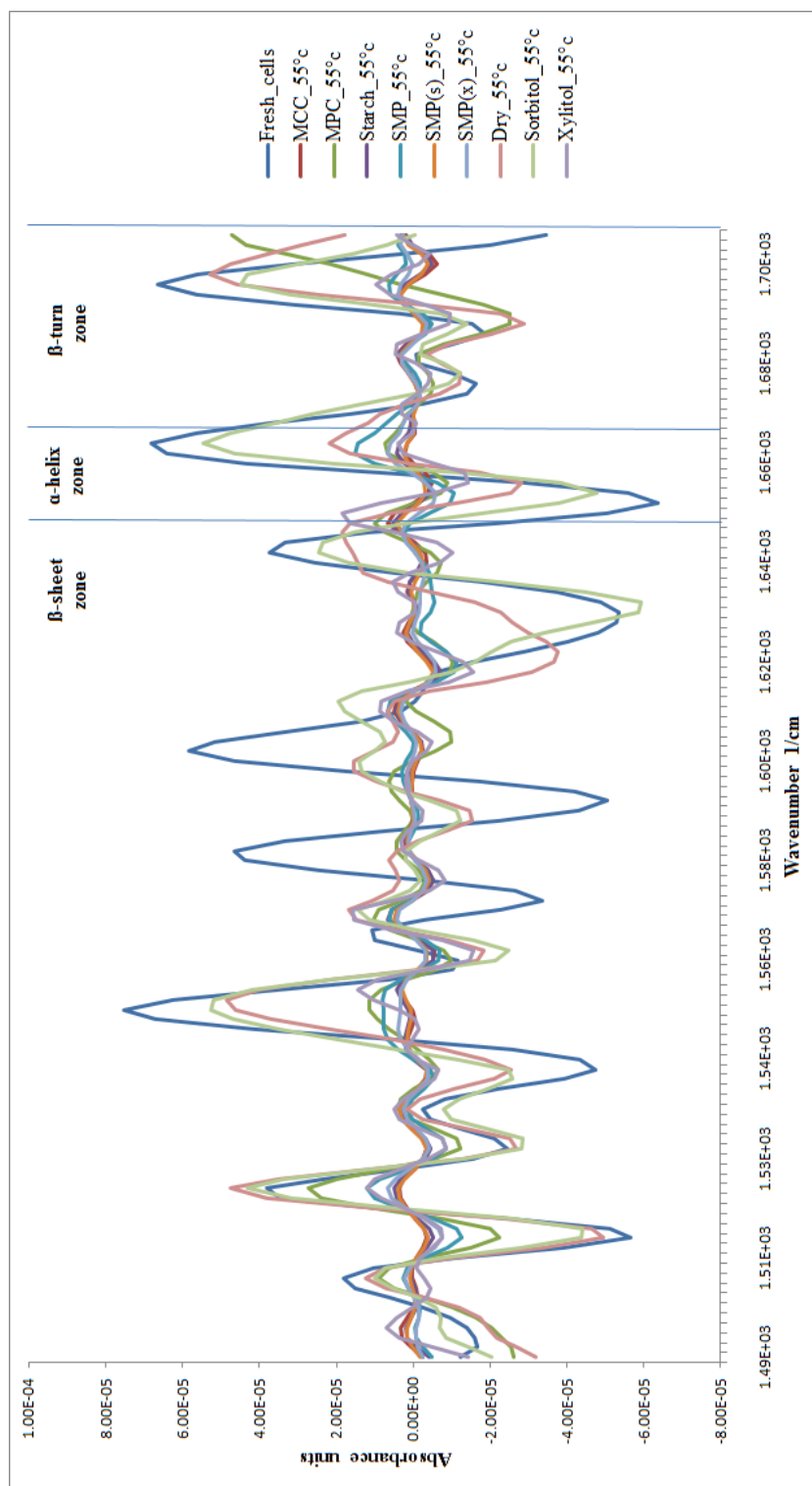
Figure 6.3: *Secondary derivative amide spectra (1700 – 1500 cm⁻¹) of the cells dried in the vacuum oven and in the FBD at 55 ˚C*
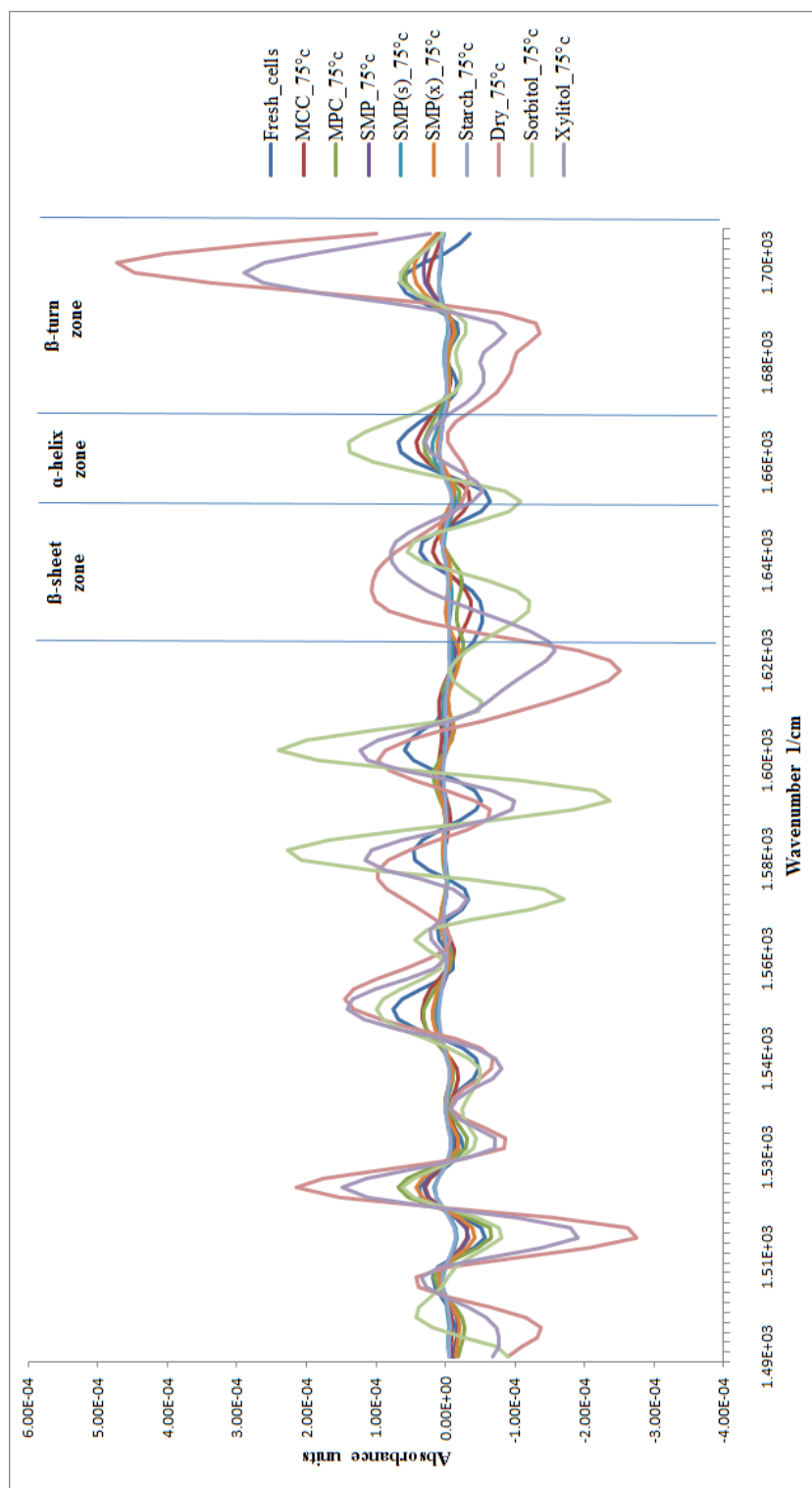
Figure 6.4: *Secondary derivative amide spectra (1700 – 1500 cm⁻¹) of the cells dried in the vacuum oven and in the FBD at 75 ˚C*

In the principal component analysis (PCA) of a multivariate data set it is important that maximum variation components are represented by the first two dimensions or sometimes referred to principal components. A Scree Plot is a simple line segment plot that shows the fraction of total variance in the data as explained or represented by each principal component (PC).
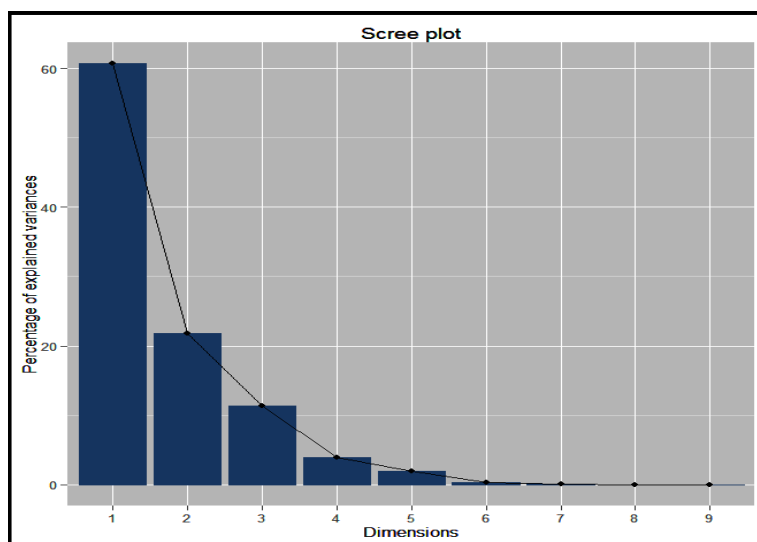
Figure 6.5: *Scree plot showing the percentage of the variations among the sample spectra contained in various dimensions. The samples analyzed were all dried at 55 ˚C*

The PCs are ordered, and by definition are therefore assigned a number label, by decreasing order of contribution to total variance. The PC with the largest fraction contribution is labeled with the label name from the preferences file. Such a plot when read left-to-right across the abscissa can often show a clear separation in fraction of the total variance where the 'most important' components cease and the 'least important' components begin. The point of separation is often called the 'elbow'. (In the PCA literature, the plot is called a 'Scree' Plot because it often looks like a 'scree' slope, where

rocks have fallen down and accumulated on the side of a mountain.) The scree plot in Fig. 6.5 for the unsupported cells shows that the first two dimensions contained 60.7% and 21.8% of the total variations among the samples which equate to 82.5% and is therefore considered satisfactory. Hence, it was not necessary to look into the rest of the dimensions as their contributions would be insignificant.

Figure 6.6 shows the contribution of individual samples towards the total variation among all the samples. In other words, higher the source of variation coming from a
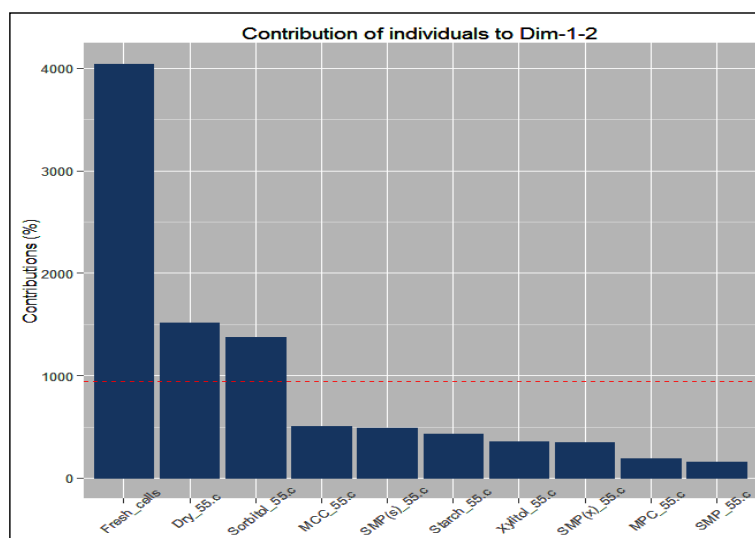
Figure 6.6 *Contribution of individual samples dried at 55 ˚C towards the total variation within dimensions 1 and 2, the red dotted line indicating important significance level*

particular sample, more dissimilar it would be considered than the rest in the group. In this group of samples dried at 55 ˚C, it can be seen that fresh cells showed the maximum difference in their IR absorbance than the rest in the group. The next significant and

nearly equal level of differences came from the sorbitol coated cells and the uncoated cells which were dried at 55 ˚C.

The other samples had comparatively less variability among themselves which is evident from their placement below the red dotted line, which indicates an important significance level. If a sample contribution on the total variability is above this line then it is considered to be of high significance. It should be noted that the three samples representing maximum variations contained therein do not necessarily imply that they are either extremely similar or dissimilar to each other. It only denotes that these samples are significantly different from the rest in the group because they contain the maximum variances within themselves.

The PCA groups or classifies the samples according to their homogeneity with each other and clubbed the similar samples under a particular color automatically assigned by the software code. According to the factor map shown in Fig. 6.7, the fresh cells
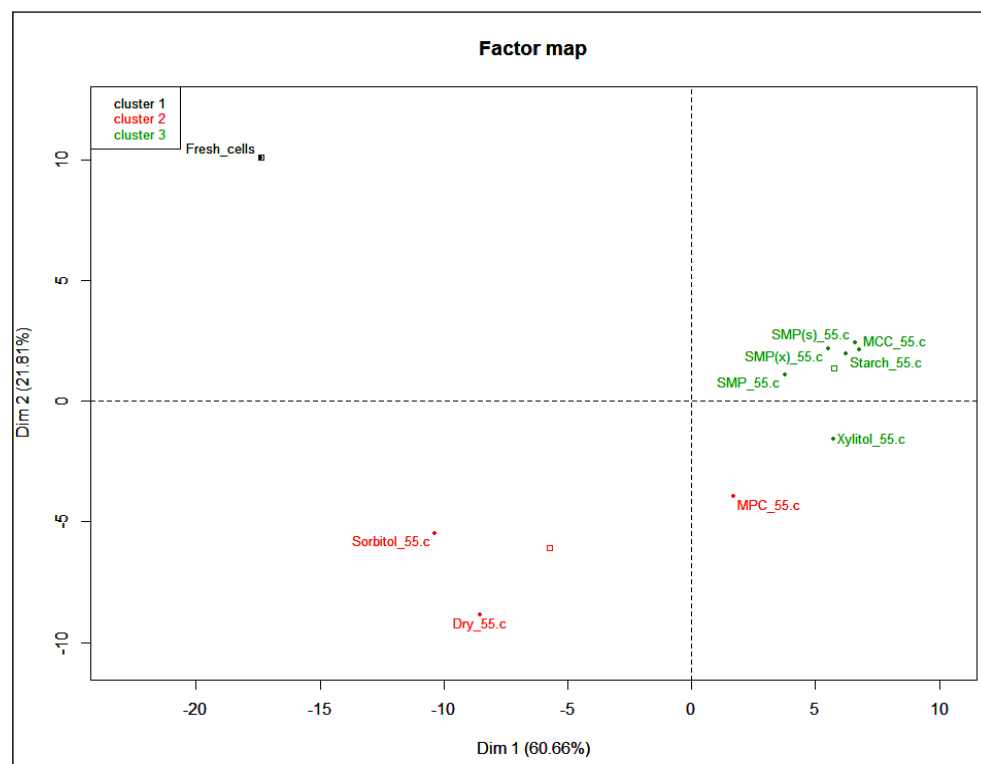
Figure 6.7 *Factor map showing principal component analysis (PCA) of the amide zone spectra of the cells dried at 55 ˚C*

emerged as an outlier and did not from any group with any of the dried samples. The red group contained three samples i.e; cells dried without any coating in the vacuum oven, with sorbitol coating in the vacuum oven and carried with MPC in FBD. According to the post-drying viability results, these three samples showed the lowest viability compared to their respective control samples. In the green group, the rest of the FB dried samples and the xylitol coated vacuum oven dried sample is shown to be more similar to each other. This group showed relatively better survival after drying despite having

some significant differences among the individual samples which are not reflected in the factor map.

Hierarchical clustering (HC) technique using Ward's algorithm was performed in order to further classify this group into narrower subclusters and the output is presented in Fig. 6.8 via a dendrogram. A dendrogram or tree diagram is popular in depicting clusters
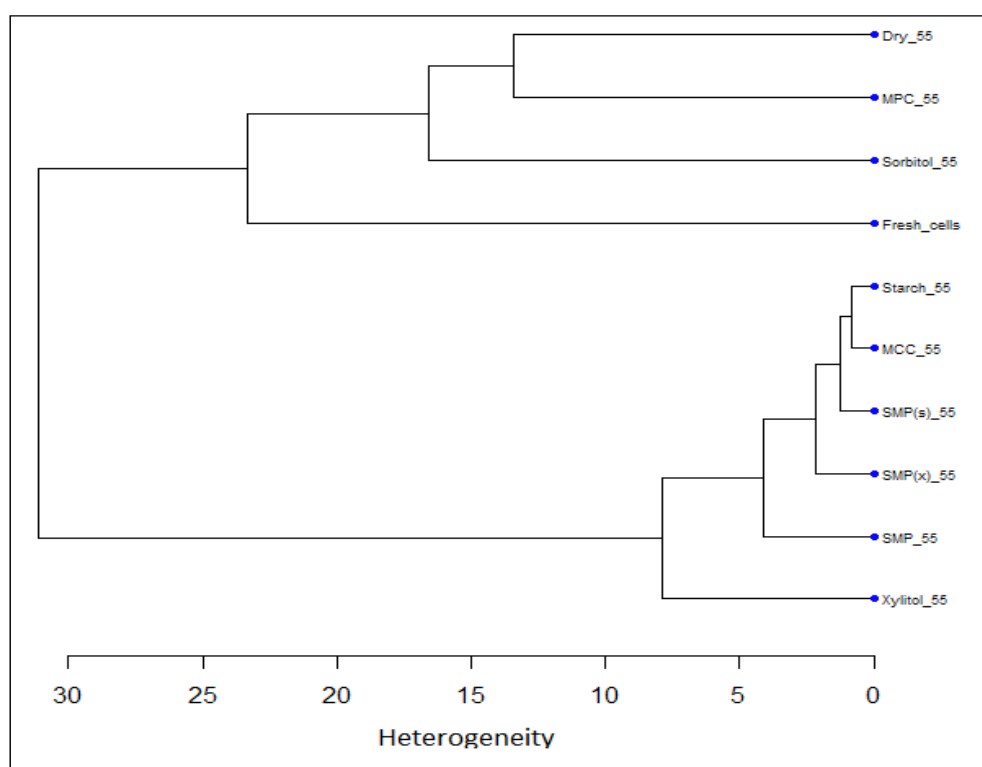
Figure 6.8 *Hierarchical cluster map showing heterogeneity of the amide zone spectra of the cells dried at 55 ˚C*

or similarities among a group of samples using an algorithm described earlier in section 6.2.1 (Davies and Mauer, 2010). Common use has been found in classifying bacterial strains under a common species by analyzing their FTIR bands (Savic *et al*., 2008).

Figure 6.8 shows two major clusters formed by the samples, the bottom one is of interest in subgrouping. The closest ones (with minimum and equal distances on the horizontal scale) indicating very similar spectral conformations were the cells carried with MCC and corn starch samples. The post-drying viable cell counts in these two types of samples were very close to each other (Fig. 5.5) and thereby appears to correlate well with the state of the secondary structures. The other samples in that cluster did not form any group in particular but remained close to each other, indicating a degree of similarity in their secondary structures but not to the extent of the closeness observed in the MCC and corn starch group. Placement of the xylitol coated samples which were dried without any excipient in this broad cluster is an interesting finding. Despite standing as an outlier, the closeness of this vacuum-dried sample (no excipient) with other FB dried samples (with excipients) clearly indicates the merit of xylitol as a superior protectant during desiccation at moderate temperature.
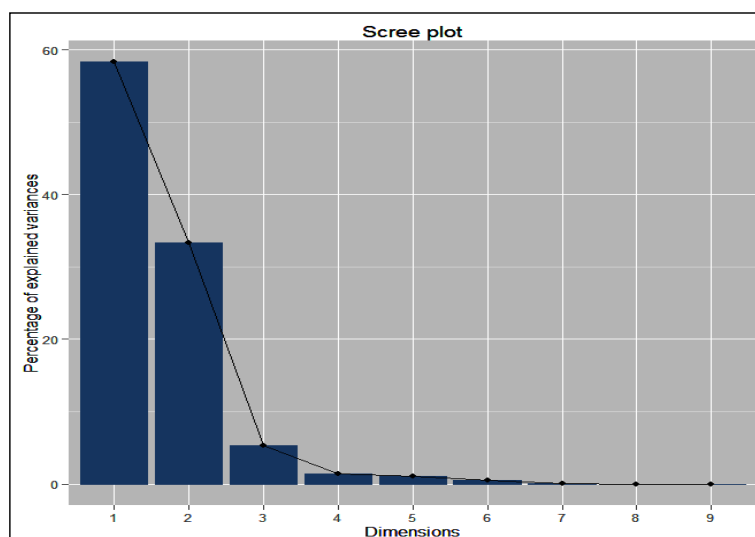
Figure 6.9: Scree plot showing the percentage of the variations among the sample spectra (amide zones) contained in various dimensions. The samples were all dried at 75 ˚C

The scree plot for the samples dried at 75 ˚C (Fig. 6.9) shows 58.3% and 33.3% of the total variances were contained in the 1st and the 2nd dimensions respectively.

The contribution map (Fig. 6.10) for the samples dried at 75 ˚C showed that the significant source of variances in the group was mainly from the three samples dried without any carrier agent. This indication correlates well with the extremely poor residual cell viability recorded in these three samples.
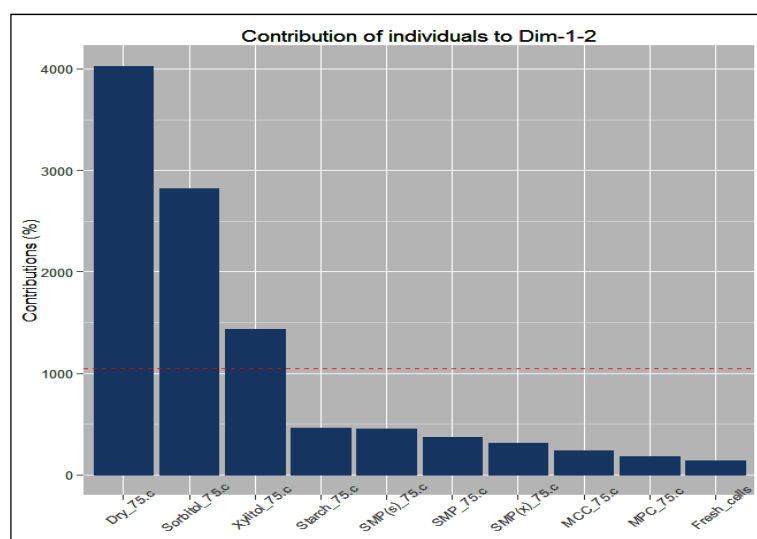
Figure 6.10: *Contribution of individual samples (amide zones) dried at 75 ˚C towards the total variation within dimensions 1 and 2, the red dotted line indicating important significance level*

The factor map in Fig. 6.11 also confirmed this observation. Out of these three unsupported drying samples, the sorbitol coated sample was an outlier with red color and the rest two were similarly grouped in black color. This group of three samples contained high concentration bacterial cells and no supporting agent, hence the marked
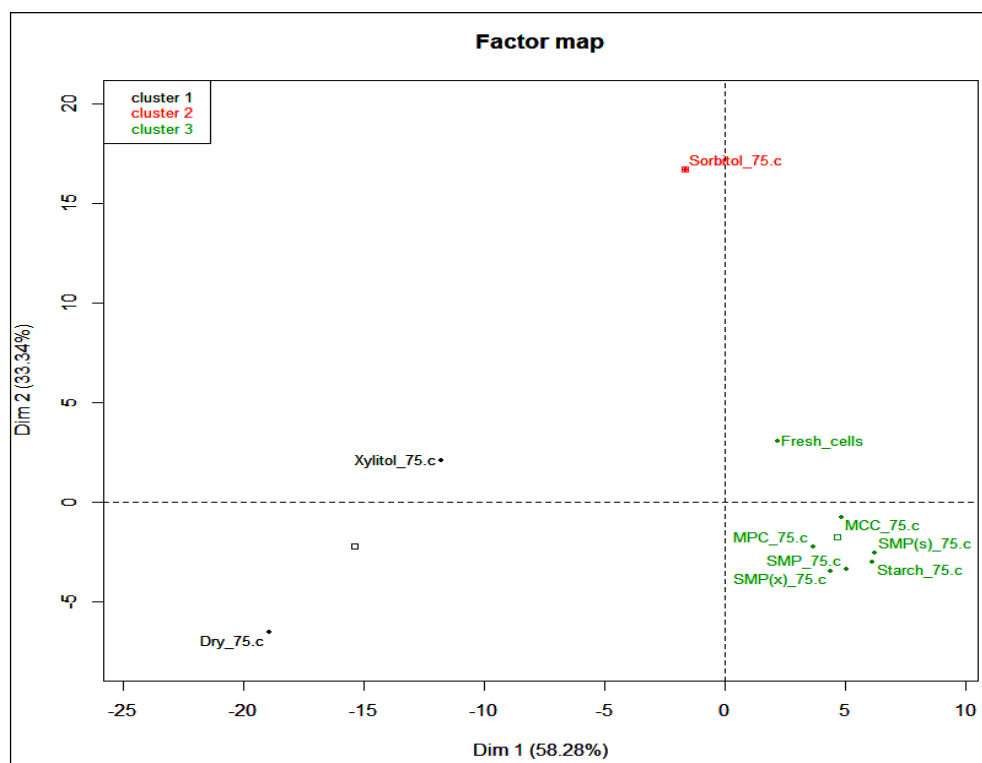
Figure 6.11: *Factor map showing principal component analysis (PCA) of the amide zone spectra of the cells dried at 75 ˚C*

difference from the supported drying samples can be easily justified. The fresh cells sample, although clubbed in the green group was placed separately from the rest. The supported drying samples were clubbed together despite having huge variations in terms of viability. This was probably due to their extreme heterogeneity with the fresh cells as well as with the unsupported drying cell samples. The composition of the supporting agents and their presence in high concentrations within the samples resulted in highly dissimilar spectral peaks compared to the unsupported drying samples.

The cluster map in Fig. 6.12 helped in isolating the narrower variations within the green cluster, i.e, the cells dried with the carrier agents at 75 ˚C. The topmost cluster showed a similarity between the cells carried by MPC and MCC. This group of two was significantly different than the next cluster formed by the rest of the 4 samples, i.e, cells
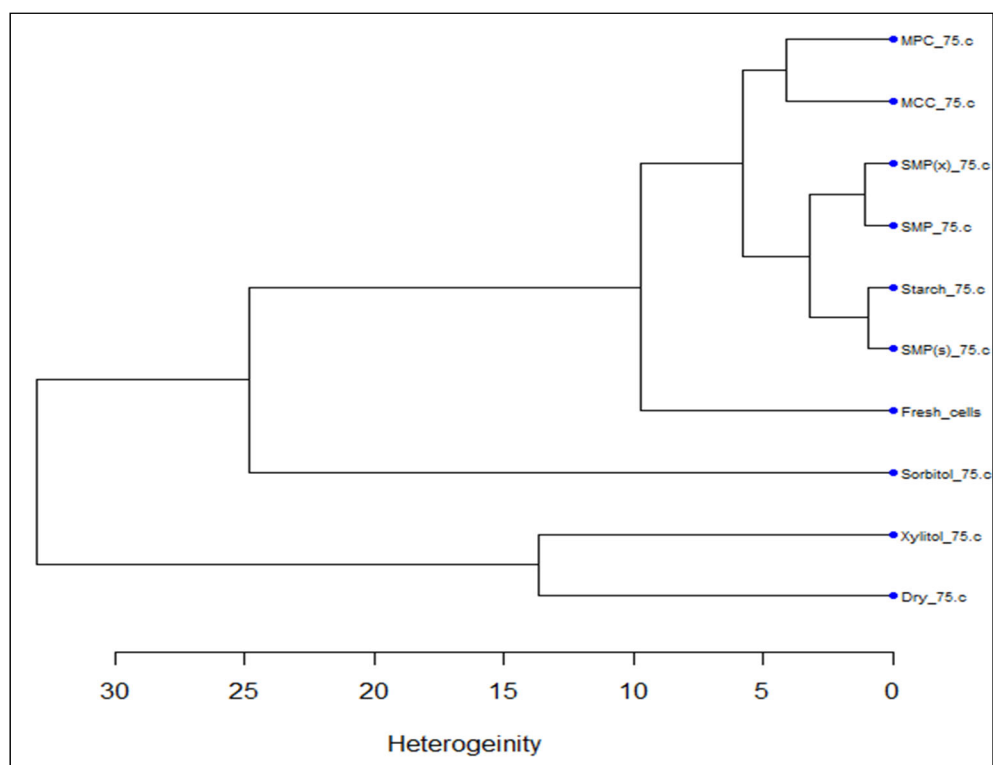
Figure 6.12: *Hierarchical cluster map showing heterogeinity of the amide zone spectra of the cells dried at 75 ˚C*

carried by starch, SMP and xylitol/sorbitol coated cells carried by SMP. Within this second cluster, there were 2 subclusters formed and that showed starch and SMP-sorbitol combination was similar to each other and the same was the case for the SMP and the SMP-xylitol samples. This clustering analysis showed almost perfect correlation with the post-drying viability counts observed (Fig. 5.5) when drying at 75 ˚C outlet

temperature yielded low viable cell population for the MCC and the MPC samples (average 8.15 log cfu/g) and the same for the other four were higher and very close to each other (average 8.53 log cfu/g).

It was previously shown that lethal damage to the bacterial cells because of heat injury is very much linked to protein denaturation and inactivation of the membrane-bound enzymes (Ikari *et al*., 2003). The heat injury can further aggravate or alleviate depending on the pH and the composition of the environment. For example, the heat inactivation process at low pH is expected to be accelerating while in presence of certain compatible osmolytes such as sugars, it has been found to be decelerating (Glaasker *et al*., 1998). In an extensive study, Kilimann *et al*. (2006) found that gradual heating of *L. lactis* cells from 40 ˚C to 65 ˚C in a milk buffer caused retarded inactivation in presence of sucrose. The amide region FTIR spectra of the viable and inactivated cell were analyzed and thermal induced alteration of spectral peaks was recorded similar to what was observed in this study. They concluded that when the bacterial cells are exposed to elevated temperatures, changes in the secondary protein structures as reflected by the amide bands spectral changes can directly be linked to protein aggregation and unfolding causing cell inactivation. The role of sucrose was found to improve the thermal stability of the intracellular proteins. In this study, it was found that the structural deformations in the cells were similar when they were desiccated at 75 ˚C in the absence of sugar or sugar alcohols (for MPC and MCC samples). These deformations could have resulted in inactivation of the cells upon drying. It was also observed that any protective effect of lactose or polyols on protein structures and its connection with cell viability is only pronounced at moderate desiccation temperature. Exposure of the cells at extremely high

temperature triggers some other detrimental factors which could not be detected from the FTIR spectra of the protein structures.

6.3 Conclusions

Desiccation of probiotic cultures is of commercial importance to preserve their functionality for a longer duration and to deliver this functionality through powdered food formulations. The importance of choosing the most effective supporting agents for desiccation is confirmed in this study. It was found that desiccation causes substantial changes in the protein secondary structures of the cell envelopes, which is correlated with the loss in cell viability. In this work, we have demonstrated that changes in bacterial protein conformations determined by analyzing the FTIR intensities of the amide bands can be correlated with the viability of desiccated cells. Such alterations can be minimized by imparting physical protection to the bacterial cells in the form of a coating of suitable food materials. Such materials should preferably possess certain characteristics, such as having plasticizing properties and being capable of forming a strong glassy matrix, as was found when using xylitol and SMP respectively.

Chapter 7.0. Storage stability of the LR6 cells and corresponding alterations in secondary protein structures during storage

The contents of this chapter have been submitted in December 2018, in the form of a manuscript, for publication in the peer-reviewed journal "Food Research International":

7.1 Introduction

Refrigerated or frozen storage of probiotic bacteria has always been found to be effective in maintaining the viability and functionality. However, such form of storage and subsequent handling involve high cost with enhanced risk of intermittent thawing (Heidebach *et al*., 2010; Bruno and Shah, 2003). A suitable stabilization technique either using a simple drying method (Miao *et al*., 2008; Kurtmann *et al*., 2009) or by complex microencapsulation (Dianawati and Shah, 2011; Donthidi *et al*., 2010) has been found to improve the stability of probiotics at moderate temperature storage in the range of 20-25 ˚C. It was reported that spray drying at low outlet temperature is more helpful in retaining the post-drying viability of probiotics but results in high moisture product due to inadequate drying. High residual moisture in free form is detrimental for storage stability because of water as a solvent aids in triggering physical and chemical reactions within the stabilization matrix. It also triggers the biological activity of the cells which is unwanted in the stabilized state. The quantity of free water in any food system is

represented by its water activity (a_w) which is directly related to the degree of bacterial biological activities, chemical, enzymatic and physical reactions, and moisture migration from one type of molecule to other (Maltini *et al*., 2003). There are several reports available citing significantly superior stability of probiotics upon storage at room temperature only when the a_w was kept at a very low level (Mugnier and Jung, 1985; Kearney *et al*., 2009; Kurtmann *et al*., 2009).

There is no general acceptance of any particular type of carrying agent, which is essential for stabilization of the probiotic cells and solely responsible for offering superior storage stability at enhanced temperatures. It is also not clear which compositional factors (proteins, lipids or carbohydrates), singly or in combination, are more important for this purpose. For example, when reconstituted skim milk as a protectant was compared with various combinations of pure disaccharides, maximum stability of freeze-dried *L. rhamnosus* at room temperature storage was observed in case of trehalose + lactose + maltose combination followed by lactose + maltose combination (Miao *et al*., 2008). The role of milk protein was not pronounced in this study. On the contrary, sodium caseinate based microcapsules were found to be more protective compared to resistant starch (which exhibited adverse effect) when *Lactobacillus* F19 and *Bifidbacterium lactis* BB12 were stored at 25 ˚C (Heidebach *et al*., 2010). Another important observation of this study was a decreased survival of the stabilized cells even at 4˚C storage when the a_w was increased to 0.30 (Heidebach *et al*., 2010). Therefore, based on several published works, a more direct correlation can be drawn between storage stability and low a_w than with the nature of the carrier agents. Sugar alcohols, such as glycerol and mannitol, have been found to have a protective effect during room

temperature storage of probiotics when compared with a few prebiotic compounds and partially skimmed milk (Savini *et al*., 2010). Similarly, sorbitol was found effective in maintaining the viability of *L. paracasei* and *L. plantarum* cells even after 150 days of storage at 25 ˚C when packed in the absence of oxygen (Coulibaly *et al*., 2010). In this project, various carrying agents for stabilization of the *Lactobacillus reuteri* LR6 cells were tried. They included polysaccharides, milk proteins, a combination of lactose and milk protein and also a combination of milk solids and sugar alcohols. The temperature of storage was 37 ˚C, not commonly chosen by other researchers and hence not much prior information is available on the effect of the mentioned carrier agents on the stability of probiotic bacteria at this storage temperature.

In order to understand the underlying mechanism partially or solely responsible for cell death during storage, some previous studies (Chavez and Ledeboer, 2007; Dianawati *et al*., 2013) have focussed on examining the alterations of secondary protein structures of the cell envelopes. The stabilization techniques and carrier agents were different in each case but they found that such alterations took place during room temperature storage even when there was no change in the glass transition temperature of the stabilization matrix. A similar approach was taken in this work by investigating such alterations in the secondary protein structures and observing how they correlate with the decay in cell viability over time. The glass transition temperatures of the samples at different a_w levels after desiccation and during the storage was also determined.

7.2 Results and discussion

7.2.1 Storage stability of the LR6 cells maintained at two different water activities

Initial viable cell populations of all the samples on average ranged from 8.34 to 9.14 log cfu/g, with the lowest recorded in MPC and the highest in corn starch samples (Fig. 7.1).
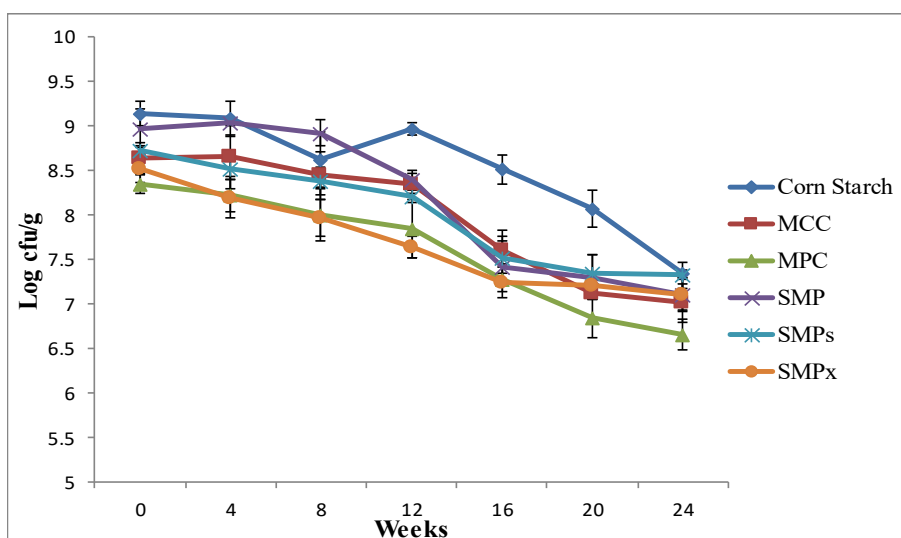
Figure 7.1: *Viable Lactobacillus reuteri LR6 populations during storage at 37 ˚C and a_w 0.11 for 24 weeks*

The trend from the slope of the graphs suggested a steady and minimal loss in viability during the first 12 weeks followed by accelerated decline in the latter half. After 24 weeks, the minimum loss was recorded for sorbitol and xylitol coated cells which were supported by SMP (1.4 log cfu/g). The maximum reduction in cell viability was observed in corn starch and SMP carried samples (1.8 and 1.9 log cfu/g respectively).

Net reductions in viable cell populations for MCC and MPC samples were 1.6 and 1.7 log cfu/g respectively.
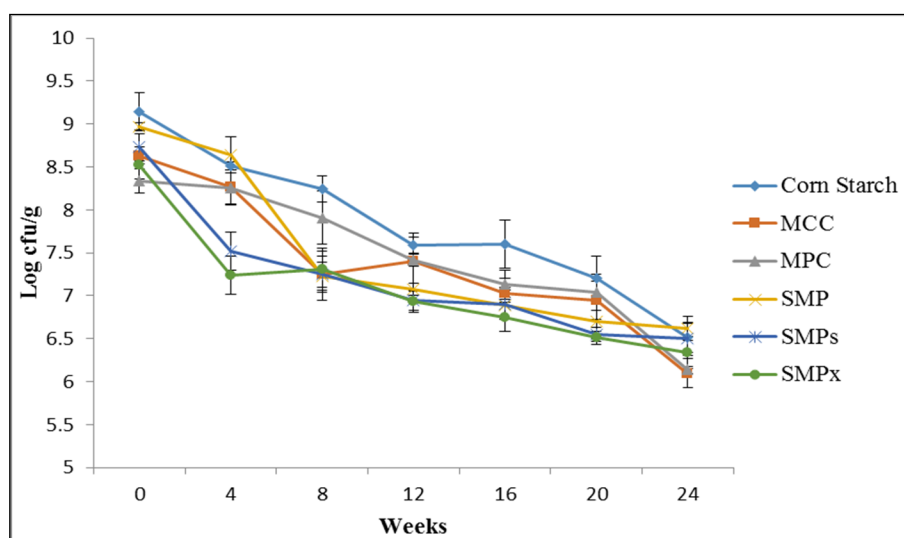
Figure 7.2: *Viable Lactobacillus reuteri LR6 populations during storage at 37 ˚C and a_w 0.30 for 24 weeks*

Fig. 7.2 shows more than 2.0 log cfu/g reductions were recorded in all the samples stored at 37 ˚C at higher 0.30 a_w. The rate of decline was more uniform throughout the storage period in contrast to the differential rate of decline observed in case of lower a_w storage. Minimum reduction in viability was recorded for MPC and sorbitol or xylitol coated cells carried by SMP (2.2 log cfu/g). SMP carried cells without any coating of polyol compound showed a slightly higher reduction of 2.35 log cfu/g at the end of 24 weeks. The maximum loss was observed for MCC and corn starch samples (2.5 and 2.6 log cfu/g). In both a_w environments coating the cells with sorbitol or xylitol proved to be beneficial, however, the effect was not significant.

Considerable variations in the stability of desiccated probiotic cells at non-refrigerated storage temperatures were observed by many researchers using different carrying agents. When spray drying was used for this purpose, which also acts as a microencapsulation process, SMP offered very good protection at 37 ˚C storage and only 0.5 log reduction in 5 weeks was recorded (Ananta *et al.*, 2005). Fermented soy milk (by the probiotic strains) was found to retain 40% of the viable cell population even after 4 months of storage at 25˚C (Wang *et al.*, 2004). Improvement in storage stability was also observed when skim milk solids were partially replaced with 10% gum Arabic (Desmond *et al.*, 2002). However, the most interesting observation was found in the work of Lian *et al.* (2002) who recorded the best protection of a bifidobacteria strain with SMP, followed by gum Arabic and gelatin mix and the stability was poorest when starch was used as the carrier agent. Similarly, O'Riordan *et al.* (2001) also observed a considerable loss in the viability of bifidobacteria (3 logs in only 5 days at ambient temperature) when waxy maize starch was used as a carrier agent. It was concluded by Ananta *et al.* (2005) that poor protection at ambient temperature may be expected if the carrying polymers do not interact in some way with the bacterial cell membrane during the desiccation process.

In the results shown above in Figs. 7.1 and 7.2, apparently a similar confirmation is evident. According to the water replacement theory of Crowe (2002), the corn starch and MCC samples did not contain any disaccharide molecule which could replace the evaporating water molecules during desiccation to form hydrogen bonds with the cell membrane proteins which in turn could prevent their denaturation. Any denaturation of membrane proteins could also result in their secondary structural changes which were analyzed using the FTIR spectroscopy and the findings will be presented in the

following sections. However, it is interesting to observe that at low a_w storage environment such damaging effects of corn starch and MCC were not very pronounced. The differences in viable cell populations in these samples compared to the best-performing ones (sorbitol and xylitol coated cells carried by SMP) were only maximum 0.5 log cfu/g after 24 weeks of storage at 37 ˚C. Therefore it can be clearly seen that damage to the bacterial cells during the desiccation process is not the only factor decisive for storage stability. The interactions between the bacterial cell and the surrounding environment (nature of carrying agents, temperature and amount of free moisture available) throughout the storage period play an equally important role.

Chavez and Ledeboer (2007) used a two-step process in stabilizing bifidobacteria cells which involved spray drying at a moderate outlet temperature followed by vacuum drying at a lower temperature. The principal carrying agents used were SMP and soy protein isolate (SPI), in pure form or in combination with various disaccharides. They found this two-step process was not only helpful in retaining more viable cells after the stabilization but was also 3 times less expensive than freeze-drying. The stabilization process used in this thesis work was similar to the above in principle where instead of adding the LR6 cells in the spray dried slurries, the bacterial cells were introduced in the intermediate processing step before the FBD (equivalent to 2nd stage drying). Chavez and Ledeboer (2007) studied the storage stability at 37 ˚C for 90 days and obtained the best results for the bifidobacteria cells carried by the combinations of SMP-gum arabic and SPI-lactose. They concluded that trehalose and sucrose exhibited poor results, whereas lactose offered very good protection. Maltodextrin was acceptable when combined with SPI but not as effective when used alone or in combination with SMP.

Similar to the findings in this study, their study proposed that pure proteins or pure carbohydrates were not effective protectants for cells during storage as compared to when they were used in the form of combinations. They had also studied the effect of increased a_w and the presence of oxygen on storage stability. It was confirmed that at a_w = 0.33 (33% RH), viable cell counts decreased to below 0.1% in only two weeks of time whereas at a_w = 0.54 absolute reduction in viable counts was observed within a few days. Use of oxygen scavengers or packaging under vacuum did not improve viability when a_w was not reduced but were helpful only when a_w was maintained at a level which yielded sufficiently high glass transition temperature, preventing the powders from caking. Teixeira *et al*. (1995) also reported that an optimum a_w between 0.11 and 0.23 prevented cell death during storage, and higher values were related to accelerated mortality of *L. delbruekii* cells.

7.2.2 Scanning electron microscopic (SEM) examination of the fresh and desiccated LR6 cells at the beginning and end of the storage period

Electron microscopy has been found as a very useful tool in revealing the direct damage on the bacterial envelope such as breakdown of the osmoregulatory capacity and changes in the intracellular DNA region caused by external physical stress or chemical actions (Hartmann *et al*., 2010).

Freshly harvested cells were found to be with smooth surface, undamaged membrane and of about 1.5 µm length (Fig 7.3a).

(a)

(b) **(c)**

(d) **(e)**
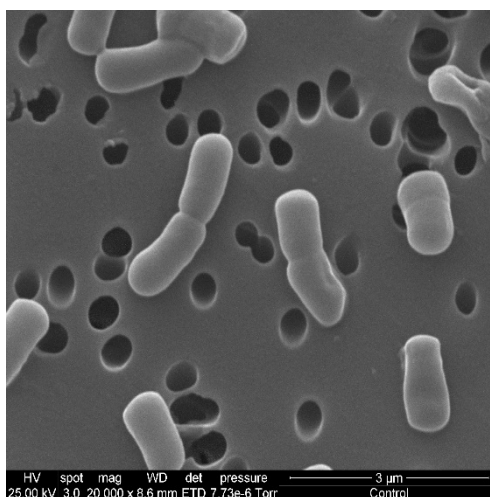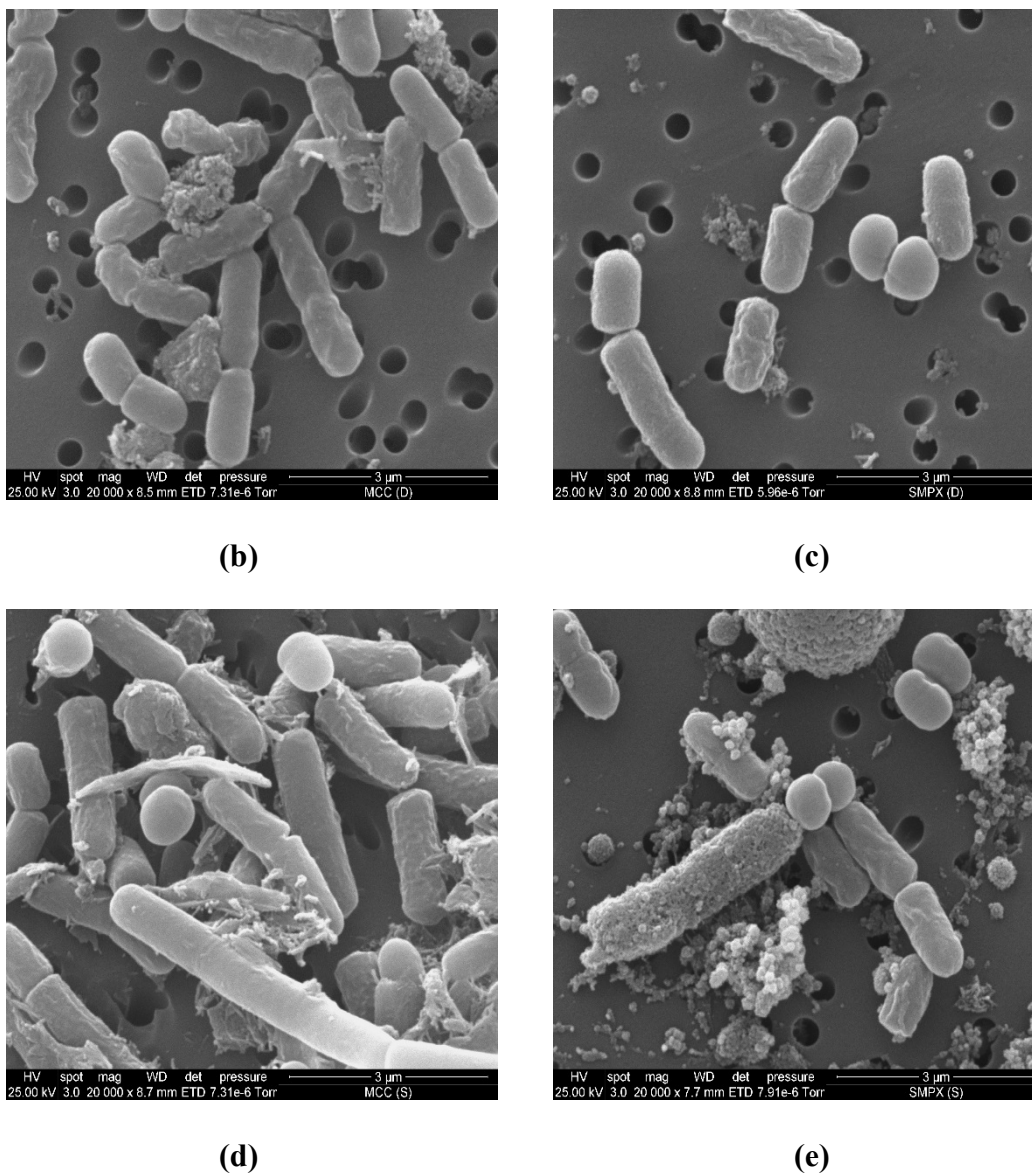
Figure 7.3: *SEM images of the surface morphology of freshly harvested LR6 cells (a) compared with cells supported with MCC (b), coated with xylitol and supported with SMP (c). Also showing the isolated cells from the same samples after the storage period in figures (d) and (e) respectively*

Desiccated cells along with MCC showed a corrugated surface with few dimple type structures (Fig. 7.3b). About 20% of the cells had a smooth surface similar to that of fresh cells. However, cells coated with xylitol and dried along with SMP showed similar deformations on the surface but to a lower extent (Fig. 7.3c). About 35% of the cells

were found to be unaffected. No visible reduction of the cell size was noticed in either of the dried samples. The rehydration step in the cell isolation process from the dried matrix did not improve the surface wrinkles indicating those changes were permanent in nature.

Such corrugated surfaces with dimples and blisters as seen in the desiccated samples were reported by Hartmann *et al*. (2010) when *S. aureus* cells were treated with antimicrobial peptides. They suggested that if the outer membrane is destabilized by some external means, in this case, heating due to desiccation process, that leads to local disruption of the inner membrane and the cytoplasmic material starts filling the periplasmic space causing blisters or corrugated type deformations without completely rapturing the outer membrane. They have also found a direct correlation of such deformations with cell death confirming this study where maximum and minimum reductions in viability of the LR6 cells immediately after desiccation were recorded in the MCC and SMP(X) samples respectively. Santivarangkna *et al*. (2006) used atomic force microscopic images to examine the cell envelopes of vacuum dried *L. helveticus* cells. Similar type of wrinkled surface with some cracks was reported and found to be well correlated with lysis of cells, loss of cell integrity, metabolic activities and residual viability. Gilbert *et al*. (1990) observed minor damages to the cell membranes occurring during the harvesting process through high-speed centrifugation which causes shear stresses. According to Wyber *et al*. (1994) and Champagne *et al*. (1991) such sub-lethally injured cells can become more susceptible to lose in viability during secondary stresses such as drying.

After the storage period when the cells were isolated from the same samples, a larger degree of damage in terms of the number of affected cells and visual quality of wrinkles were noticed. Almost all the cells dried with MCC developed further membrane deformations during the storage period (Fig. 7.3d) whereas a few cells were found to retain smooth surface alike fresh cells when coated with xylitol and dried with SMP (Fig. 7.3e). According to Sikkema *et al*. (1994), these morphological alterations could have occurred due to aberrations in membrane lipid composition, altered membrane fluidity and/or membrane integrity resulting in cell wall lysis and loss of intracellular dense material.

7.2.3 Transmission electron microscopic examination of the fresh and desiccated LR6 cells

Figure 7.4 shows the LR6 cell bodies when viewed under transmission electron microscopy. Freshly harvested cells (Fig. 7.4a) showed normal shapes with no damage in the inner membrane structure. An almost intact but slightly waved outer membrane structure was observed. The thin periplasmic space showed uniform appearance.
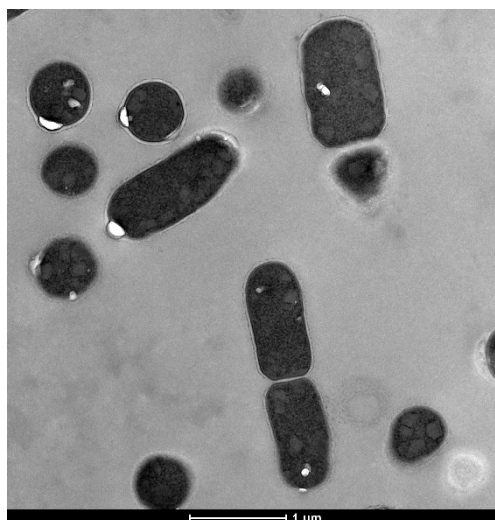
(a)
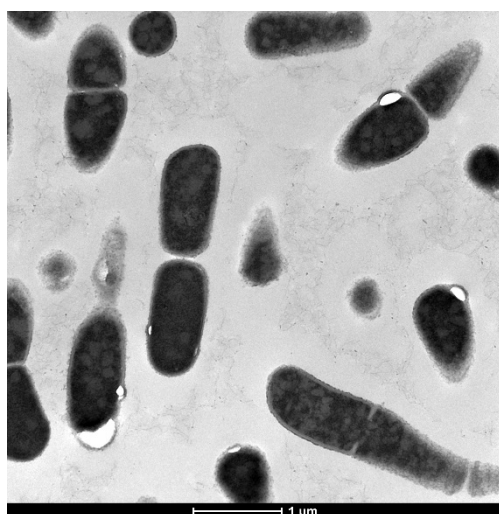
(b)
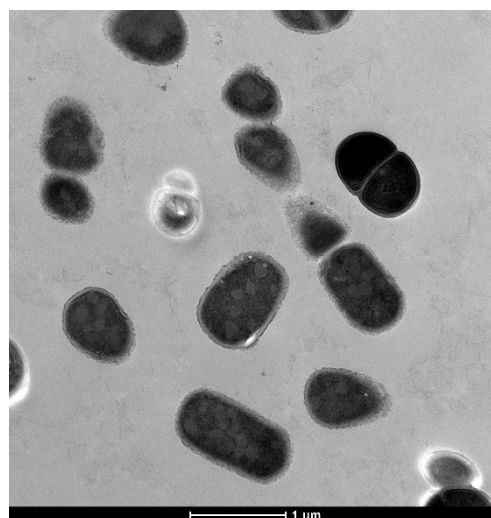
(c)

Figure 7.4 TEM *images of freshly harvested LR6 cells (a) compared with cells supported with MCC (b), coated with xylitol and supported with SMP (c)*

Most of the xylitol coated desiccated cells supported by SMP captured in the image presented in Fig. 7.4 (c) showed a minor increase in the electron density of the cytosol, seen as grey spot like shapes (Hartmann *et al*., 2010). The periplasmic place appeared hyperhydrated but with intact inner and outer membranes. When the cells were

desiccated in presence of MCC, almost all the cells captured in the sample image (Fig. 7.4 b) showed much larger electron density, as evident from the lighter shades (i.e., more grey spots). The blurred periplasmic spaces indicated clear damages in the cell membranes in the form of faded boundaries, indicating structural alterations as confirmed by FTIR studies reported earlier. A few lysed cells were noticed in both the desiccated samples which were not present in the freshly harvested cell sample. It was suggested that the increased electron density of the cytosol could be an indication of the reduced osmoregulatory capability of the cells which prevent further propagation when rehydrated (Hartmann *et al.*, 2010). Reduction in osmoregulation ability causes inflow or outflow of water within the cell bodies depending upon the external liquid media in which the cells are exposed (in this case peptone water was used for rehydration). This is reflected in the increase or decrease in electron density of the cytosol (the grey spots) (Hartmann *et al.*, 2010).

7.2.4 Principal component analysis of the FTIR spectra of the LR6 cells isolated at the beginning and end of the storage period

PCA of the spectral data showed that 85.6% of the total variations among the samples stored at a_w 0.11 were captured by the first two principal components, which can be considered to be satisfactory. The MPC samples, both before and after storage, were
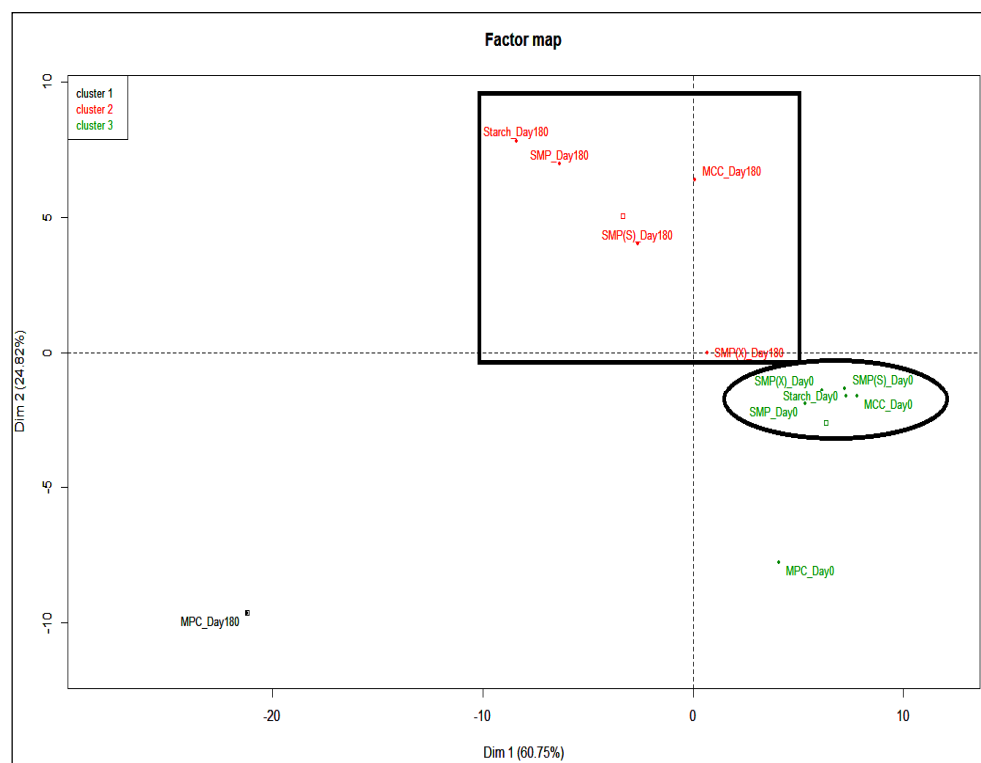
Figure 7.5: *Factor map showing principal component analysis (PCA) of the amide zone spectra of the cells stored at a_w 0.11*

substantially different from the rest of the group. This is reflected in the factor map (Fig. 7.5), in which both samples (codes MPC_Day0 and MPC_Day180) were found to be outliers, the former being placed separately within the green cluster (all day 0 samples) and the latter not being a part of any cluster.

This difference observed in the case of these two outliers was probably because of a limitation of the cell isolation protocol, in which the cells were separated from the dried MPC matrix by the two-stage gravitational separation process. The supernatant was collected in the first stage, with the expectation of it containing no insoluble compounds that would contaminate the samples in the second-stage high-speed centrifugation, which was intended to isolate the pure cell bodies only. If the aqueous suspension of the

MPC samples contained any suspended and denatured milk protein fractions that were not precipitated out by gravity, there was the risk that these protein fractions would contaminate the cell bodies during the second-stage high-speed centrifugation. This was probably true for the SMP group samples as well, but to a lesser extent because of the relatively lower protein content of SMP.

The other samples were broadly grouped (within the rectangle and the ellipse) according to the storage time, i.e. the spectra of all samples at the beginning of storage were dissimilar to the spectra obtained for the same samples at the end of storage. This indicated a substantial alteration in the secondary structures of the cell envelope proteins during the storage period.

The hierarchical clustering map in Fig. 7.6 showed the heterogeneity among individual samples within a cluster, which was useful in explaining the differences in viable cell counts in different samples and whether these differences were directly correlated with protein structural alterations.
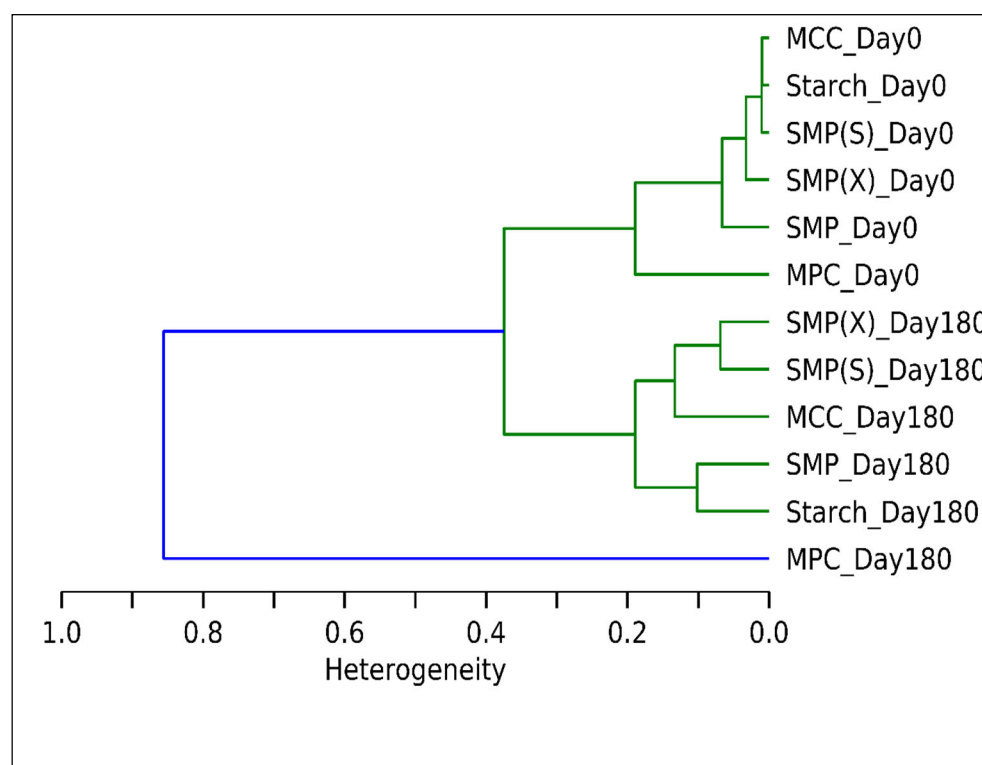
Figure 7.6: *Hierarchical cluster map showing heterogeneity of the amide zone spectra of the cells stored at a$_w$ 0.11*

The broad cluster (lower half) containing the stored samples included two small subclusters each with two samples (Fig. 7.6). The sorbitol- and xylitol-coated cells (SMP(S)_Day180 and SMP(X)_Day180) were grouped together, indicating maximum similarity between them, and the cells carried by corn starch and SMP formed another subcluster. Cells stabilized with MPC or MCC did not group with any other sample, indicating maximum dissimilarity in their secondary protein structures to those of the other group samples.

These results correlated well with the residual viable cell counts in these samples after 180 days of storage at 37 °C and a$_w$ 0.11 (Fig. 7.1). The LR6 cells were best protected in the sorbitol- and xylitol-coated samples and the clustering of these two sample spectra

indicated the similarity in their secondary protein structures at the end of storage. The maximum reduction in cell viability was observed in the corn starch and SMP samples, which also formed a cluster. The MCC and MPC samples were moderately affected by the long duration, non-refrigerated storage and were not part of any cluster.

Storage at a_w 0.30 resulted in a further loss in cell viability in all samples, compared with storage at a_w 0.11 (Fig. 7.2). This was reflected in the factor map in Fig. 7.7, in which all the day 0 samples were clustered (within the circle) on the left-hand side and showed considerable distance in the horizontal dimension (representing approximately 62% of the total variance) from the other two clusters (within the ellipse and the rectangle).
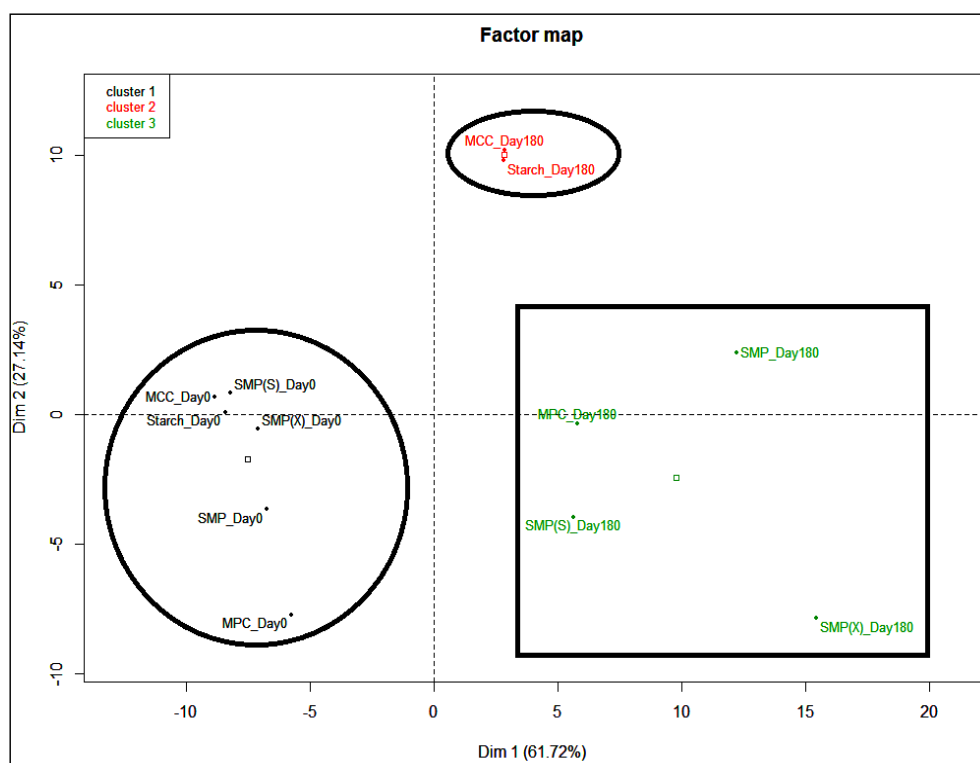
Figure 7.7: *Factor map showing principal component analysis (PCA) of the amide zone spectra of the cells stored at a_w 0.30*

The rectangle cluster was scattered, which represented the moderately affected cells in the four samples, as was shown in Fig. 7.2. The cluster within the ellipse contained the maximum affected cells carried by MCC and corn starch. This cluster was displaced by a small distance vertically from the rectangle cluster, indicating minor differences from other stored samples. As the vertical scale represented only approximately 27% of the total variance, it can be considered to be minor. The distances between samples within the circled cluster (day 0 samples) and those within the ellipse (day 180 samples) were substantial in both vertical and horizontal directions, indicating significant alterations of the secondary protein structures in the cells carried by MCC and corn starch during the storage period.

The cluster map for the samples stored at a_w 0.30 in Fig. 7.8 shows three subclusters within the stored samples. These subclusters consisted of (1) the worst affected group, containing MCC and corn starch samples; (2) cells carried by MPC and sorbitol-coated cells carried by SMP; (3) cells carried by SMP only and the xylitol-coated cells carried by SMP. As the last four samples [(2) and (3)] contained almost identical levels of residual viable cells (range 2.2–2.35 log cfu/g), these two subclusters did not reveal any
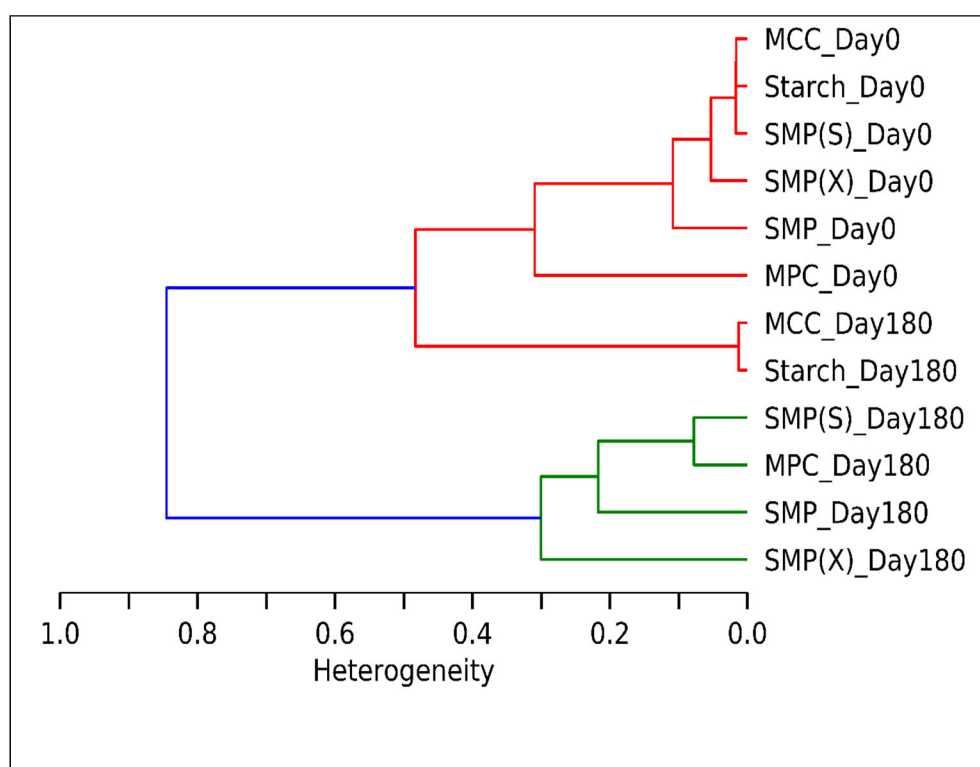
Figure 7.8: *Hierarchical cluster map showing heterogeneity of the amide zone spectra of the cells stored at a_w 0.30*

additional information in explaining the role of these carrier excipients as the protectant during storage. All the day 0 samples were grouped together at the bottom part of the cluster map. This suggests that other factors might need to be included to correctly predict cell viability in a relatively high a_w storage environment.

7.2.5 Role of glassy/rubbery state of the stabilizing matrix on the viability of LR6 cells during storage

The FTIR analysis detected the deformations took place in the secondary protein structures of the LR6 cell envelopes and such deformations were found to be well correlated with residual cell viability at the beginning and end of the storage period. It could not be revealed from this analysis if such deformations were minor in nature or resulted in thermodynamic destabilization and thereby unfolding of the proteins. When the same samples were adjusted to two different a_w levels and stored under similar conditions, a major difference anticipated in the physical properties of the samples was the glassy state at which the cell bodies were adhered to the carrying agents. It was hypothesized that a living organism, when embedded in a glassy matrix during dehydration, supported better storage stability (Bruni & Leopold, 1991; Sun & Leopold, 1997). To confirm this, Oldenhof *et al*. (2005) spray dried lactobacilli cells and found improved survival during ambient storage when carried by a 50:50 mixture of maltodextrin and sucrose. It was confirmed that maltodextrin caused strengthening of the glassy matrix which correlated well with the survival statistics. Such glassy structure in the external environment is thought to offer an effective barrier against oxygen permeability and also helpful in restricting the molecular mobility (Ananta *et al*., 2005).

Each thermogram presented in Fig. 7.9 displays a single observable glass transition (T_g) phase as detected by the change in slope when the samples were heated from 20 ˚C to 120 ˚C at a consistent rate.

7.9 (a)
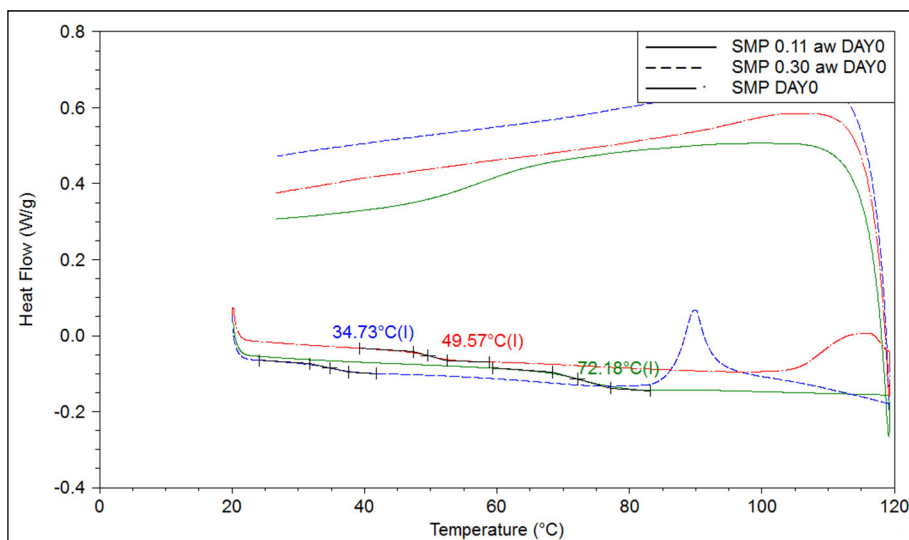
7.9 (b)
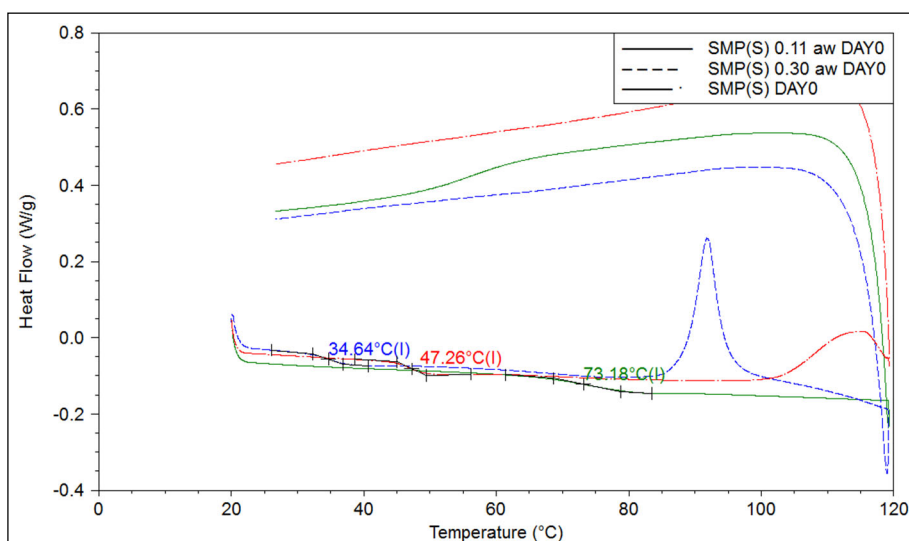
7.9 (c)
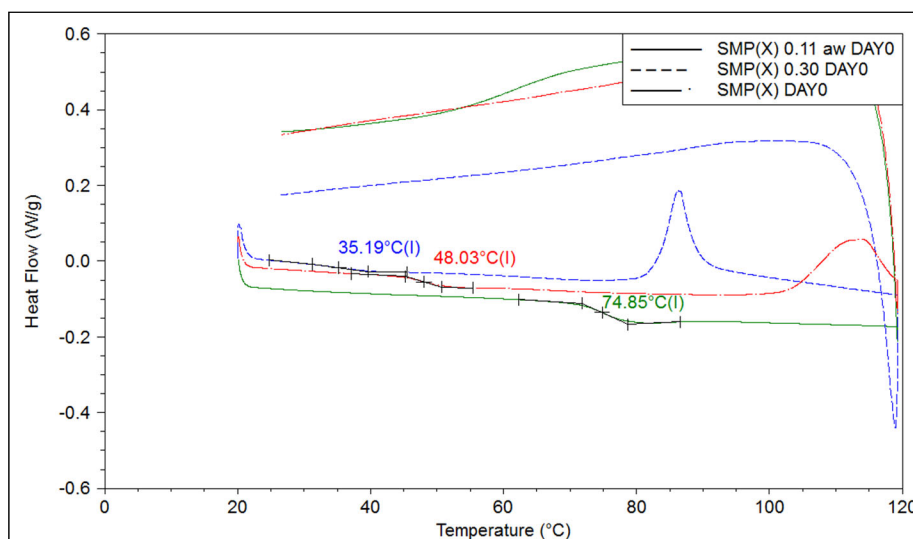
Figure 7.9: *Representative DSC thermograms showing glass transition temperatures at the beginning of the storage trial for the LR6 cells stabilized with only SMP (a), SMP with sorbitol (b) and SMP with xylitol (c). Each figure displays three thermograms for the same sample after FBD drying and after adjustments of the a_w at 0.11 and 0.30 levels*

Immediately after the drying operation, when the a_w values of the samples were closer to 0.20 to 0.23, the T_g was found to be 49.6 °C for the SMP and were slightly reduced to 47.3 °C and 48 °C for the sorbitol and xylitol containing samples respectively. When the a_w values of the samples were adjusted to 0.30, the T_g for all the samples was depressed to around 35 °C, which was lower than the storage temperature during the stability trial. This indicates these samples were not in a glassy state during the course of the trial. On the other end, when the a_w was adjusted to 0.11, the T_g for all the samples was shifted upwards closer to 72-75 °C, indicating the samples in a strong glassy physical state during the storage period.
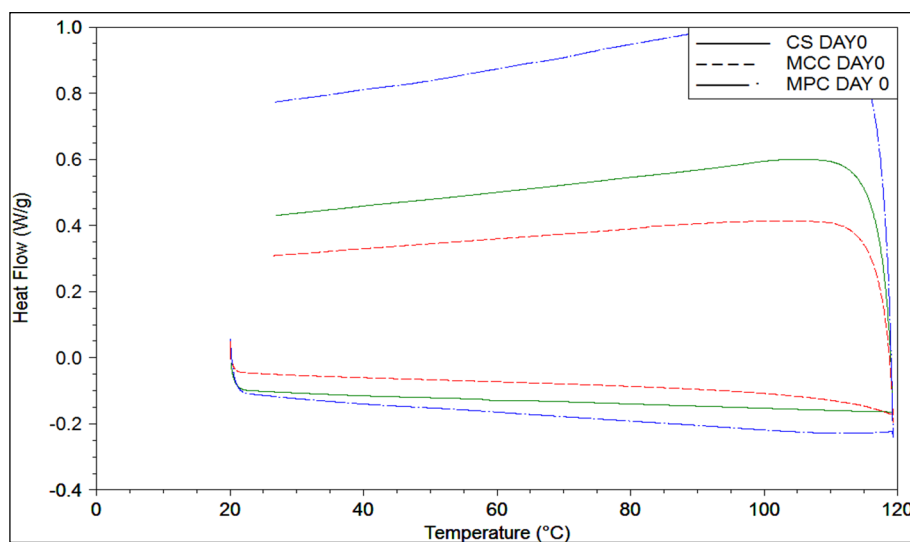
Figure 7.10: *DSC thermograms of the LR6 cells stabilized with corn starch (green), MCC (red) and MPC (blue)*

The thermograms shown in Fig. 7.10 represent the samples made with corn starch, MCC and MPC. No detectable slope change in the measured temperature range was observed in any of them. Therefore, it is hard to speculate if the LR6 cells were at all trapped into any kind of strong or weak glassy matrix in these samples during the storage period. From the stability results shown earlier in Figs. 7.1 and 7.2, it can be seen that the overall performance of these compounds in terms of storage stability at low as well as the high a_w environment was poorer compared to the SMP group. This could be correlated with the absence of any strong glassy state in these samples, as found by the DSC analysis.

The role of a suitable stabilizing agent is extremely important for protecting the cell envelope proteins during dehydration and also to provide a strong glassy matrix for long term storage stability (Remmele *et al*., 1997; Allison *et al*., 1999). A popular choice has been the common disaccharides, such as sucrose and trehalose, for such purpose because

these compounds in the amorphous state are able to form hydrogen bonds with the proteins by replacing the water molecules which become excluded from the system during dehydration (Allison *et al.*, 1999). Although the formation of a glassy matrix by these sugars is an important requirement to protect the proteins during the dehydration step, for ensuring long term storage stability the glass transition must take place at a temperature much higher than the storage temperature of the proteins (Garzon-Rodriguez *et al.*, 2003). In Fig 7.9, an upshift of the T_g for all the samples (irrespective of the sorbitol or xylitol coating over the cells) from ~48 ˚C to ~73 ˚C was noticed when the a_w was lowered from ~0.23 to 0.11 level. The high differential between the storage temperature (37 ˚C) and the T_g was probably an important factor responsible for the superior stability of LR6 cells in these samples. On the other hand, the T_g was depressed to ~35 ˚C (lower than storage temperature) when the same samples were adjusted to a higher a_w of 0.30 which indicates the LR6 cells in these samples were not entrapped into a glassy matrix. The rubbery state of the stabilizing agent might have facilitated easy moisture migration to and from the protein molecules (Ananta *et al.*, 2005) resulting into the deformation in their secondary structures as evident from the FTIR analysis reported earlier.

7.2.6 Changes in T_g during the storage period

Figure 7.11 (a to f) shows the DSC thermograms of the same samples presented in figures 7.9 and 7.10 after storage at 37 °C for 121 days. For each sample, the measured T_g was slightly lower than what was recorded at the beginning. The depression of T_g

7.11 (a)
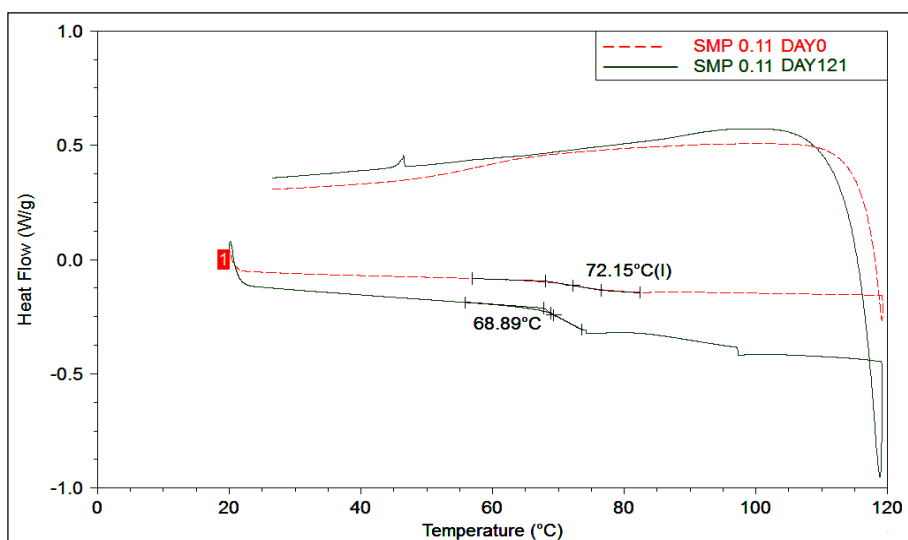
7.11 (b)
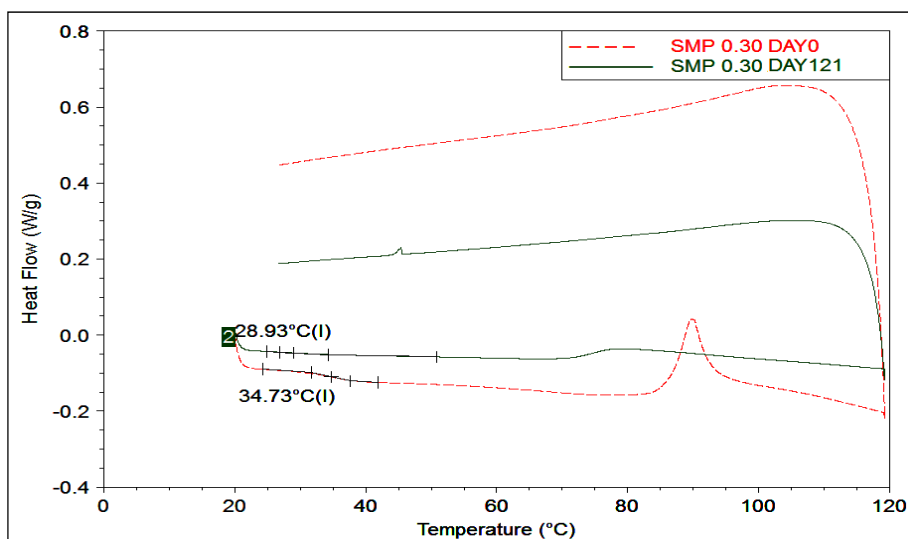
for the samples maintained at a_w 0.11 ranged from 3.3 °C to 7.1 °C and the same for the samples maintained at a_w 0.30 ranged from 5.8 °C to 8.5 °C.

7.11 (c)
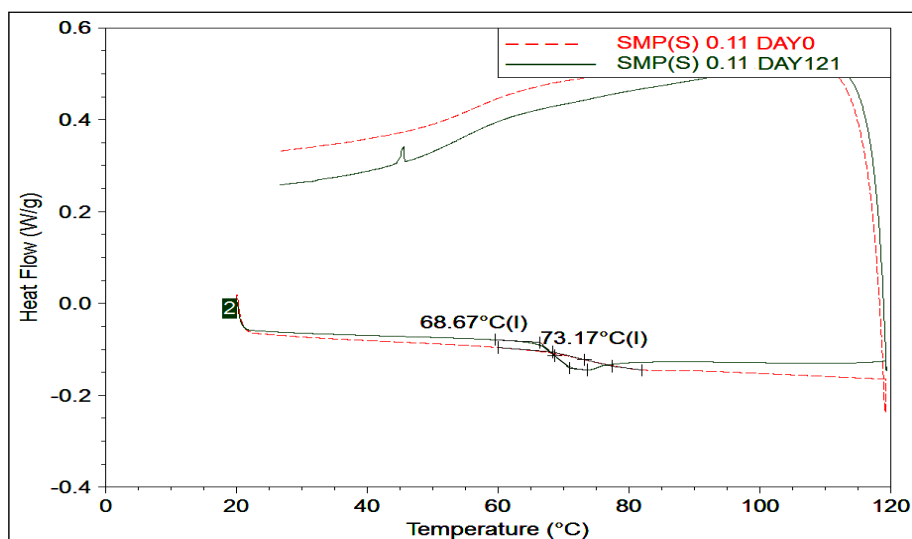
7.11 (d)
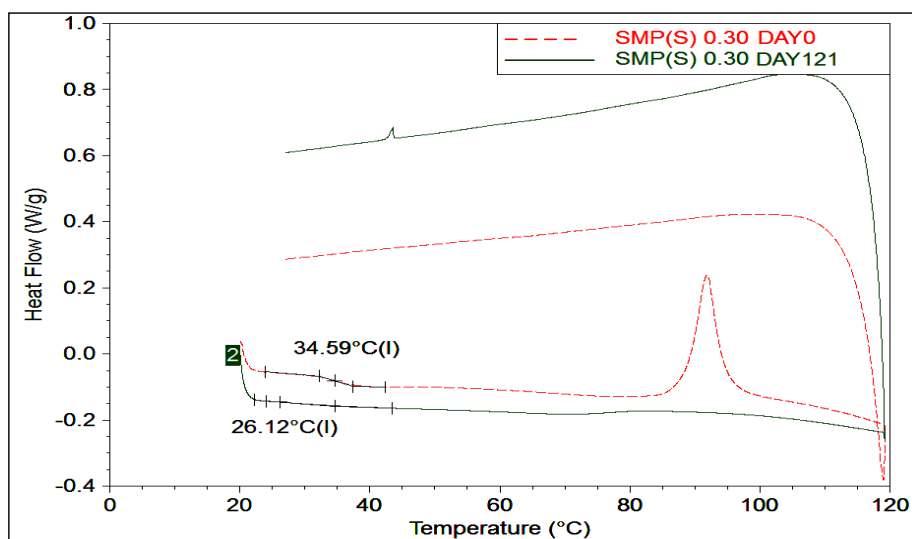
Lapsiri *et al.* (2013) found a direct relationship between the stability of spray-dried *L. plantarum* cells and the storage temperatures as well as with the relative humidity which in effect control the a_w of the stabilization matrix. They observed diminishing stability

7.11 (e)
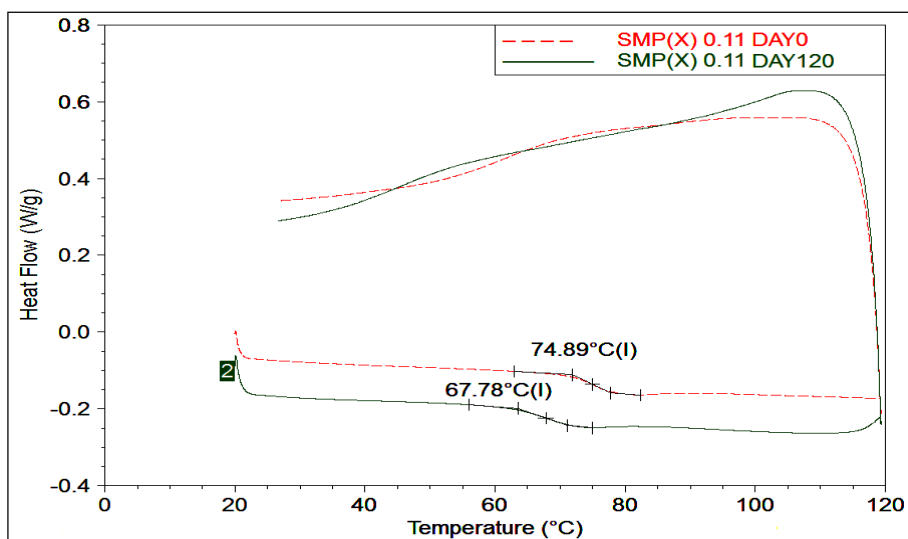
7.11 (f)
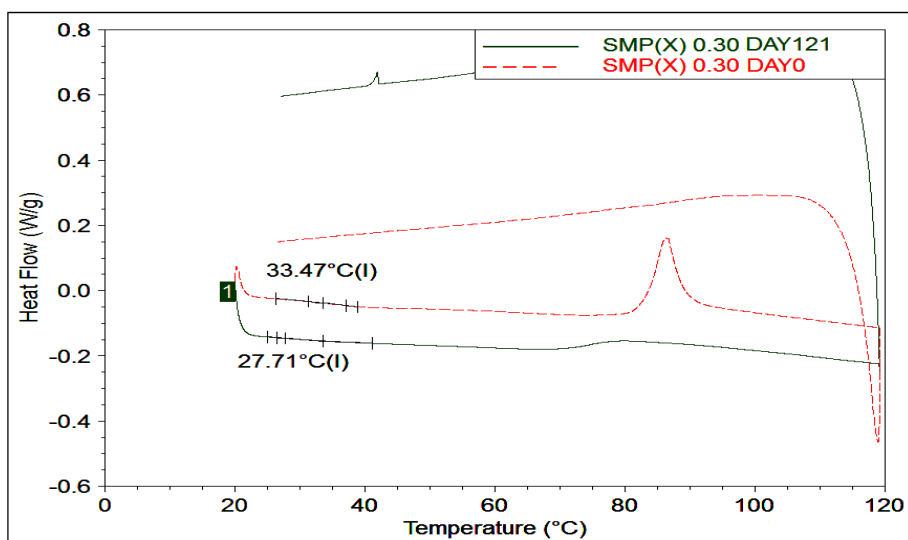
Fig. 7.11: *DSC thermograms showing changes in T_g during storage at 37 ˚C for samples stored at a_w 0.11 and 0.30*

with increased storage temperatures and relative humidity and concluded that these two factors influence the T_g of the samples and consequently the degree of protection available to the viable cells. Increasing storage temperature at a constant RH of 33% was found to be aiding in lowering the T_g probably because of the ease of water mobility

within the matrix (Bhandari and Adhikari, 2009). Water was found to be an effective plasticizer (Shrestha *et al.*, 2007) which when gradually adsorbed by the matrix constituents at high temperature, helped in lowering the T_g of the particles (Pehkonen *et al.*, 2008). This observation was found to be in agreement with the results shown in Figs. 7.9 and 7.11 where a decrease of T_g was recorded with increasing a_w of the matrix and also during the storage period at 37°C.

7.2.7 Visual inspection of the Amide 1 regions of the FTIR spectra

As shown in Figs. 7.1 and 7.2, comparatively superior storage stability for the LR6 cells were recorded for the sorbitol or xylitol coated cells which were carried by SMP and relatively poorer stability was observed in the MCC carried cell samples. A further look at the amide 1 bands of particularly these samples revealed some alterations which may have taken place during the storage period (Fig. 7.12 and 7.13) and accordingly may be attributed to the extent of cell survivability. Amide 1 region of the FTIR spectrum of any protein containing sample is dominated by a band at wavenumber 1656 cm^{-1} representing α-helix structures. To find out if any alterations in the secondary structures of the cell envelope proteins are taking place during storage, the FTIR spectra of the amide 1 region as recorded at the beginning and end of the trial period were compared. Figs. 7.12 (a) for sorbitol and 7.12 (b) for xylitol containing samples did not detect any such alteration. The spectral peaks were identified to be located at ~1651 cm^{-1}

(a)
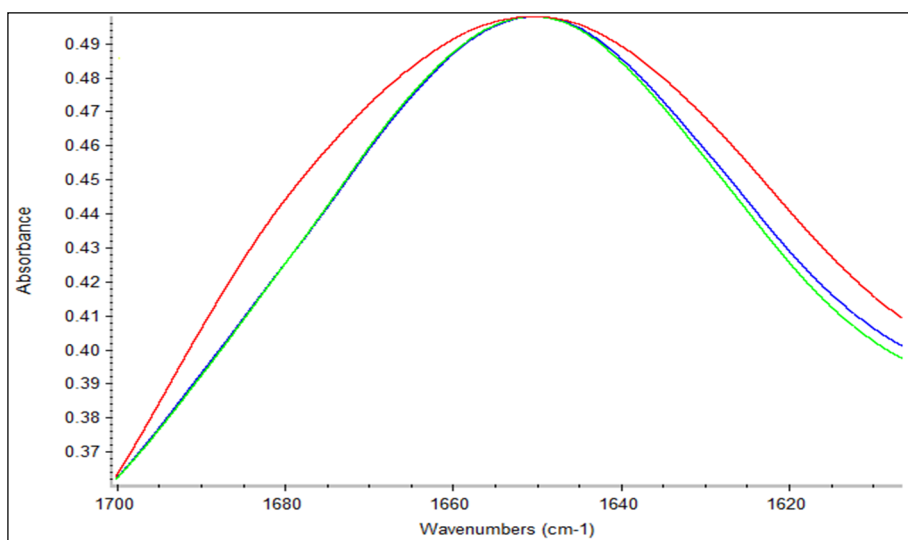
(b)
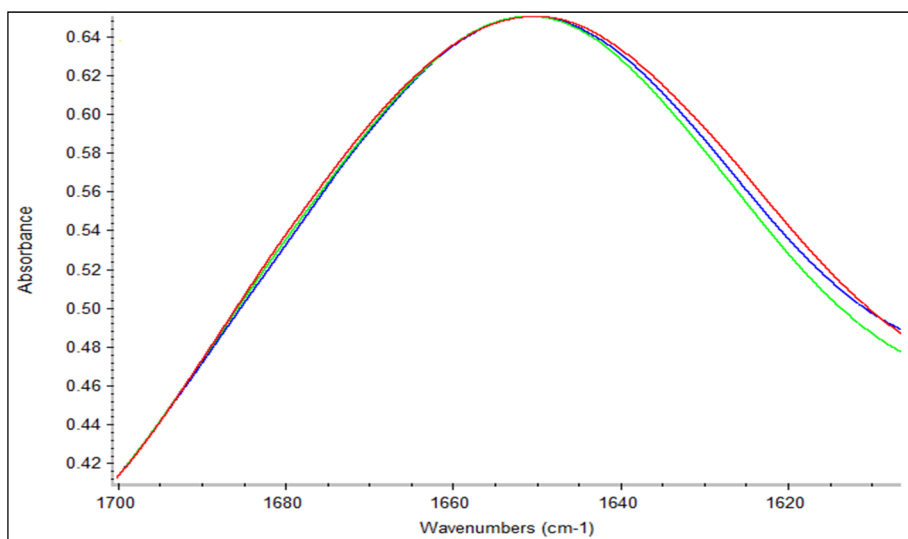
(b)

Figure 7.12: *Area normalized, baseline adjusted Amide 1 spectra of sorbitol (a) and xylitol (b) coated LR6 cells carried by SMP at the beginning (blue), after 24 weeks of storage at a_w 0.11 (green) and at 0.30 (red)*

wavenumber for both types of samples and remained constant throughout the storage period. The adjustments in a_w at two different levels did not seem to have any impact on the structural integrity. When the amide 1 region of the cells isolated from the MCC samples was compared (Fig. 7.13), distinctive shifts in the spectral peaks were noticed in the samples stored for 24 weeks at 37 ˚C. After dehydration i.e, at the beginning of storage, the amide 1 peak was noticed at wavenumber 1636 cm^{-1} which indicated a perturbation in secondary structures with a partial loss in native α-helix content and an increase in non-native β-sheet structures (Garzon-Rodriguez *et al.*, 2003). At the end of the storage trial, this peak was shifted to ~1647 cm^{-1} for both the low and high a_w environments. Apart from this peak shift, the stored samples also exhibited significant changes in the absorbance levels near wavenumber 1620 cm^{-1} indicating the formation of protein aggregration and increase in the clevage products during the rehydration of
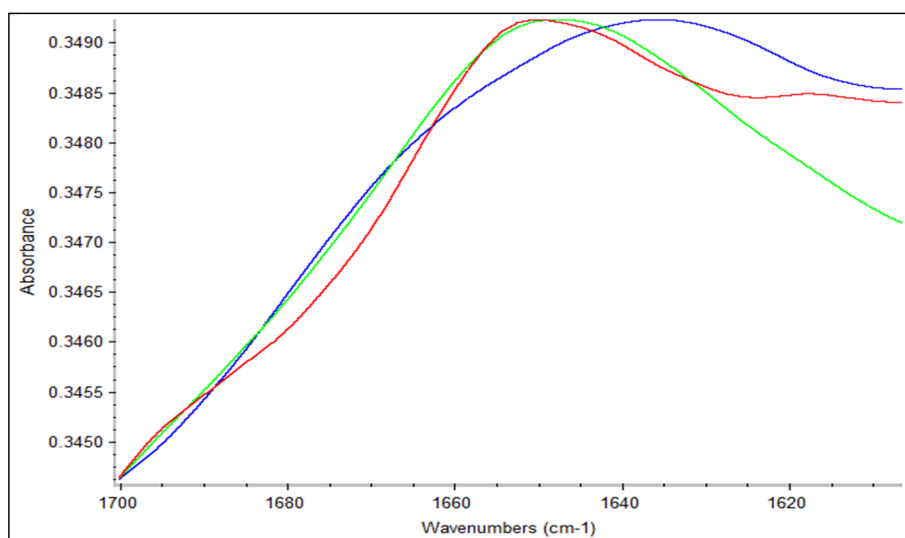
Figure 7.13: *Area normalized, baseline adjusted Amide 1 spectra of LR6 cells carried by MCC at the beginning (blue), after 24 weeks of storage at a_w 0.11 (green) and at 0.30 (red)*

the cells prior to FTIR analysis (Garzon-Rodriguez *et al.*, 2003). These structural perturbations were probably partially responsible for the relatively poorer storage stability of the LR6 cells recorded in the MCC carried samples. However, it should be kept in mind that although no such major alterations were detected for the best performing samples containing sorbitol or xylitol, still there were on average 1.4 (for a_w 0.11) to 2.2 (for a_w 0.30) log cfu/g reduction in viable cell populations in those samples during the storage period. This decay cannot be explained by the above analysis which prompts us to believe that there are other factors involved in the process of gradual decay in cell viability over prolonged storage at elevated temperatures and needs to be investigated further.

7.2.8 Effect of the degree of agglomeration of the stabilized matrix on the storage stability

Figs. 7.1 and 7.2 looked at the storage stability by analyzing a bulk sample of powders in which the bacterial cell distributions (both viable and non-viable) were expected to be non-homogenous. This is because of the wet granulation technique used in preparing the samples where the ratio of the powdered carrier agents to the bacterial cell mass was very high. A dry blending system might still be able to guarantee a uniform mixing cum distribution of the minor components if all the components are free flowing in nature. However that was not the case in the present experiment.

In the wet granulation process used, when ~5 g of the wet biomass was mixed to the 200 g carrier agents, particularly because of the sticky nature of them, the uniform distribution of the bacterial cells throughout the matrix could not be ensured. From physical inspection as well as from analyzing the particle size distribution in the Malvern Mastersizer (Fig. 4.4, Chapter 4), it could be seen that a wide distribution of particle sizes after the drying was present. Since it is of interest examining the protection available to the bacterial cell membranes through protein stabilization coming from the carrier agents, it became important to find out if the efficiency of the agglomeration process has a role to play in this regard. Therefore, the bulk powder sample was segregated into 4 fractions based on average particle diameters using a sieving process.

It was found that the maximum mass fraction (81.2%) was passed through the 250μ sieve (Table 7.1). This was followed by 13.2% of the sample mass which passed through the 500μ sieve but was retained by the 250μ sieve. A minor portion of 3.2% had mean diameters between 500μ to 1000μ and only 2.4% of the powders was retained by the 1000μ sieve. These fractions were then analyzed for the viable lactobacilli counts at day 0 and stored at 37 °C but without any adjustment in a_w. This is because the importance of low a_w matrix in offering superior storage stability was already established in the previous storage study (Figs. 7.1 and 7.2) whereas in this experiment the objective was to investigate the effect agglomeration on the same. Table 7.1 shows that the degree of agglomeration had a very important role in offering physical protections to the LR6 cells during the desiccation process. The viable cell populations in the higher particle size groups (above 500μ and 1000μ) were between 9.5 to 9.9 log cfu/g whereas the same for the lower particle size (below 500μ but above 250μ) group was only 7.8 log cfu/g. The

minimum viable cell concentration was recorded (7.3 log cfu/g) in the finer particles having less than 250µm diameter but having the maximum mass fraction. However, at the same time, it is not clear if the higher concentration of residual viable cells recorded within the larger granules is solely because of the physical protection obtained or because of the higher distribution of the cells into these granules during the mixing/granulation process itself. Since bigger granules are formed when more liquid or wet biomass is available to the surrounding powder particles, it can be naturally assumed that the cell distribution throughout the entire matrix was never uniform but was skewed towards the coarse granules being formed.

To understand this process in a better way, the storage stability test was important. If the physical protection in the form of bigger sized granules was not a contributing factor then the decline in viability during storage should have been uniform for all the sample
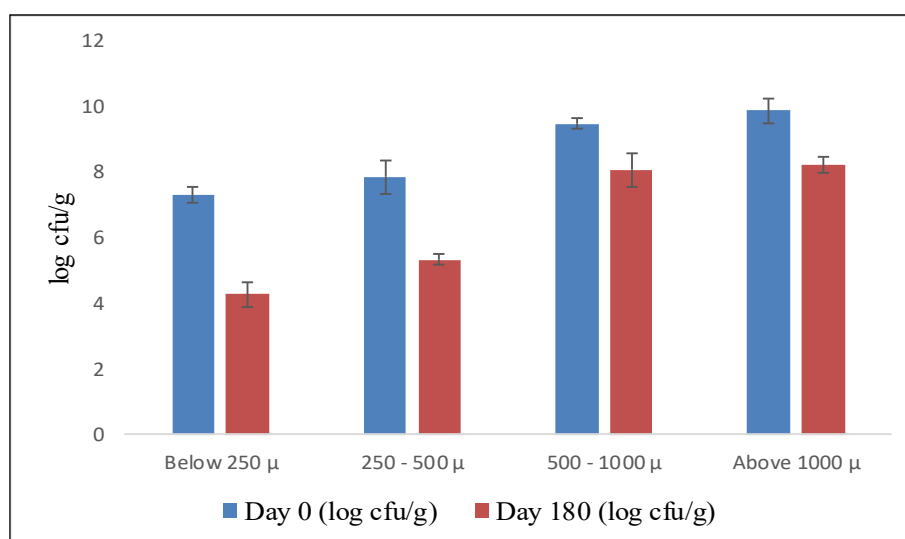
Fig. 7.14: *Comparison of viability loss during storage among the stabilized LR6 samples having different granule sizes*

groups. However, that was not the case and after 180 days of storage at 37 °C it was found that the bacterial calls adhered to the finest particles suffered maximum loss in viability (41.4%) whereas the minimum loss (14.9%) was within the particles with average diameter above 500µm (Figure 7.14, Table 7.1). This indicates that the higher population of live cells recorded in the bigger sized granules after desiccation as well as after the storage period was a result of the physical protection obtained by the granulation process and not only because of the higher cell distributions in those granules.

This view is in agreement with the findings from the FTIR study on the cells post desiccation (Chapter 6) and post-storage (Section 7.3.5) where the role of the supporting agents in preserving the membrane protein structures was found directly correlated with the residual cell viability. It can be assumed that within the relatively bigger sized granules, the bacterial cells were better surrounded and covered by the SMP particles whereas on the extreme another end, the cells were merely adhered to the single powder particles making them more exposed to the stressful environment during desiccation and storage. There have been many efforts in stabilizing probiotic bacteria into different matrices and physical characterization of the same. Particle size analysis was a common investigation in these studies, however, the effect of different sized particles on storage stability was not performed (Ding & Shah, 2009; Anekella & Orsat, 2014; Yonekura *et al*., 2014). This was probably due to the spray drying process used in these studies which generally yields very uniform particle size distributions with small variability. No previous work has been found which used a wet granulation process to generate bigger agglomerates exactly as done in this experiment. Also, no such previous work has been

found looking into the effect of particle sizes on storage stability. A recent work by Park *et al*. (2016) demonstrated that probiotics-loaded pellets in a tablet form offered superior storage stability under ambient conditions and concluded that the improvement was due to the physical barriers in the form of pellets/tablets irrespective of the carrier agents used by them.

Table 7.1 shows 81% mass fraction of the sample was of average diameter below 250µm and this fraction contained the lowest vible cell population after the stabilization as well as after the storage. Because of the maximum weightage contributed by this group to the overall sample mass, the average population of viable cells in the overall

Table 7.1: *Summary data for the results shown in Fig. 7.14*

Particle size Range	Weight (g)	Mass Fraction (%)	Viable cells Day 0 (log cfu/g)	Viable cells Day 180 (log cfu/g)	Loss in Viability (%)
Below 250 µ	203	81.2	7.3 ± 0.24	4.28 ± 0.38	41.37
250 - 500 µ	33	13.2	7.85 ± 0.51	5.34 ± 0.19	31.97
500 - 1000 µ	8	3.2	9.48 ± 0.17	8.07 ± 0.48	14.87
Above 1000 µ	6	2.4	9.88 ± 0.38	8.22 ± 0.27	16.80

sample was substantially lower. This was undesirable, hence to ensure higher viable cell population at the end of the storage period, the granulation efficiency must be improved. Apparently, this could be achieved by adding more probiotic slurries into a given powdered mass which would aid binding these residual smaller particles into bigger

agglomerates. However, this has been found to be of practical difficulty because increasing on the slurry volume has been found to cause bigger chunks particularly with the SMP based samples which are difficult to break and form smaller granules. Accordingly, the process was optimized to add only enough quantity of probiotic slurry to obtain a moisture content of 11-13% in the wet granulated mass. A probable solution to this without adding additional slurry could be to recycle the fines (below 250µ size) back to the process by segregating them using a sieve and putting back into the next batch. However, the effect of such repeated re-wetting and drying process on the bacterial cell viability post desiccation and storage is unknown. No published work on this effort has been found in the literature and is being recommended.

7.3 Conclusions

This study confirmed the importance of maintaining a lower a_w of the stabilization mix when enhanced temperature storage is required. However, the stability in a high a_w environment was not very encouraging. Therefore, the challenge to the food industry in delivering stabilized probiotics at around a_w 0.30 remains and more research in finding a solution is recommended. The useful correlation that was found between the alteration in secondary protein structures (as obtained from the FTIR analysis) and the loss in cell viability is novel. This is one of the few studies that has investigated the storage of probiotics at 37 °C, which is very often the ambient temperature in countries with tropical weather; hence, the importance of shelf-stable probiotic products at this temperature is paramount.

Chapter 8.0. Effect of stabilization and enhanced temperature storage on the probiotic attributes (*in vitro*) of the *Lactobacillus reuteri* LR6 cells

8.1 Introduction

According to De-Vries *et al*. (2006) probiotic bacteria should possess certain common characteristics such as the ability to favorably alter the intestinal microflora, inhibit the growth of harmful pathogens, proven safety for the host etc. but most importantly, should be resistant to the stresses encountered in the gastro-intestinal (GI) tract. The survival and subsequent colonization in the digestive tract are considered to be very important to impart the physiological functions expected from a probiotic strain. Such properties have always received special attractions while selecting new probiotics for human consumption. The expected resistance in the GI tract is tested via the tolerance of a particular organism towards low pH and bile toxicity (Singh *et al*., 2014). Similarly, colonization ability is another important criterion for probiotic strain selection and to evaluate its robustness. This is tested in an *in vitro* model by evaluating the surface hydrophobicity and aggregation properties of a probiotic strain (Collado *et al*., 2007).

The *Lactobacillus reuteri* LR6 strain used in this study was earlier characterized and validated for its common probiotic properties by Singh *et al*. (2014). However, in that study, the freshly isolated strain from the human infant feces was grown in the selective media and no other processing similar to the ones used in this study for stabilization was

applied. Therefore, it was of high interest to verify if the most common probiotic attributes expected from this LR6 strain are retained after stabilization and high-temperature storage. Without this verification, it will probably not be possible to recommend the consumption of food products fortified with this probiotic strain. In the previous sections, with the help of FTIR spectroscopy and electron microscopy, it was found that stabilization and high-temperature storage caused substantial alterations in cell membrane protein structures and also physical damages in the form of corrugated surfaces with blister formations. Therefore, it was planned to isolate the freshly stabilized as well as stored LR6 cells from the dried matrices and compare with the freshly grown cells for acid tolerance, bile tolerance, and surface hydrophobicity properties.

It was important to test the particular cells which were subjected to the stabilization related processes and not their subsequent generations. Hence, no subculturing of the isolated cells were performed. The cell isolation protocol was the same as described in section 3.3.1. The fresh cells sample as control was prepared as per the method described in section 3.1.1. It was made sure that the initial viable cell populations in all the samples were close to each other by adjusting the dilutions where necessary. For acid and bile tolerance tests, the same protocol was followed as per sections 3.3.9 and 3.3.10 respectively by exposing the cells to simulated gastric and intestinal fluids (SGX and SIF). The only changes made in the protocol followed for this particular experiment was the use of three different pH levels in the SGF (pH 2.0, pH 3.0, pH 6.5) and three different bile salts concentration levels (0.5%, 1.0%, 2.0%) in the SIF. The method for testing surface hydrophobicity is described in section 3.3.9.

8.2 Results and discussion

8.2.1 Acid tolerance of the LR6 cells after desiccation and storage at 37°C for 12 weeks

Table 8.1 lists and Figure 8.1 summarizes the effect of desiccation (week 0) on the acid tolerance of LR6 cells at different pH levels. When incubated for 120 min at pH 2.0, the viable population in the freshly harvested cells gradually came down from 9.3 log cfu/ml to 5.5 log cfu/ml on average, resulting in a loss of 59%.

Table 8.1: *Changes in viable cell populations in the acid environment (2-h exposure at different pH levels) at week 0*

Sample	Acid tolerance (Week 0)											
	pH 2.0				pH 3.0				pH 6.5			
	0 min	60 min	90 min	120 min	0 min	60 min	90 min	120 min	0 min	60 min	90 min	120 min
Fresh cells	9.3 ± 0.6	7.6 ± 0.4	6.7 ± 0.7	5.5 ± 0.8	9.2 ± 0.6	8.4 ± 0.4	8.0 ± 0.5	8.1 ± 0.6	9.4 ± 0.2	9.3 ± 0.3	9.1 ± 0.6	9.5 ± 0.7
SMP	8.8 ± 0.3	6.1 ± 0.3	5.2 ± 0.4	4.1 ± 0.4	8.9 ± 0.2	8.2 ± 0.3	7.8 ± 0.1	7.4 ± 0.4	8.9 ± 0.2	8.9 ± 0.3	8.8 ± 0.2	9.1 ± 0.2
SMP + Sorbitol	8.8 ± 0.5	6.2 ± 0.4	5.3 ± 0.5	4.0 ± 0.3	8.7 ± 0.5	7.9 ± 0.1	7.2 ± 0.5	7.3 ± 0.5	8.7 ± 0.5	8.8 ± 0.3	8.8 ± 0.2	9.0 ± 0.3
SMP + Xylitol	8.9 ± 0.4	6.6 ± 0.1	5.3 ± 0.4	4.2 ± 0.4	8.7 ± 0.3	8.3 ± 0.3	7.5 ± 0.4	7.4 ± 0.3	8.7 ± 0.3	8.6 ± 0.4	8.8 ± 0.1	9.1 ± 0.2

Average values ± standard deviation (n = 3)
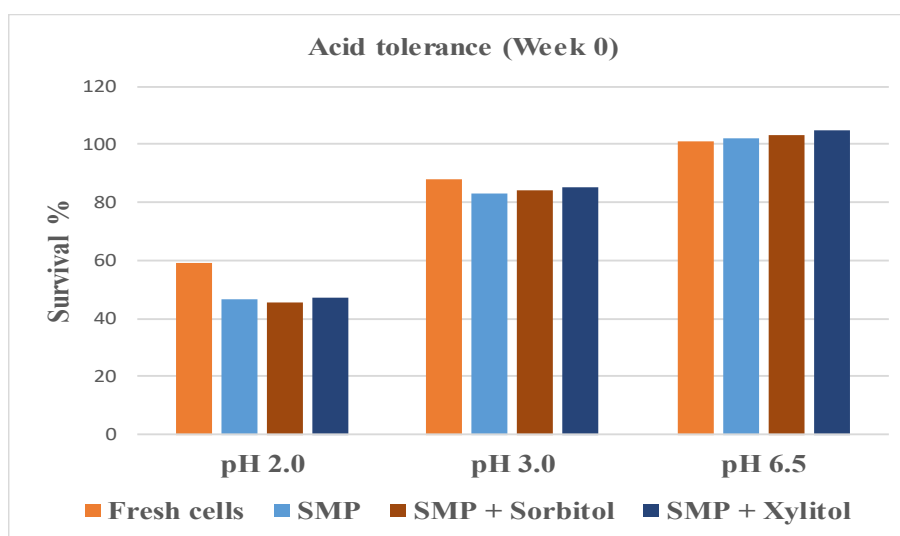
Fig. 8.1: *Survival of LR6 cells in the acid environment (2-h exposure at different pH levels) at week 0*

The desiccated LR6 cells isolated from the 3 experimental samples (carried by SMP, coated with sorbitol, xylitol and carried by SMP) were adjusted to about 8.8 log cfu/ml. After 120 min of incubation, the viable cell population in these 3 samples was on average 4.1 log cfu/ml and remained very close to each other. The survival was in the range of 46% to 47%, much less than the fresh cells. At pH 3.0, the final viable count for the fresh cells was on average 8.1 log CFU/ml and the same for the desiccated cells was 7.4 log cfu/ml with little variations among the 3 samples. As expected, the survival percentages were also higher, 88% for the fresh cells and in the range of 83% to 85% for the desiccated cells. The difference between the fresh and desiccated cells in terms of survival was less pronounced when the pH was raised from 2.0 to 3.0. At pH 6.5, no loss in viable cell populations was recorded in any of the samples, probably because of the favorable pH environment.

The desiccated samples were adjusted to a$_w$ 0.11 and 0.30 by controlling the relative humidity inside desiccators as described in section 3.3.4, packed in double layered LDPE followed by heat sealed alluminum foils and stored at 37 °C for 12 weeks. After the storage, the cells were isolated and incubated in the SGF exactly following the protocol used for week 0 experiment and compared with the freshly grown cells. Table 8.2 shows the reductions in viable cell populations throughout the incubation period at different pH levels and Figure 8.2 summarizes the results in the form of survival percentages.

Table 8.2: Changes in viable cell populations in the acid environment (2-h exposure at different pH levels) after 12 weeks of storage at 37 °C

		Acid tolerance (Week 12)											
Sample		pH 2.0				pH 3.0				pH 6.5			
		0 min	60 min	90 min	120 min	0 min	60 min	90 min	120 min	0 min	60 min	90 min	120 min
Fresh cells		9.1 ± 0.2	7.7 ± 0.6	6.5 ± 0.2	5.7 ± 0.5	9.2 ± 0.4	8.9 ± 0.1	8.4 ± 0.4	8.3 ± 0.3	9.4 ± 0.3	9.3 ± 0.2	9.4 ± 0.1	9.1 ± 0.1
SMP	aw 0.11	8.6 ± 0.4	6.9 ± 0.3	4.2 ± 0.4	3.8 ± 0.3	8.6 ± 0.2	8.3 ± 0.3	7.0 ± 0.1	7.0 ± 0.2	8.3 ± 0.4	8.1 ± 0.2	8.1 ± 0.3	8.1 ± 0.1
	aw 0.30	7.1 ± 0.3	6.1 ± 0.2	3.9 ± 0.4	2.7 ± 0.2	7.4 ± 0.4	7.0 ± 0.4	6.3 ± 0.2	5.9 ± 0.4	7.6 ± 0.3	7.6 ± 0.1	7.8 ± 0.4	7.8 ± 0.3
SMP + Sorbitol	aw 0.11	8.7 ± 0.2	7.3 ± 0.6	4.5 ± 0.5	3.9 ± 0.3	8.6 ± 0.4	8.1 ± 0.4	8.0 ± 0.1	6.9 ± 0.3	8.3 ± 0.1	8.3 ± 0.3	8.4 ± 0.4	8.5 ± 0.3
	aw 0.30	6.5 ± 0.5	6.0 ± 0.1	4.1 ± 0.3	2.6 ± 0.1	7.0 ± 0.2	7.1 ± 0.3	6.5 ± 0.3	5.4 ± 0.3	6.8 ± 0.2	6.4 ± 0.3	6.5 ± 0.1	6.7 ± 0.4
SMP + Xylitol	aw 0.11	8.8 ± 0.5	8.2 ± 0.5	5.7 ± 0.4	4.1 ± 0.4	8.6 ± 0.4	8.2 ± 0.1	7.4 ± 0.4	7.1 ± 0.3	8.8 ± 0.3	8.8 ± 0.4	8.8 ± 0.2	8.5 ± 0.2
	aw 0.30	7.8 ± 0.3	6.3 ± 0.4	3.7 ± 0.3	3.2 ± 0.4	7.3 ± 0.6	7.2 ± 0.2	6.1 ± 0.5	6.0 ± 0.4	7.7 ± 0.4	7.9 ± 0.2	7.9 ± 0.2	7.7 ± 0.1

Average values ± standard deviation (n = 3)

(a)

(b)
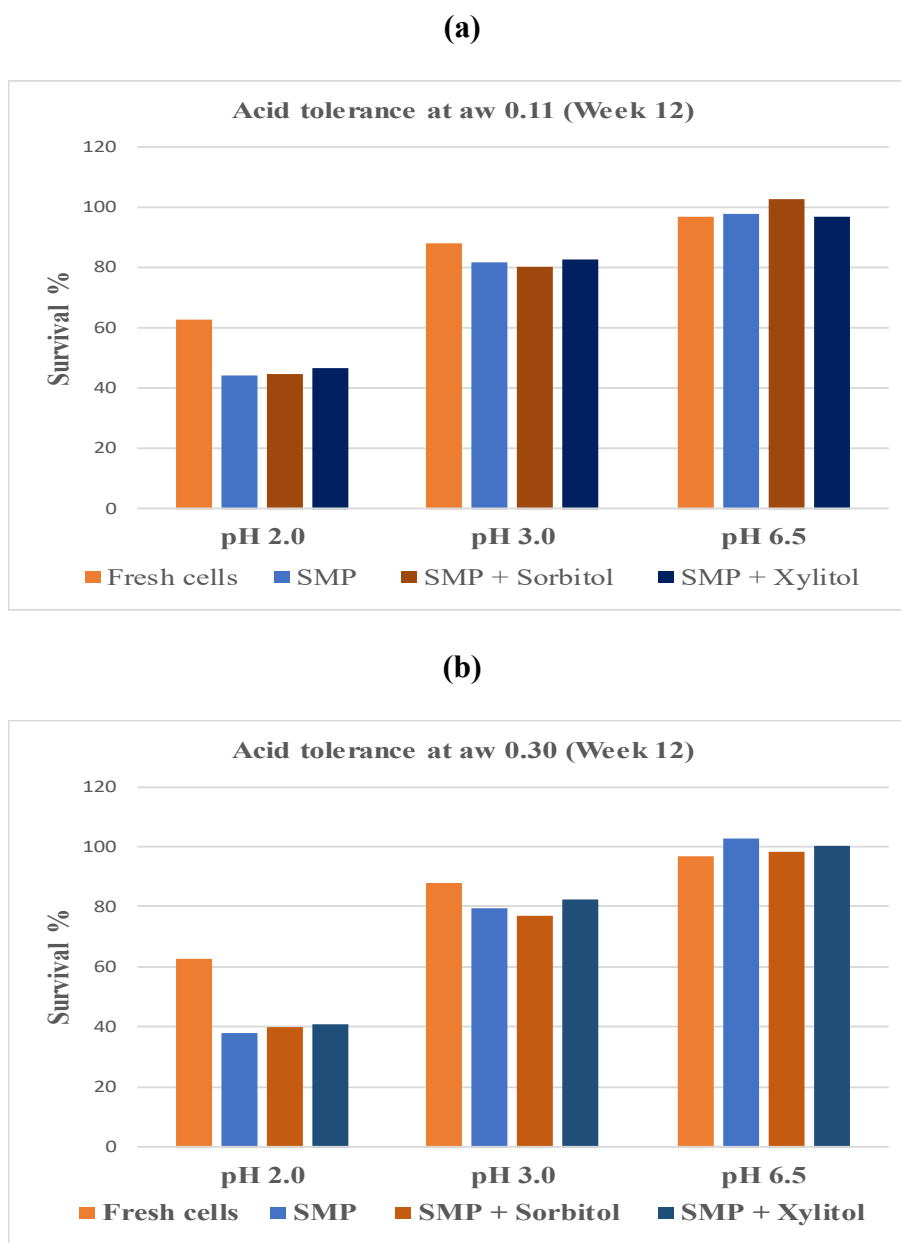
Fig. 8.2: *Survival of LR6 cells after 12 weeks of storage at a_w 0.11 (a) and a_w 0.30 (b) in the acidic environment (2-h exposure at different pH levels)*

At pH 2.0, the freshly grown cell population was reduced from 9.1 log cfu/ml on average to 5.7 log cfu/ml. Cells stored in the desiccated samples at a_w 0.11 did not lose viability to any great extent during the storage period. Using identical protocol for the cell isolation and the dilution process followed at week 0, the initial viable cell counts

for the desiccated samples were in a narrow range of 8.6 to 8.8 log cfu/ml. After 120 min incubation at pH 2.0, the final populations in these samples were recorded in the range of 3.8 to 4.1 log cfu/ml. In summary, the 63% of the freshly grown cells survived the tolerance test whereas the survival rate of the desiccated cells with SMP, SMP + sorbitol and SMP + xylitol were 44%, 45%, and 47% respectively. Similar comparison at other pH levels indicated that at pH 3.0, 88% of the fresh cells survived whereas the survival of the desiccated cells were in the range of 80% to 83% with minor variations. A clear trend of low survival rates for the desiccated cells at these two pH levels was found with slightly better results for xylitol coated samples in each case.

By contrast, incubation at pH 6.5 did not show any distinct variation between the fresh and desiccated samples when 97% of the fresh cells were recovered and the recovery for the experimental samples was from 97% to 102%. When the samples were adjusted to a_w 0.30, as expected, the viable cell populations in the desiccated samples after isolation were reduced in the range of 6.5 to 7.8 log cfu/ml. It was found that the high a_w environment not only affected the cells during the 12 week storage period but was also detrimental for their acid resistance. Figure 8.2 (b) shows that at pH 2.0 as well as at pH 3.0 these samples showed considerably lower survival rate than the low a_w samples. The rate of survival for the cells stabilized with SMP, SMP + sorbitol and SMP + xylitol were 38%, 40%, and 41% respectively at pH 2.0. The same trend was seen at pH 3.0 were 80%, 77%, and 82% respectively. At both the pH levels, the cells stored at a_w 0.11 showed slightly superior survival in the range of 44% to 47% at pH 2.0 and 80% to 83% at pH 3.0. No clear trend in this context was observed during incubation at pH 6.5.

8.2.2 Bile salts tolerance of the LR6 cells after desiccation and storage at 37°C for 12 weeks

A similar trend was observed when the freshly grown and desiccated cells within the same set of samples were subjected to incubation in the simulated intestinal fluid (SIF) containing various concentrations of bile salts.

Table 8.3: *Changes in viable cell populations in bile salts environment (8-h exposure at different concentration levels) at week 0*

Sample	Bile tolerance (Week 0)								
	0.5%			1.0%			2.0%		
	0 h	4 h	8 h	0 h	4 h	8 h	0 h	4 h	8 h
Fresh cells	8.9 ± 0.5	6.8 ± 0.2	6.3 ± 0.3	8.9 ± 0.2	5.8 ± 0.4	5.3 ± 0.5	9.1 ± 0.2	6.4 ± 0.3	5.0 ± 0.4
SMP	8.7 ± 0.3	6.1 ± 0.3	5.6 ± 0.4	8.9 ± 0.4	6.0 ± 0.3	5.0 ± 0.2	8.7 ± 0.2	5.7 ± 0.4	4.9 ± 0.4
SMP + Sorbitol	8.9 ± 0.5	6.2 ± 0.4	5.8 ± 0.5	8.7 ± 0.5	7.1 ± 0.1	5.6 ± 0.3	8.9 ± 0.1	6.2 ± 0.3	5.1 ± 0.6
SMP + Xylitol	8.7 ± 0.4	6.6 ± 0.1	5.8 ± 0.4	9.2 ± 0.3	7.0 ± 0.3	5.7 ± 0.4	9.0 ± 0.3	6.6 ± 0.4	5.3 ± 0.2

Average values ± standard deviation (n = 3)

At the beginning of the storage trial (week 0 results, Table 8.3 and Figure 8.3), the desiccated cells supported by SMP, SMP + sorbitol and SMP + xylitol samples when exposed to 0.50% bile salts concentration, showed 64%, 65% and 67% survival respectively, compared to 71% survival recorded in the fresh cells sample. Increase in bile concentration to 1.0% resulted in a slightly higher loss in cell viability with 56% to 62% survival in the desiccated samples and 60% in the fresh cells sample. Further

increment of bile concentration to 2.0% had a moderately incremental effect on cell mortality.
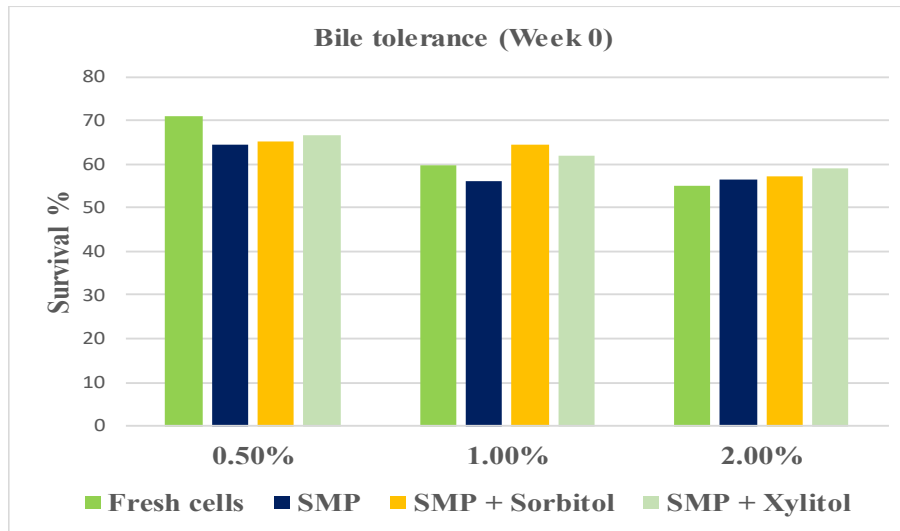
Fig. 8.3: *Survival of LR6 cells in the presence of bile salts (8-h exposure at different concentration levels) at week 0*

The survival for the fresh cells was reduced to 55% and the same for the desiccated cells ranged within 56% to 59%. In most cases, at different bile concentration levels, xylitol coated cells supported by SMP showed better survival after incubation of 8 hours in the SIF.

After the storage period of 12 weeks (Table 8.4 and Figure 8.4), the desiccated cells maintained at a_w 0.11 showed superior survival compared to the ones maintained at a_w 0.30 irrespective of the bile salts concentrations.

Table 8.4: *Changes in viable cell populations in bile salts environment (8-h exposure at different concentration levels) after 12 weeks of storage at 37 °C*

Sample		0.5%			1.0%			2.0%		
		0 h	4 h	8 h	0 h	4 h	8 h	0 h	4 h	8 h
Fresh cells		8.8 ± 0.5	6.9 ± 0.2	6.5 ± 0.3	8.9 ± 0.3	6.0 ± 0.4	5.5 ± 0.5	9.0 ± 0.4	6.7 ± 0.3	5.3 ± 0.4
SMP	**aw 0.11**	8.6 ± 0.3	5.8 ± 0.3	5.2 ± 0.4	8.3 ± 0.4	6.0 ± 0.3	5.0 ± 0.4	8.5 ± 0.2	5.9 ± 0.4	4.9 ± 0.1
	aw 0.30	7.3 ± 0.2	4.1 ± 0.3	3.8 ± 0.6	7.4 ± 0.4	5.0 ± 0.1	3.5 ± 0.3	7.6 ± 0.3	5.3 ± 0.5	3.0 ± 0.3
SMP + Sorbitol	**aw 0.11**	8.9 ± 0.6	4.3 ± 0.5	4.9 ± 0.4	8.7 ± 0.2	5.0 ± 0.2	5.0 ± 0.4	8.7 ± 0.2	6.2 ± 0.1	4.5 ± 0.3
	aw 0.30	6.8 ± 0.7	3.9 ± 0.4	3.1 ± 0.3	6.9 ± 0.2	4.2 ± 0.4	3.0 ± 0.5	7.1 ± 0.3	4.6 ± 0.2	2.8 ± 0.6
SMP + Xylitol	**aw 0.11**	9.0 ± 0.2	6.2 ± 0.2	5.6 ± 0.4	8.7 ± 0.1	5.9 ± 0.2	5.2 ± 0.3	8.8 ± 0.3	6.5 ± 0.5	4.8 ± 0.3
	aw 0.30	7.0 ± 0.2	5.1 ± 0.5	4.0 ± 0.3	7.3 ± 0.4	5.1 ± 0.4	3.7 ± 0.3	7.3 ± 0.1	4.9 ± 0.1	3.2 ± 0.2

Average values ± standard deviation (n = 3)

The survival range for the former group at 0.50% bile concentration was from 55% to 62% and 46% to 57% for the latter group, compared to 74% survival for the fresh cells.

(a)

(b)
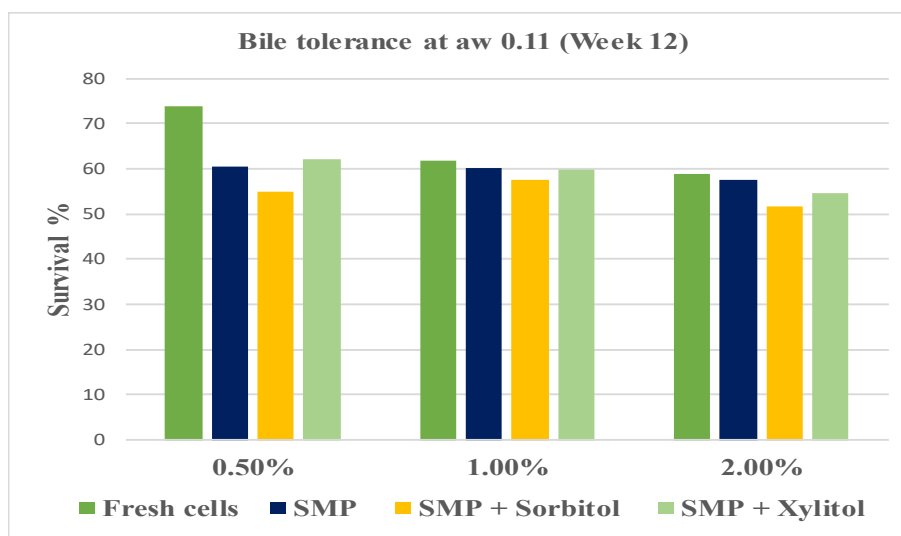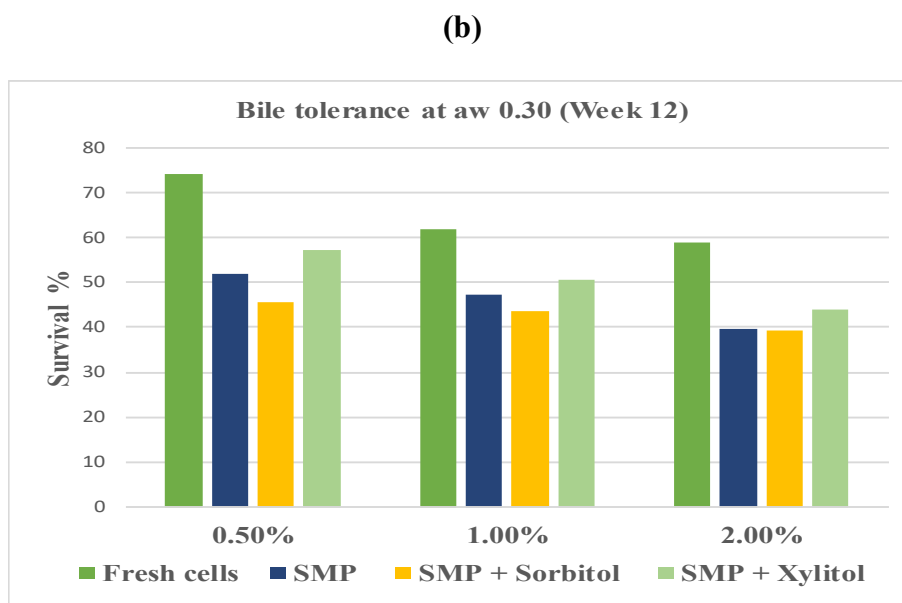
Fig. 8.4: *Survival of LR6 cells after 12 weeks of storage at a_w 0.11 (a) and a_w 0.30 (b) in the presence of bile salts (8-h exposure at different concentration levels)*

At 1.0% bile concentration, the difference between the two groups was more pronounced. The low a_w group yielded a survival between 57% to 60%, whereas elevated a_w environment was found responsible for only 43% to 51% survival compared to 62% in case of fresh cells.

8.2.3 Surface hydrophobicity of the LR6 cells after desiccation and storage at 37°C for 12 weeks

The cell surface hydrophobicity was measured by the affinity of the LR6 cells towards three hydrocarbons namely, n-hexadecane, xylene, and n-octane, following the protocol suggested by Singh *et al.* (2014). According to the results shown in Table 8.5, freshly harvested LR6 cells exhibited weaker hydrophobicity (18%) towards n-hexadecane

whereas the same towards the other two organic solvents were higher (56% and 28% for xylene and n-octane respectively). These values differ considerably from the ones reported by Singh *et al.* (2014), however, the trend remained the same. It was found that desiccation had a slightly adverse effect on the surface hydrophobicity. Overall, each sample tested showed lower values than the fresh cells corresponding to each solvent except for the SMP + Sorbitol towards xylene, which was slightly better.

Table 8.5: *Surface hydrophobicity of the LR6 cells at week 0*

Sample	Surface hydrophobicity (%) Week 0		
	n- Hexadecane	Xylene	n-Octane
Fresh cells	18.5 ± 1.7	56.3 ± 1.7	28.6 ± 2.1
SMP	17.4 ± 3.1	49.7 ± 0.8	27.4 ± 1.0
SMP + Sorbitol	16.8 ± 2.5	58.3 ± 4.1	24.3 ± 2.1
SMP + Xylitol	17.6 ± 2.1	51.6 ± 2.2	26.3 ± 3.2

Average values ± standard deviation (n = 3)

After storage at 37 °C for 12 weeks, the same samples shown a further reduction in hydrophobicity (Table 8.6) in this property.

Table 8.6: *Surface hydrophobicity of the LR6 cells at week 12*

Sample		Surface hydrophobicity (%) Week 12		
		n- Hexadecane	Xylene	n-Octane
Fresh cells		23.2 ± 2.1	53.5 ± 3.1	31.5 ± 1.9
SMP	**aw 0.11**	13.9 ± 2.3	32.3 ± 2.4	18.1 ± 0.7
	aw 0.30	10.4 ± 0.9	28.5 ± 1.9	14.2 ± 1.4
SMP + Sorbitol	**aw 0.11**	14.4 ± 2.2	38.1 ± 3.0	14.1 ± 1.1
	aw 0.30	11.8 ± 1.4	33.3 ± 3.0	10.9 ± 4.6
SMP + Xylitol	**aw 0.11**	13.2 ± 1.7	35.9 ± 2.1	17.1 ± 1.4
	aw 0.30	10.9 ± 2.0	31.7 ± 0.8	12.6 ± 2.7

Average values ± standard deviation (n = 3)

For ease of comparison, the retention of hydrophobicity over the storage period was calculated (Figure 8.5) as per the formula described in section 3.3.9, which showed considerable reduction occurring in the cells stored at a_w 0.30, a trend similar to the acid and bile resistance properties. Among the experimental samples, SMP + Sorbitol showed maximum retention with n-hexadecane at both a_w storage. No distinct trend was observed when the samples were treated with xylene or n-octane.

(a)
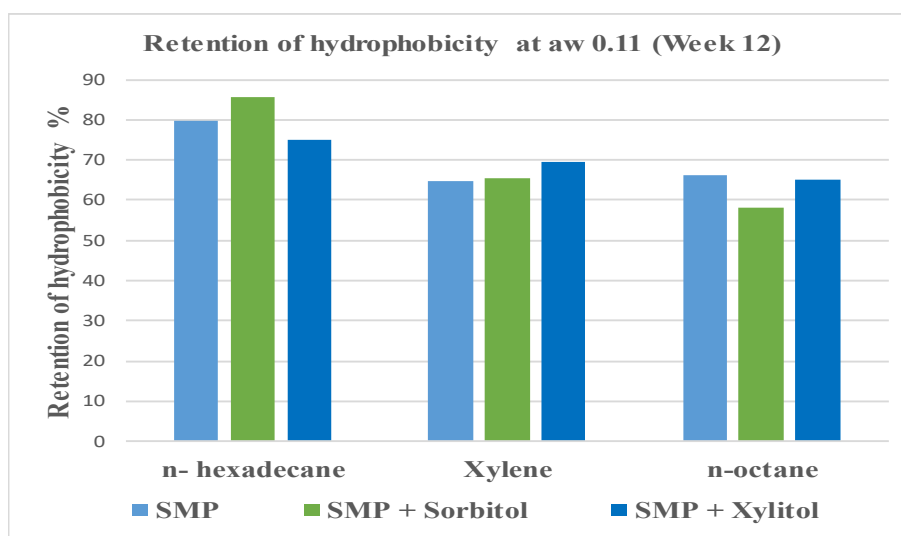
(b)
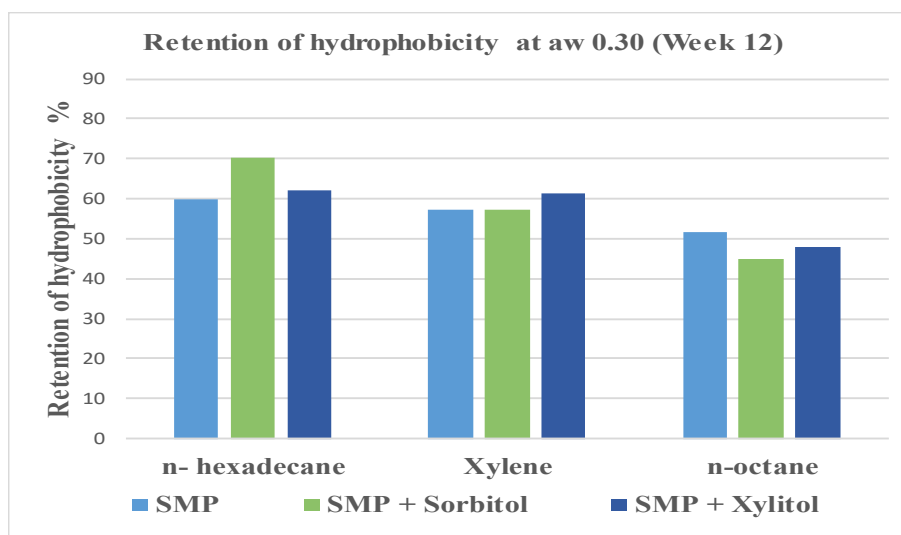
Fig. 8.5: *Retention of surface hydrophobicity of the LR6 cells over storage at a_w 0.11 (a) and at a_w 0.30 (b)*

In order to deliver the targeted health benefits to the host, the consumed probiotic bacteria must remain viable until they reach the intestine and allowed to colonize there under favorable conditions. During this delivery process, the consumed probiotics encounter several biological barriers to survive such as salivary lysozyme, hydrochloric

acid and bile salts secreted in the stomach and duodenum respectively (Saarela *et al*., 2000). The hardest barrier among them has been reported as the acidic stomach environment where the pH ranges between 1.5 and 3.0 (Charteris *et al*., 1998). The primary aim of the study reported in section 7.3.11 was to find out if the intrinsic tolerance of the particular probiotic strain under study against these barriers remains similar to the scenario when they are consumed via any of the common fermented foods/drinks (simulated situation for the freshly grown cells) or deteriorates when the cells are stabilized and stored for a longer duration. Similarly, the purpose of the cell hydrophobicity test was to evaluate the potential adhesion and subsequent colonization properties as an *in vitro* biochemical marker (Rosenberg *et al*., 1980). The hydrophobic nature of the outermost surface has been thought to be aiding in the attachment of the bacteria to the host tissue and offers a competitive advantage over other microorganisms present in the GI tract (Schillinger *et al*., 2005).

The results presented above indicate three major findings in these aspects. Firstly, in terms of tolerance towards acidic and bile environments, the freshly grown cells showed superior properties over the desiccated or stored cells at acidic pH conditions and bile concentration levels tested. The second important finding was slightly superior acid and bile tolerance properties of the xylitol coated desiccated cells (SMP + Xylitol) over the non-coated (SMP) as well as the sorbitol coated cells (SMP + Sorbitol). Better tolerance properties were maintained throughout the storage period as well. The third interesting observation was the detrimental effects of high a_w storage not only applies to the viability of the cells but also on the acid, bile tolerance, and the surface hydrophobicity. At pH 2.0 the differences in acid tolerance of these cells were more pronounced

irrespective of the type of supporting agents in which they were desiccated. In the bile salts experiment, the relatively poor survival of the cells stored at high a_w was recorded at all the three concentration levels chosen. A marginal improvement in bile tolerance at all levels was noticed for the xylitol coated cells after desiccation and storage as well. The LR6 cells showed a poorer affinity towards n-Hexadecane and were found more hydrophobic against the other two solvents used. This type of variation to different solvents by different bacterial strains of probiotic nature has been reported earlier (de Ambrosini *et al*., 1998). The higher retention of surface hydrophobicity at a_w 0.11 can be explained by the presence of water molecules in a monolayer state via a hydrogen bond with the supporting agents which might have prevented the molecular mobility and consequently reducing the surface lipid oxidation (Bhandari and Howes, 1999).

While searching for the previous work similar to this experiment to compare these findings, only one published study by Dianawati and Shah (2011) was found. In that study, freeze-dried cultures of microencapsulated *B. lactis* Bb12 cells were compared with freshly grown cells for acid, bile tolerance and surface hydrophobicity, immediately after drying and also throughout the storage for 10 weeks under ambient conditions. However, the cells were not isolated from the dried matrix before subjecting to the acid and bile environments because they wanted to examine the protective effects of the encapsulation technique used. The authors had recorded only 51-56% and 36-39% survival after freeze-drying and storage respectively when the cells were exposed to pH 2.0 media. The corresponding survival rates for bile salts incubation tests were 67-78% and 52-70% respectively. Superior retention of surface hydrophobicity was recorded when the cells were stored in a low a_w matrix. Their study confirmed the process of

gradual decay in the cell membrane integrity during ambient storage which was thought to be reducing the intrinsic strength of the bacterial cells against the stressful elements and also causing physicochemical changes in the outermost surfaces of the bacterial cells reducing the hydrophobicity. In the FTIR analysis of the major cell membrane components reported in sections 6.2.3, 6.2.4 and 7.3.5 and also through the microscopic images in sections 7.3.2 and 7.3.3, it was confirmed that membrane integrity following desiccation and storage for the LR6 cells was compromised. In those sections, it was also shown that xylitol coating had a minor protecting role. The acid/bile tolerance and the surface hydrophobicity results supported this finding.

8.3 Conclusions

Resistance towards acidic environment in the stomach and bile salts present in the small intestine is considered important property of any probiotic strain because in absence of this intrinsic strength, the viability of the cells is expected to be compromised. In effect, the cells are not expected to reach the large intestine, multiply and colonize, and be functional as per their individual characteristics. In order to colonize, it is also important that the hydrophobic nature of the cell surface remains intact. This study has highlighted an important shortcoming of the stabilization process and high-temperature storage of the experimental probiotic strain. It was found that all the three desired properties mentioned above were compromised with enhanced detrimental effects from the high a_w storage conditions.

The alterations in the cell membrane structures due to such type of processing and storage, as revealed in chapter 6.0 and 7.0, might have caused easy penetration of the acid and bile salt components inside the cell bodies. Microscopic observations revealed that stabilization resulted into wrinkled cell surface with blister formations and compromised integrity with enhanced electron density of the cytosol. These changes were probably responsible for the reduction of the cell surface hydrophobicity.

Chapter 9.0. Concluding discussion and recommendations

Over the last two decades, maintaining structural properties and propagation abilities of the useful microorganisms during long term storage have drawn considerable attention to the research communities. The intended applications are commercial scale production of functional foods, nutraceuticals, biofuels, and biochemicals. To achieve cost competitiveness and production efficiency, a robust upstream cell propagation technique and subsequent processing stages are very important. This is reflected by the ongoing efforts being seen in the form of numerous publications related to the development of novel strategies to ensure both higher storage stability and functionality of the useful microorganisms, particularly the ones having probiotic attributes. Probiotic bacterial cell preservation using a suitable stabilization technique is therefore of paramount importance not only to maintain the viability during the long term storage but also to ensure the functionality, optimum propagation ability and robustness against gastric transition when they are consumed. Though many attempts have been made to increase microorganisms' stability during various downstream processing stages, improving the survival rates is still one of the major challenges in industrial starters and probiotics production (Yildirim *et al*., 2007). Apart from their long-term storage, cellular stability of probiotics constitutes another challenge to provide beneficial health effects.

Through extensive literature search and also from analyzing the samples of stabilized probiotic products procured from the market, it was found that superior stability and

other characteristics as mentioned above were achievable if the probiotics were carried by a matrix maintained at a very low water activity level. This was being achieved by using the freeze-drying technique through which it was possible to obtain a very low water activity level (near 0.10) but with some disadvantages of imparting cellular injuries during the freezing process. This was a feasible technique for preserving the probiotics in the ingredient form with controlled packaging. However, the real challenge was to maintain this superior stability after fortifying such ingredient into a powdered food formulation, mostly done through dry blending. In any powdered food, the probiotic ingredient constitutes of only 0.1 to 1.0% whereas the bulk is a spray dried formulation having water activity range of 0.20 to 0.30. Once the probiotic ingredient is mixed into this bulk and stored for some period, the equilibrium water activity is bound to go up and is thought to be the primary reason of deterioration of losing the cell viability over long term storage. Therefore, the primary objective of this project was to come up with a novel stabilization technique through which an ingredient with intermediate level of water activity (between 0.20 to 0.30) can be produced without significantly compromising the storage stability.

The first part of this project focused on developing a novel stabilization technique which would offer superior storage stability to a range of probiotic strains when compared to the freeze-drying process generally employed for this purpose. After thorough searching of previous literature (Chapter 2.0) on this subject area, it was found that dairy proteins in a combination with polysaccharides could be a good potential carrier material for stabilizing a lactobacillus strain, namely *L. casei* 431. Fluidized bed drying, due to the moderate temperature drying feasibility, was chosen as a prospective technique to

stabilize this particular strain. A combination of milk powder a carrier agent, fluidized bed drying and sub-lethal stress (osmotic, heat) adaptation was proved to be most effective. Upon getting significantly better stability for this strain at 25 °C storage for 9 months, other two lactobacilli and bifidobacterium strains were treated under the same conditions and their storage stability evaluated. As expected, osmotically stressed and fluidized bed dried cells in all the 4 samples showed significantly better stability when compared to the same strains stabilized by freeze drying without any stress adaptation (Chapter 4.0).

In the second part of the project (Chapter 5.0), another probiotic strain with proven efficacy, *Lactobacillus reuteri* LR6, was stabilized using the developed protocol but this time an enhanced storage temperature of 37 °C was used. Also, the fluidized bed drying parameters were further optimized to obtain the maximum survivability of the stabilized probiotic cells. Apart from milk solids, a few other food grade carrier agents of different and unique physicochemical properties were investigated. An additional treatment given to the probiotic cells prior to stabilization was a thin film coating of either of the two common polyol compounds, sorbitol and xylitol. Overall, the best survival was recorded for the cells coated with xylitol and carried by skim milk powder.

The current consensus is that freeze drying needs a lyoprotectant to prevent ice-crystal formation related damages, but this may be less important in the case of low temperature air drying. The damage inflicted by spray drying is generally attributed to the high temperature. In low temperature air drying, as was the case in FBD, the principal concern was to avoid the osmotic shock. A good example of this is the adverse effect of

mixing with very dry casein on the viability of bacteria (Mille *et al.,* 2004). In ideal conditions of desiccation, it can be imagined that only the exact or maximum feasible quantity of moisture should be removed from the surface of the cells i.e from the external environment without affecting the internal water content (schematically presented in Fig. 9.1).
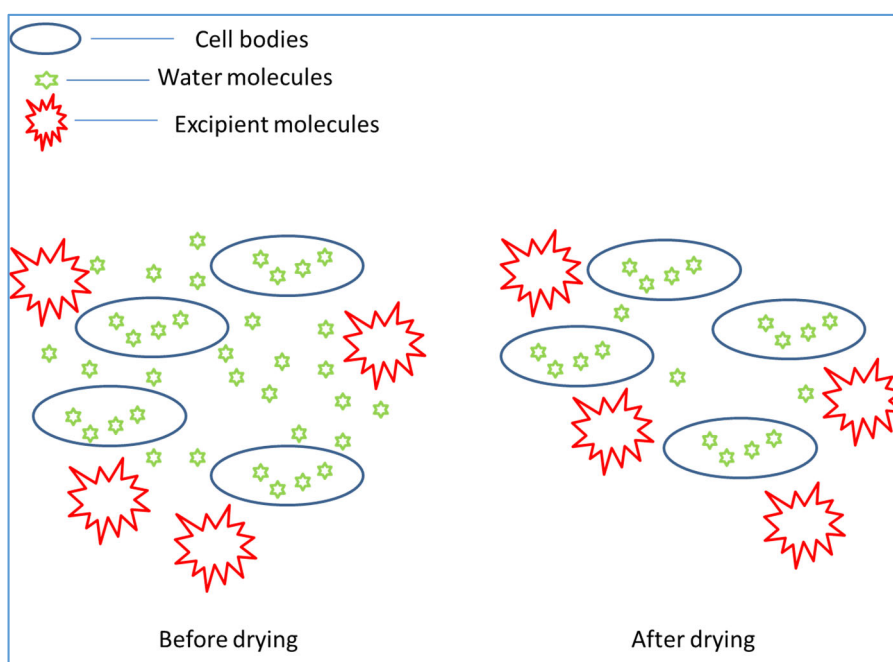
Fig. 9.1: Schematic diagram of ideal desiccation mechanism for bacterial cells

At the same time the osmotic shock from the external environment should be maintained at minimum to avoid cell lysis or other damages to the membrane. To mimic such theoretically ideal condition, a coating of appropriate plasticising material around the cells was thought to be essential which was the rationale behind the choice of the polyols as they were known to have good humectant properties. The very similar results obtained in case of the SMP samples indicate that this plasticising effect may also be

coming from the SMP agglomeration process. Roos (2010) suggested that in skim milk powder, lactose and in some cases its hydrolysis products were significantly plasticised by water, which was observed from a rapidly decreasing T_g with increasing water content. He emphasized that particularly during the agglomeration process of SMP, the formation of clusters is followed by removal of water and cooling to solidify the surfaces into a glassy state.

The third part of the research (Chapter 6.0 and 7.0) focused on to explain the results obtained so far by inspecting the changes occurred to the bacterial cell bodies due to the adverse processing and storage conditions and correlating the same with the loss in cell viability during stabilization and storage. This was tried by exposing the isolated bacterial cells to an infra-red environment and recording the molecular vibrations of the cell membrane components caused by this exposure. Any deformation or alteration from the natural configurations, particularly of the proteins present therein, was inspected by statistically analyzing the obtained reflection of the infra-red beam in the form of a spectrum. It was found that despite the moderate heat treatment given to the cells through fluidized bed drying, deformations in the secondary structures of the membrane proteins were inevitable. The spectral analysis and periodic enumeration of the viable bacterial cells by standard microbial plate counting suggested a direct correlation between the degree of protein deformations and loss in bacterial cell viability. Figure 9.2 illustrates the summerized findings of chapter 6.0 showing the importance of stabilizing the probiotic cells in the presence of suitable protectants in order to minimize the alterations in the membrane protein structures.
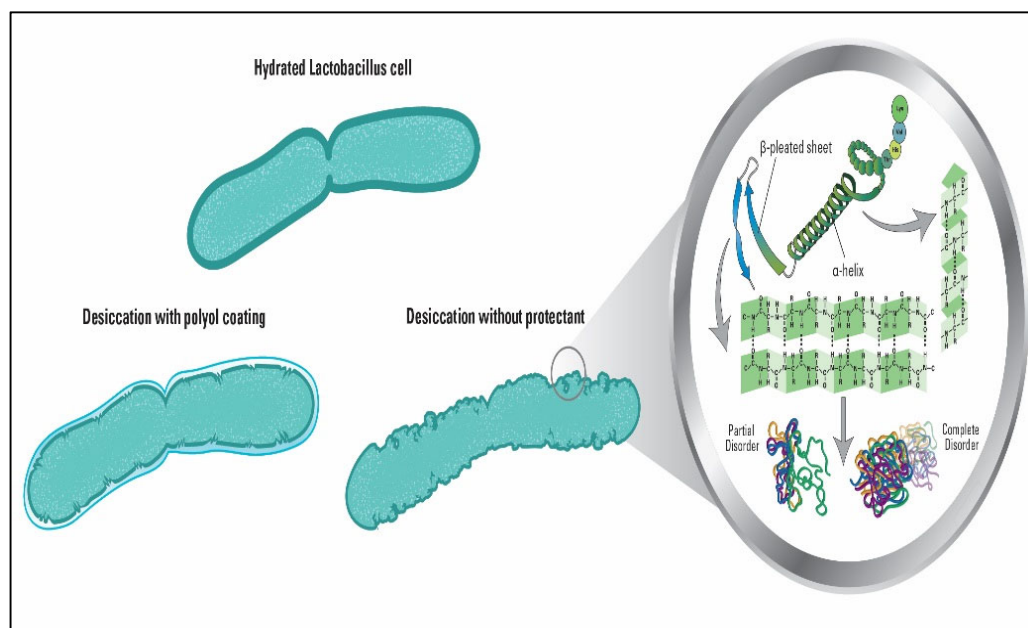
Fig. 9.2: *Illustration of the cell desiccation process without excipients*

It was also found that such deformations were not limited to the stabilization process but continued to develop during non-refrigerated storage. Therefore, the objective was to identify any type of carrier materials with specific physicochemical properties which aided in reducing such deformations and were able to retain more viable cells. It was earlier established by Crowe (2002) that during dehydration, proteins were better stabilized and the structures remained intact in presence of sugar or sugar-like compounds. In line with that hypothesis, a combination of xylitol, which is a sugar alcohol, and skim milk powder, a combination of milk carbohydrate and proteins, were found to be most effective compared to the others tested. During the storage period, the structural deformations and consequent loss in viability were also found correlated with the free moisture content of the stabilization matrix, measured as water activity (a_w).
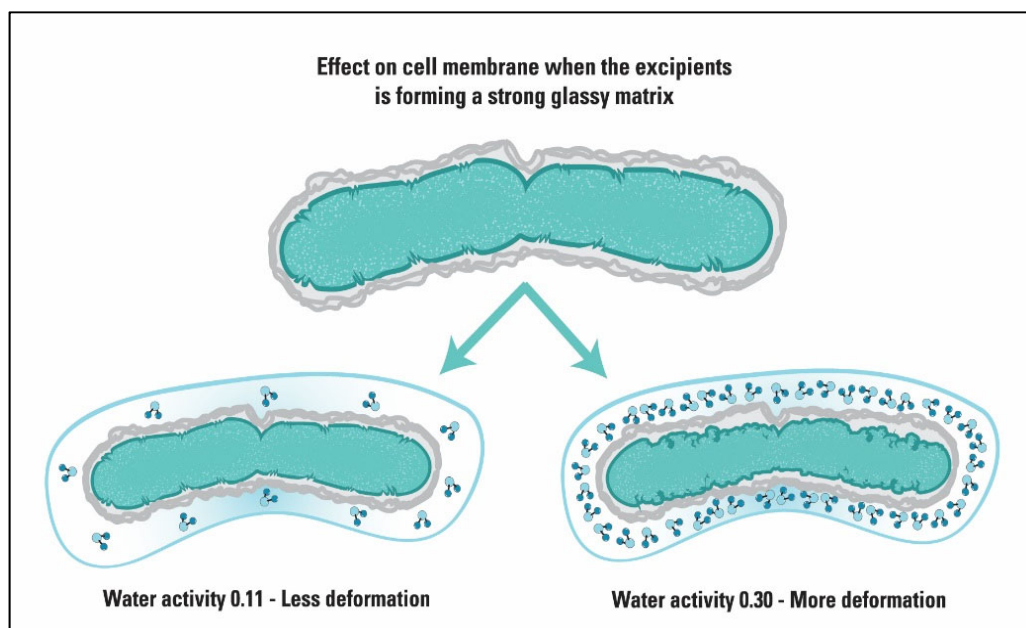
Fig. 9.3: *Illustration showing the advantage of strong glassy matrix formation by the excipients and the effect of matrix water activity on cell membrane integrity*

A high a_w was found to be yielding a very low glass transition temperature (T_g) for the milk solids based matrices. This was proven to be detrimental for the cell viability because a less glassy matrix and higher mobility of free water molecules might have induced hydration of the solutes aiding oxidation and formation of reactive oxygen species (ROS) through chemical reactions with the membrane components, particularly at high storage temperature. This might also have resulted in imparting increased osmotic stress to the bacterial cells (Mugnier and Jung; 1985). Therefore, it can be concluded that while choosing a carrier agent to support the probiotic bacterial cells for stabilization, the focus should be on a combination which can offer lower T_g at the intended storage temperature and a_w level. This becomes more important when an intermediate a_w level around 0.20 to 0.25 in the final product is targeted. This selection must also ensure to offer a better protective effect on the cells during the stabilization

process of choice. Figure 9.3 illustrates the importance of a strong glassy matrix around the bacterial cells and also the advantages of storage in a low a_w matrix in minimizing the structural deformations in the membrane proteins and thereby prolongling the shelf stability, as reported in chapter 7.0.

Figure 9.4 depicts a graphical abstract of the processing steps used for stabilization, storage results, and the summary findings from the analytical techniques used in understanding the mechanisms behind loss in cell viability over storage.
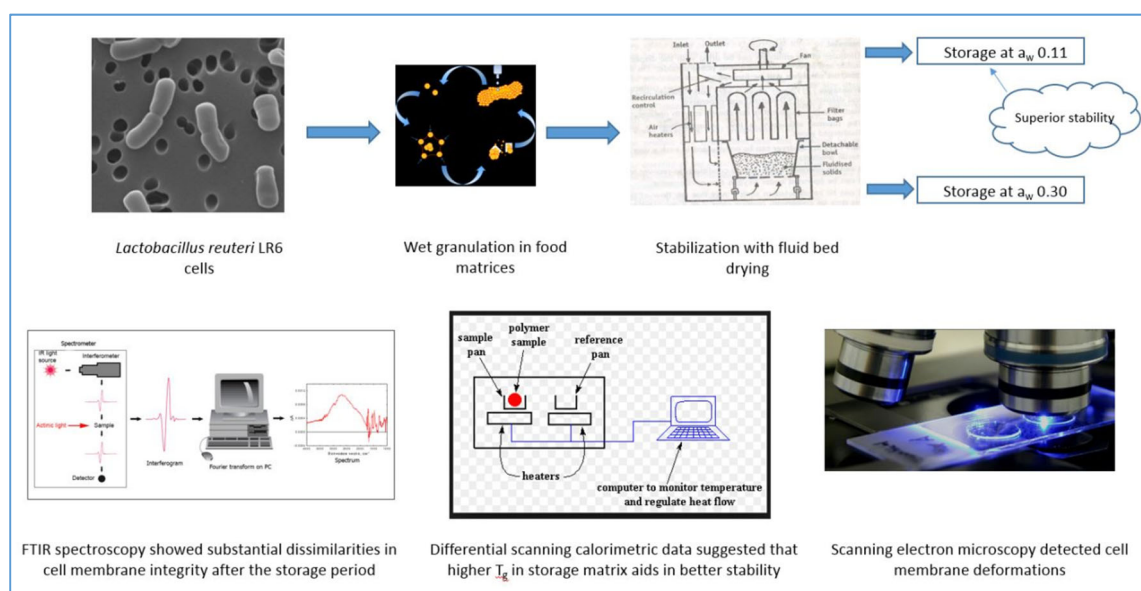
Fig. 9.4: *Graphical summary of the findings reported in the thesis chapters.*

The study also revealed two very important aspects regarding the optimum particle size of the stabilizing matrix for getting the desired effects and the robustness of the stabilized and stored bacterial cells in terms of their intrinsic probiotic attributes. It was found that the bigger particles formed during the wet agglomeration and the fluidized

bed drying processes were able to offer considerably higher protection to the cells during stabilization and storage as well. This was probably due to the placement of the cells within the core of bigger agglomerates causing less exposure to the adverse environment compared to the fine particles where the cells were expected to only adhere to the particle surfaces. Secondly, the robustness of the stabilized and stored bacterial cells were found to be highly compromised against stomach acid and pancreatic bile environments. The hydrophobic nature of the cells surface, which is thought to be helpful in intestinal colonization were also found to be compromised because of the stabilization process and subsequent long term storage. This emphasized the importance of consuming freshly grown probiotics within fermented foods such as yogurt over stabilized probiotics in the form of nutraceuticals and other powdered food formulations in order to receive maximum health benefits.

However, the approach taken in this project for understanding the underlying mechanisms responsible for cell death over processing and storage has some limitations. The bacterial cells were isolated from the same fermentation cycle at a particular time point of their growth phase and were subjected to the stabilization process by applying uniform processing stresses to each individual cells. However, it can be assumed that the physiological state of all the bacterial cells at the point of harvesting was not identical. When the harvesting is done at the early stationary phase of the growth cycle, the growth rate gradually approaches the cell death rate towards an equilibrium which is represented by a flat, almost horizontal growth curve (Fig. 3.1). At this point of harvesting, a portion of the viable cells is present in their full form (just after the cell division took place). Another portion remains at the verge of a fresh division to occur and a third fraction is

about to experience death (not reproducible) due to nutrient depletion and sometimes due to the presence of inhibitory compounds produced during fermentation. Therefore, the cell biomass collected at any particular time point cannot be considered homogenous in terms of their physiological conditions. Hence it can be hypothesized that their intrinsic robustness towards processing and storage related stresses would vary considerably, which in turn would affect their reproducibility after stabilization and storage. Unfortunately, it was not possible to segregate the harvested cell biomass based on this assumption and inspect each fraction individually. The argument against this could be that each sample of stabilized probiotics in different carrier materials contained the same fractions in almost equal proportions and therefore the experimental design gave perfect comparative analysis. However, a better and absolute scientific understanding could be gathered if the cell biomass could be purified based on these fractions and investigated thereafter (similar to the concept used in the sieve analysis experiment reported in section 7.2.8). This could tell us more precisely if the post-processing and storage survivability are more dependent on the intrinsic physiological strength or the external protection in the form of encapsulation or stabilization technology, choice of carrier materials etc.

Further recommendations

Further areas for research are listed below.

1) Foods containing no dairy ingredients and vegan lifestyle are getting popular nowadays. The non-dairy supporting agents used in this study i.e, corn starch and MCC did not prove to be superior over milk solids. Hence more of such ingredients should be searched for and tested. This research has been able to narrow down the search criteria i.e, should preferably have a plasticizing property and be able to restrict the water mobility even at slightly higher a_w level (example mannitol) (Mugnier and Jung; 1985). A dried emulsion comprising of vegetable oils, pea protein, mannitol, and maltodextrin may be a good example.

2) The *in vitro* tests performed on the stabilized LR6 cells to evaluate the quality of probiotic attributes can be further validated using an *in vivo* model via suitable animal trials, in which fresh and stabilized probiotics can be fed and isolated from the fecal samples. The quantitative difference between the two groups should indicate the survival and colonization potency of the stabilized cells.

3) The ultimate application of this technology is aimed at fortification into suitable food products. Hence, thorough product stability studies and sensory evaluation would be important as a preliminary step towards successful commercialization of this technology.

4) Apart from the structural alterations of the cell membrane proteins, membrane lipid oxidation during stabilization and storage could be an important factor for the loss of membrane integrity. This should be examined by developing a

suitable protocol and a comparative correlation analysis be done with the results reported in this thesis.

Overall, the current research introduces a promising alternative technique for stabilization of probiotic bacteria intended to be delivered through shelf stable powdered foods. Further development and experimental designs can be conceived based on the suggestions listed above. The health benefits of probiotic-rich foods are well accepted and clinically proven. But the regulatory authorities throughout the world are becoming stricter and the food manufacturers in today's world will have to ensure that the minimum stipulated quantity of viable bacteria is delivered properly. Successful delivery of probiotic bacteria in an ambient stable food format is still a challenge. Therefore, the successful targeted delivery of probiotic bacteria to the human intestine has tremendous market potential and it will be highly welcomed by the industry if a complete solution can be offered at an affordable cost.

References

Ahn, J. B., Hwang, H. J. and Park, J. H. (2001) Physiological responses of oxygen tolerant anaerobic *Bifidobacterium longum* under oxygen. *Journal of Microbiological Biotechnology,* **11***,* 443-451.

Ainsley-Reid, A., Vuillemard, J. C., Britten, M., Arcand, Y., Farnworth, E. and Champagne, C. P. (2005) Microentrapment of probiotic bacteria in a Ca^{2+} -induced whey protein gel and effects on their viability in a dynamic gastro-intestinal model. *Journal of Microencapsulation,* **22**(6), 603-619.

Alderbeth, I., Cerquetti, M., Poilane, I., Wold, A. E. and Collingon, A. (2000) Mechanisms of colonisation and colonisation resistance of the digestive tract. *Microbial Ecology of Health and Disease,* **12,** 223-239.

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. *Archives of Biochemistry and Biophysics,* **365**, 289–298.

Alpert P (2005) The limits on frontiers of desiccation-tolerant life. *Integr. Comp. Biol.,* **45**, 685–695

Anal, A. K. and Singh, H. (2007) Recent advances in microencapsulation of probiotics for industrial applications and targeted delivery. *Trends in Food Science & Technology,* **18** (5), 240-251.

Ananta, E., Volkert, M., and Knorr, D. (2005) Cellular injuries and storage stability of spray-dried *Lactobacillus rhamnosus* GG. *International Dairy Journal,* **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. *Scandinavian Journal of Nutrition,* **45**, 58-75.

Anekella, K., & Orsat, V. (2014) Shelf life stability of lactobacilli encapsulated in raspberry powder: Insights into non-dairy probiotics. *International Journal of Food Sciences and Nutrition*, **65**(4), 411-418.

Audet, P., Paquin, C., and Lacroix, C. (1988) Immobilized growing lactic-acid bacteria with κ-carrageenan - locust bean gum gel. *Applied Microbiology and Biotechnology,* **29**(1), 11-18.

Audet, P., Paquin, C., and Lacroix, C. (1990) Batch fermentations with a mixed culture of lactic-acid bacteria immobilized separately in kappa-carrageenan locust bean gum gel beads. *Applied Microbiology and Biotechnology,* **32**(6), 662-668.

Audet, P., Paquin, C., and Lacroix, C. (1991) Effect of medium and temperature of storage on viability of lactic-acid bacteria immobilized in kappa-carrageenan-locust bean gum gel beads. *Biotechnology Techniques,* **5**(4), 307-312.

Axelsson, L. T. (1993) Lactic acid bacteria: classification and physiology. *Lactic acid bacteria*, 1-63.

Baba, E., Nagaishi, S., Fukata, T., and Arakawa, A. (1991) The role of intestinal microflora on the prevention of sulmonella colonization in gnotobiotic chickens. *Poultry Science,* **70**, 1902-1907.

References

Ballongue, J. (2004) Bifidobacteria and probiotic action. *Lactic acid bacteria: microbiology and functional aspects,* **(Ed.3)**, 67-123.

Banker, M. C., Layne, J. R., Hicks, G. L., and Wang, T. C. (1992) Freezing preservation of the mammalian cardiac explant - Comparing the protective effect of glycerol and polyethylene-glycol. *Cryobiology*, **29**(1), 87-94.

Basso, P. J., Camara, N. O. S., and Sales-Campos, H. (2019) Microbial-based therapies in the treatment of inflammatory bowel disease - an overview of human studies. *Frontiers in Pharmacology*, **9**, 11.

Baugher, J. L., & Klaenhammer, T. R. (2011) Invited review: Application of omics tools to understanding probiotic functionality. *Journal of Dairy Science*, **94**(10), 4753-4765.

Bayles, D. O. and Wilkinson, B. J. (2000) Osmoprotectants and cryoprotectants for *Listeria monocytogenes*. *Letters in Applied Microbiology,* **30**(1), 23-27.

Bayrock, D., & Ingledew, W. M. (1997) Mechanism of viability loss during fluidized bed drying of baker's yeast. *Food Res. Int.*, **30** (6), 417–425.

Bayrock, D., & Ingledew, W. M. (1997a) Fluidized bed drying of baker's yeast: moisture levels, drying rates, and viability changes during drying. *Food Res. Int.*, **30** (6), 407–415.

Bhandari, B. R., & Howes, T. (1999) Implication of glass transition for the drying and stability of dried foods. *Journal of Food Engineering*, **40**(1-2), 71-79.

Bhandari , B., & Adhikari , B. (2009) Glass-transition based approach in drying of foods. In C. Ratti (Ed.), *Advances in food dehydration* (pp. 38–62). London: CRC Press.

Booyens, J., & Thantsha, M. S. (2014) Fourier transform infra-red spectroscopy and flow cytometric assessment of the antibacterial mechanism of action of aqueous extract of garlic (*Allium sativum*) against selected probiotic Bifidobacterium strains. *BMC Complementary and Alternative Medicine*, **14**, 289

Bozoglu, T. F., Ozilgen, M., and Bakir, U. (1987) Survival kinetics of lactic acid starter cultures during and after freeze drying. *Enzyme Microbiology Technology*, **9**, 531–537

Brennan, M., Wanismail, B., Johnson, M. C., and Ray, B. (1986) Cellular damage in dried *Lactobacillus acidophilus*. *Journal of Food Protection*, **49** (1986), 47–53

Bruno, F. A., & Shah, N. P. (2003) Viability of two freeze-dried strains of Bifidobacterium and commercial preparations at various temperatures during prolonged storage. *Food Microbiology and Safety*, **68**, 2336–2339

Buitink, J., van den Dries, I. J., Hoekstra, F. A., Alberda, M., and Hemminga, M. A. (2000) High critical temperature above T_g may contribute to the stablity of biological systems. *Biophys. J.,* **79** (2) 1119–1128

Burns, P., Lafferriere, L., Vinderola, G., and Reinheimer, J. (2014) Influence of dairy practices on the capacity of probiotic bacteria to overcome simulated gastric digestion. *International Journal of Dairy Technology*, **67**(3), 448-457.

Cacela, C., & Hincha, D. K. (2006) Low amounts of sucrose are sufficient to depress the phase transition temperature of dry phosphatidylcholine, but not for lyoprotection of liposomes. *Biophysical Journal*, **90**(8), 2831-2842.

Cardona, T. D., Driscoll, R. H., Paterson, J. L., Srzednicki, G. S., and Kim, W. S. (2002) Optimizing conditions for heat pump dehydration of lactic acid bacteria. *Drying Technology,* **20**(8), 1611-1632.

References

Carpenter, J. F., & Crowe, J. H. (1989) An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. *Biochemistry*, **28**(9), 3916-3922.

Carvalho, A. S., Silva, J., Ho, P., Teixeira, P., Malcata, F. X. and Gibbs, P. (2004) Relevant factors for the preparation of freeze-dried lactic acid bacteria. *International Dairy Journal,* **14**(10), 835-847.

Chadwick, V. S., Chen, W. X., Shu, D. R., Paulus, B., Wilson, I., Bethwaite, P., and Tie, A. (2003) Immune activation in irritable bowel syndrome: Wrong title or wrong disease? Part 2 - Reply. *Gastroenterology*, **124**(4), 1163-1164.

Champagne, C. P., Gardner, N., Brochu, E., and Beaulieu, Y. (1991) The freeze-drying of lactic-acid bacteria – a review. *Canadian Institute of Food Science and Technology Journal*, **24**(3-4), 118-128.

Champagne, C. P., & Fustier, P. (2007) Microencapsulation for delivery of probiotics and other ingredients in functional dairy products. *Functional dairy products,* **2**, 404-426.

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

Chavez, B. E., & Ledeboer, A. M. (2007) Drying of probiotics: optimization of formulation and process to enhance storage survival. *Drying Technology,* **25**, 1193–1201.

Chen, L. Y., Remondetto, G. E., and Subirade, M. (2005) Food protein-based materials as nutraceutical delivery systems. *Paper presented at the 1st International*

Symposium on Delivery of Functionality in Complex Food Systems, Lausanne, SWITZERLAND.

Chen, L. Y., & Subirade, M. (2007) Effect of preparation conditions on the nutrient release properties of alginate-whey protein granular minicrospheres. *European Journal of Pharmaceutics and Biopharmaceutics,* **65**(3), 354-362.

Clark, P. A., & Martin, J. H. (1994) Selection of bifidobacteria for use as dietary adjuncts in cultured dairy foods: Tolerance to simulated bile concentrations of human small intestines. *Cultured Dairy Products Journal,* **29**(3), 18, 20-21.

Clementi, F., & Rossi, J. (1984) Effect of drying and storage-conditions on survival of *Leuconostoc oenos*. *Am. J. Enol. Vitic.*, **35**(3), 183–186.

Collado, M. C., Surono, I., Meriluoto, J., and Salminen, S. (2007) Indigenous dadih lactic acid bacteria: Cell-surface properties and interactions with pathogens. *Journal of Food Science*, **72**(3), M89-M93.

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. *Journal of Applied Microbiology*, **96**(2004), 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 metabolizable sugars. *Applied and Environmental Microbiology,* **71**(6), 3060-3067.

Costa, E., Usall, J., Teixido, N., Garcia, N., and Vinas, I. (2000) Effect of protective agents, rehydration media and initial cell concentration on viability of *Pantoea agglomerans* strain CPA-2 subjected to freeze-drying. *Journal of Applied Microbiology,* **89**(5), 793-800.

References

Coulibaly, I., Dubois-Dauphin, R., Destain, J., Fauconnier, M.-L., Lognay, G., and Thonart, P. (2010) The resistance to freeze-drying and to storage was determined as the cellular ability to recover its survival rate and acidification activity. *International Journal of Microbiology,* **2010**, 625239.

Crowe, J. H., Carpenter, J. F., and Crowe, L. M. (1998) The role of vitrification in anhydrobiosis. *Annual Review of Physiology*, **60**(1), 73–103.

Crowe, L. M. (2002) Lessons from nature: the role of sugars in anhydrobiosis. *Comparative Biochemistry and Physiology a – Molecular and Integrative Physiology,* **131**, 505–513.

Daly, M. J., Gaidamakova, E. K., Matrosova, V. Y., Vasilenko, A., Zhai, M., Venkateswaran, A., Hess, M., Omelchenko, M. V., Kostandarithes, H. M., Makarova, K. S., *et al*. (2004) Accumulation of Mn(II) in Deinococcus radiodurans facilitates gamma-radiation resistance. *Science*, **306**, 1025–1028.

Dave, R. I. & Shah, N. P. (1997) Viability of yoghurt and probiotic bacteria in yoghurts made from commercial starter cultures. *International Dairy Journal,* **7**(1), 31-41.

de Ambrosini, V. M., Gonzalez, S., Holgado, A. P. D., and Oliver, G. (1998) Study of the morphology of the cell walls of some strains of lactic acid bacteria and related species. *Journal of Food Protection*, **61**(5), 557-562.

de Angelis, M., and Gobbetti, M. (2004) Environmental stress responses in lactobacillus: A review. *Proteomics,* **4**(1), 106-122.

Delcour, J., Ferain, T., Deghorain, M., Palumbo, E., and Hols, P. (1999) The biosynthesis and functionality of the cell-wall of lactic acid bacteria. *International Journal of General and Molecular Microbiology*, **76**(1-4), 159-184.

Desai, K. G. H., & Park, H. J. (2005) Recent developments in microencapsulation of food ingredients. *Drying Technology,* **23**, 1361-1394.

Desmond, C., Ross, R. P., O'Callaghan, E., Fitzgerald, G. and Stanton, C. (2002) Improved survival of *Lactobacillus paracasei* NFBC 338 in spray-dried powders containing gum acacia. *Journal of Applied Microbiology,* **93**(6), 1003-1011.

deUrraza, P., & DeAntoni, G. (1997) Induced cryotolerance of *Lactobacillus delbrueckii subsp. bulgaricus* LBB by preincubation at suboptimal temperatures with a fermentable sugar. *Cryobiology*, **35**(2), 159-164.

Devaldez, G. F., Degiori, G. S., Holgado, A. and Oliver, G. (1983) Protective effect of adonitol on lactic-acid bacteria subjected to freeze drying. *Applied and Environmental Microbiology,* **45**(1), 302-304.

de Vries, M. C., Vaughan, E. E., Kleerebezem, M., and de Vos, W. M. (2006) *Lactobacillus plantarum*- survival, functional and potential probiotic properties in the human intestinal tract. *International Dairy Journal*, **16**(9), 1018-1028.

de Vos, P., Faas, M. M., Spasojevic, M., and Sikkema, J. (2010) Encapsulation for preservation of functionality and targeted delivery of bioactive food components. *International Dairy Journal*, **20**(4), 292-302.

Dianawati, D., & Shah, N. P. (2011) Survival, acid and bile tolerance, and surface hydrophobicity of microencapsulated *B. animalis ssp lactis* Bb12 during storage at room temperature. *Journal of Food Science,* **76**, M592–M599.

Dianawati, D., Mishra, V., and Shah, N. P. (2013) Stability of microencapsulated *Lactobacillus acidophilus* and *Lactococcus lactis ssp. cremoris* during storage at room temperature at low a_w. *Food Research International,* **50**, 259–265.

References

Dianawati, D., Lim, S. F., Ooi, Y. B. H., and Shah, N. P. (2017) Effect of Type of Protein-Based Microcapsules and Storage at Various Ambient Temperatures on the Survival and Heat Tolerance of Spray Dried *Lactobacillus acidophilus*. *Journal of Food Science*, **82**(9), 2134-2141.

Diether, N. E., & Willing, B. P. (2019) Microbial Fermentation of Dietary Protein: An Important Factor in Diet⁻Microbe⁻Host Interaction. *Microorganisms*, **7**(1).

Ding, W. K., & Shah, N. P. (2007) Acid, bile, and heat tolerance of free and microencapsulated probiotic bacteria. *Journal of Food Science,* **72**(9), M446-M450.

Ding, W. K., & Shah, N. P. (2009) Effect of Homogenization Techniques on Reducing the Size of Microcapsules and the Survival of Probiotic Bacteria Therein. *Journal of Food Science*, **74**(6), M231-M236.

Doleyres, Y., Paquin, C., LeRoy, M. and Lacroix, C. (2002) *Bifidobacterium longum* ATCC 15707 cell production during free- and immobilized-cell cultures in MRS-whey permeate medium. *Applied Microbiology and Biotechnology,* **60**(1-2), 168-173.

Doleyres, Y., Fliss, I. and Lacroix, C. (2004) Continuous production of mixed lactic starters containing probiotics using immobilized cell technology. *Biotechnology Progress,* **20**(1), 145-150.

Dong, A., Huang, P., and Caughey, W. S. (1992) Redox-dependent changes in beta-extended chain and turn structures of cytochrome-C in water solution determined by 2nd derivative amide-1 infrared-spectra. *Biochemistry*, **31**(1), 182-189.

Donthidi, A. R., Tester, R. F. and Aidoo, K. E. (2010) Effect of lecithin and starch on alginate-encapsulated probiotic bacteria. *Journal of Microencapsulation,* **27**(1), 67-77.

Dziuba, B., Babuchowski, A., Nalecz, D., and Niklewicz, M. (2007) Identification of lactic acid bacteria using FTIR spectroscopy and cluster analysis. *International Dairy Journal,* **7**, 183–189.

Efiuvwevwere, B. J. O., Gorris, L. G. M., Smid, E. J., and Kets, E. P. W. (1999) Mannitol-enhanced survival of *Lactococcus lactis* subjected to drying. *Appl. Microbiol. Biotechnol.* **51**(1), 100–104.

Elliott, A., & Ambrose, E. J. (1950) Evidence of chain folding in polypeptides and proteins. *Discussions of the Faraday Society*, (9), 246

Espina, F., & Packard, V. S. (1979) Survival of *Lactobacillus acidophilus* in a spray-drying process. *J. Food Prot.*, **42** (2), 149–152.

FAO/WHO (2001). Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. [Report of a joint FAO/WHO expert consultation]. *Cordoba, Argentina, 1-4 October, 2001*.

FAO/WHO (2003). Standard for Fermented Milks. *CODEX STAN 243.*

Fatemeh, S., Mustafa, S., Ariff, A., and Manap, Y. A. (2011) Optimization of a cryoprotective medium and survival of freeze-dried *Bifidobacterium infantis* 20088 throughout storage, rehydration and gastrointestinal tract transit for infant formula probiotic applications. *African Journal of Microbiology Research,* **5**(21), 3373-3384.

Faye, T., Tamburello, A., Vegarud, G. E., and Skeie, S. (2012) Survival of lactic acid bacteria from fermented milks in an in vitro digestion model exploiting

sequential incubation in human gastric and duodenum juice. *Journal of Dairy Science*, **95**(2), 558-566.

Ferreira, V., Soares, V., Santos, C., Silva, J., Gibbs, P., 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. *Biotechnology Letters*, **27**(4), 249-252.

Fijan, S. (2014) Microorganisms with Claimed Probiotic Properties: An Overview of Recent Literature. *International Journal of Environmental Research and Public Health*, **11**(5), 4745-4767.

Foerst, P., Kulozik, U., Schmitt, M., Bauer, S., and Santivarangkna, C. (2012) Storage stability of vacuum-dried probiotic bacterium *Lactobacillus paracasei* F19. *FOOD AND BIOPRODUCTS PROCESSING*, **90**(2), 295-300.

Fonseca, F., Beal, C., and Corrieu, G. (2000) Method of quantifying the loss of acidification activity of lactic acid starters during freezing and frozen storage. *Journal of Dairy Research,* **67**(1), 83-90.

Foye, O. T., Huang, I. F., Chiou, C. C., Walker, W. A., and Shi, H. N. (2012) Early administration of probiotic *Lactobacillus acidophilus* and/or prebiotic inulin attenuates pathogen-mediated intestinal inflammation and Smad 7 cell signaling. *Fems Immunology and Medical Microbiology,* **65**(3), 467-480.

Fowler, A., & Toner, M. (2005) Cryo-injury and biopreservation. *Annals of New York Academy of Sciences*, **1066**, 119–135.

Franca, M. B., Panek, A. D., and Eleutherio, E. C. A. (2007) Oxidative stress and its effects during dehydration. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology,* **146**(4), 621-631.

Fredrickson, J. K., Li, S. W., Gaidamakova, E. K., Matrosova, V. Y., Zhai, M., Sulloway, H. M., Scholten, J. C., Brown, M. G., Balkwill, D. L., and Daly, M. J. (2008) Protein oxidation: key to bacterial desiccation resistance? *ISME J*. **2**, 393–403.

Fuller, R. (1989) Probiotics in man and animals. *Journal of Applied Bacteriology,* **66**(5), 365-378.

Fuller, R. (1992) History and development of probiotics. *In: R. Fuller (ed) Probiotics. The scientific basis, Chapman and Hall, London.*, 1-8.

Ganji-Arjenaki, M., & Rafieian-Kopaei, M. (2018) Probiotics are a good choice in remission of inflammatory bowel diseases: A meta analysis and systematic review. *Journal of Cellular Physiology*, **233**(3), 2091-2103.

Gaon, D., Garcia, H., Winter, L., Rodriguez, N., Quintas, R., Gonzalez, S. N. and Oliver, G. (2003) Effect of lactobacillus strains and *Saccharomyces boulardii* on persistent diarrhea in children. *Medicina-Buenos Aires,* **63**(4), 293-298.

Garcia, A. H. (2011) Anhydrobiosis in bacteria: From physiology to applications. *Journal of Biosciences*, **36**(5), 939-950.

Gardiner, G. E., O'Sullivan, E., Kelly, J., Auty, M. A. E., Fitzgerald, G. F., Collins, J. K., *et al*. (2000) Comparative survival rates of human-derived probiotic *Lactobacillus paracasei* and *L. salivarius* strains during heat treatment and spray drying. *Applied and Environmental Microbiology*, **66**(6), 2605-2612.

Garzon-Rodriguez, W., Koval, R. L., Chongprasert, S., Krishnan, S., Randolph, T. W., Warne, N. W., and Carpenter, J. F. (2004) Optimizing storage stability of lyophilized recombinant human interleukin-11 with disaccharide/hydroxyethyl starch mixtures. *Journal of Pharmaceutical Sciences,* **93**, 684–696.

References

Gauger, D. R., Binder, H., Vogel, A., Selle, C., and Pohle, W. (2002) Comparative FTIR-spectroscopic studies of the hydration of diphytanoylphosphatidylcholine and -ethanolamine. *Journal of Molecular Structure*, **614**(1-3), 211-220.

George, S. D. St., Cenkowoski, S., and Muir, W. E. (2004) A review of drying techniques for the preservation of nutritional compounds in waxy skinned fruit. URL:http://www.docentes.unal.edu.co/hamartinezco/docs/Tecnicas%20de%20Preservacion.pdf

Gibbs, B. F., Kermasha, S., Alli, I. and Mulligan, C. N. (1999) Encapsulation in the food industry: a review. *International Journal of Food Sciences and Nutrition,* **50**(3), 213-224.

Gilbert, P., Pemberton, D., and Wilkinson, D. E. (1990) Synergism within polyhexamethylene biguanide biocide formulations. *Journal of Applied Bacteriology,* **69**, 593–598.

Gionchetti, P., Rizzello, F., Venturi, A., Brigidi, P., Matteuzzi, D., Bazzocchi, G., *et al*. (2000) Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: A double-blind, placebo-controlled trial. *Gastroenterology,* **119**(2), 305-309.

Gismondo, M. R., Drago, L. and Lombardi, A. (1999) Review of probiotics available to modify gastrointestinal flora. *International Journal of Antimicrobial Agents,* **12**(4), 287-292.

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 nonelectrolyte stress. *Journal of Bacteriology*, **180**, 4718–4723.

Goderska, K., & Czarnecki, Z. (2008) Influence of micro encapsulation and spray drying on the viability of Lactobacillus and Bifidobacterium strains. *Polish Journal of Microbiology,* **57**(2), 135-140.

Gorry, P. A. (1990) General least-squares smoothing and deferentiation by the convolution (Savitzky-Golay) method. *Analytical Chemistry*, **62**(6), 570-573.

Goodrich, R. P., Crowe, J. H., Crowe, L. M., and Baldeschwieler, J. D. (1991) Alterations in membrane surfaces induced by attachment of carbohydrates. *Biochemistry*, **30**(21), 5313-5318.

Gouin, S. (2004). Microencapsulation: industrial appraisal of existing technologies and trends. *Trends in Food Science & Technology,* **15**(7-8), 330-347.

Gomez, Z. A., Tymczyszyn, E., De Antoni, G., and Anibal, D. E. (2003) Action of trehalose on the preservation of *Lactobacillus delbrueckii ssp. bulgaricus* by heat and osmotic dehydration. *Journal of Applied Microbiology*, **95**, 1315–1320

Grdadolnik, J., & Hadzi, D. (1998) FT infrared and Raman investigation of saccharide-phosphatidylcholine interactions using novel structure probes. *Spectrochimica Acta Part a-Molecular and Biomolecular Spectroscopy*, **54**(12), 1989-2000.

Guerzoni, M. E., Lanciotti, R. and Cocconcelli, P. S. (2001) Alteration in cellular fatty acid composition as a response to salt, acid, oxidative and thermal stresses in *Lactobacillus helveticus*. *Microbiology-Sgm,* **147**, 2255-2264.

Guslandi, M., G. Mezzi, Sorghi, M. and Testoni, P. A. (2000) *Saccharomyces boulardii* in maintenance treatment of Crohn's disease. *Digestive Diseases and Sciences* **45**(7), 1462-1464.

References

Guslandi, M., Giollo, P., and Testoni, P. A. (2003) A pilot trial of Saccharomyces boulardii in ulcerative colitis. *European Journal of Gastroenterology & Hepatology,* **15**(6), 697-698.

Haller, D., Colbus, H., Ganzle, M. G., Scherenbacher, P., Bode, C. and Hammes, W. P. (2001) Metabolic and functional properties of lactic acid bacteria in the gastro-intestinal ecosystem: A comparative in vitro study between bacteria of intestinal and fermented food origin. *Systematic and Applied Microbiology,* **24**(2), 218-226.

Hansen, J. M., Go, Y. M., and Jones, D. P. (2006) Nuclear and mitochondrial compartmentation of oxidative stress and redox signaling. *Annual Review of Pharmacology and Toxicology*, **46**(3), 215-234.

Haris, A., Ramasamy, K., Julianto, T., Sieo, C., and Ho, Y. (2012) Effect of heat, pH and coating process with stearic acid using a fluidized bed granulator on viability of probiotic *Lactobacillus reuteri* C 10. *African Journal of Biotechnology*, **11**(26), 6857-6865.

Hartmann, M., Berditsch, M., Hawecker, J., Ardakani, M. F., Gerthsen, D., and Ulrich, A. S. (2010) Damage of the bacterial cell envelope by antimicrobial peptides gramicidin S and PGLa as revealed by transmission and scanning electron microscopy. *Antimicrobial Agents and Chemotherapy,* **54**, 3132–3142.

Havenaar and Huis In't Veld (1992) Probiotics: a general view. *Lactic acid Bacteria in Health and Disease, (ed. B.J.B Wood), Elsevier Applied Science Publishers, Amsterdam*, **1,** 151-170.

Heckly, R. J. (1985) Principles of preserving bacteria by freeze-drying. *Developments in Industrial Microbiology,* **26**, 379-395.

254

Heidebach, T., Forst, P. and Kulozik, U. (2009) Transglutaminase-induced caseinate gelation for the microencapsulation of probiotic cells. *International Dairy Journal,* **19**(2), 77-84.

Heidebach, T., Forst, P. and Kulozik, U. (2010) Influence of casein-based microencapsulation on freeze-drying and storage of probiotic cells. *Journal of Food Engineering,* **98**(3), 309-316.

Hernández A, Weekers F, Mena J, Zamora J, Borroto C and Thonart P (2007) Culture and Spray-drying of *Tsukamurella paurometabola* C-924: stability of formulated powders. *Biotechnol. Lett*. **29**, 1723–1728

Heyman, M., R. Ducroc, Desjeux, J. F. and Morgat, J. L. (1982) Horseradish-peroxidase transport across adult-rabbit jejunum *in vitro. American Journal of Physiology,* **242**(6), G558-G564.

Hicky, M. (2005) Current legislation of probiotic products. *Probiotic Dairy Products (ed. Adnan Tamine),* Blackwell Publishing Limited, 73-97.

Hidaka, H. & Eida, T. (1988) Roles of fructooligosaccharides for the improvements of intestinal flora and suppression of production of putrid substances. *Shokuhim-Kogyo,* **31**, 52.

Hod, K., Dekel, R., Cohen, N. A., Sperber, A., Ron, Y., Boaz, M., and Maharshak, N. (2018) The effect of a multispecies probiotic on microbiota composition in a clinical trial of patients with diarrhea-predominant irritable bowel syndrome. *Neurogastroenterology and Motility*, **30**(12), 9.

Holzapfel, W. H., Haberer, P., Snel, J., Schillinger, U. and Huis in't Veld, J. H. J. (1998) Overview of gut flora and probiotics. *International Journal of Food Microbiology,* **41**(2), 85-101.

References

Hoover, D. G. & Chen, H. (2005) Bacteriocins with Potential for Use in Foods. *In: P. M. Davidson, J. Sofos and A. L. Branen (eds.), Antimicrobials In Foods* (3rd Edition), **143**, 389-428.

Hubalek, Z. (2003) Protectants used in the cryopreservation of microorganisms. *Cryobiology,* **46**(3), 205-229.

Husson, F., & Josse, J. (2010). Multivariate data analysis. Special focus on clustering and multiway methods. Retrieved from https://www.r-project.org/conferences/useR-2010/tutorials/Husson+Josse.pdf.

Ibanez, M. D., del Rio, P. R., Alsina, D. G. S., and Iglesias, V. V. (2018) Effect of synbiotic supplementation on children with atopic dermatitis: an observational prospective study. *European Journal of Pediatrics*, **177**(12), 1851-1858.

In't Veld, G., Driessen, A. J. M., and Konings, W. N. (1992) Effect of the unsaturation of phospholipid acyl chains on leucine transport of *Lactococcus lactis* and membrane-permeability. *Biochim. Biophys. Acta*, **1108**(1), 31–39.

Ikari, A., Nakano, M., Ishibashi, M., Kawano, K., Suketa, Y., Harada, H., and Takagi, K. (2003) Recovery from heat shock injury by activation of Na+-glucose cotransporter in renal epithelial cells. *Biochimica et Biophysica Acta - Molecular Cell Research,* **1643**, 47–53.

Ishikawa, H., Akedo, I., Umesaki, Y., Tanaka, R., Imaoka, A. and Otani, T. (2003) Randomized controlled trial of the effect of bifidobacteria-fermented milk on ulcerative colitis. *Journal of the American College of Nutrition,* **22**(1), 56-63.

Isolauri, E. & Salminen, S. (2005) Probiotics, gut inflammation and barrier function. *Gastroenterology Clinics of North America,* **34**(3), 437-+.

Jay, J. M. (1991) Modern Food Microbiology. *5th Ed., New York: Van Nostrand Reinhold*.

Jazayeri, O., Daghighi, S. M., and Rezaee, F. (2017) Lifestyle alters GUT-bacteria function: Linking immune response and host. *Best Practice & Research in Clinical Gastroenterology*, **31**(6), 625-635.

Joshi, V. S., & Thorat, B. N. (2011) Formulation and cost-effective drying of probiotic yeast. *Drying Technology,* **29**(7), 749-757.

Kailasapathy, K., & Supriadi, D. (1996) Effect of whey protein concentrate on the survival of *Lactobacillus acidophilus* in lactose hydrolysed yoghurt during refrigerated storage. *Milchwissenschaft-Milk Science International,* **51**(10), 565-569.

Kailasapathy, K., & J. Chin (2000) Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and bifidobacterium spp. *Immunology and Cell Biology*, **78**(1), 80-88.

Kailasapathy, K. (2002) Microencapsulation of probiotic bacteria: technology and potential applications. *Curr Issues Intest Microbiol,* **3**(2), 39-48.

Kashket, E. R. (1987) Bioenergetics of lactic-acid bacteria - cytoplasmic pH and osmotolerance. *Fems Microbiology Reviews,* **46**(3), 233-244.

Katelaris, P. H., Salam, I. and Farthing, M. J. G. (1995) Lactobacilli to prevent travelers' diarrhea. *New England Journal of Medicine,* **333**(20), 1360-1361.

Kearney, N., Meng, X. C., Stanton, C., Kelly, J., Fitzgerald, G. F., and Ross, R. P. (2009) Development of a spray dried probiotic yoghurt containing *Lactobacillus paracasei* NFBC 338. *International Dairy Journal*, **19**(11), 684-689.

References

Kets, E. P. W., Galinski, E. A., deWit, M., deBont, J. A. M. and Heipieper, H. J. (1996) Mannitol, a novel bacterial compatible solute in *Pseudomonas putida* S12. *Journal of Bacteriology,* **178**(23), 6665-6670.

Kets, E. P. W., Teunissen, P. J. M. and DeBont, J. A. M. (1996) Effect of compatible solutes on survival of lactic acid bacteria subjected to drying. *Applied and Environmental Microbiology,* **62**(1), 259-261.

Kilimann, K. V., Doster, W., Vogel, R. F., Hartmann, C., and Ganzle, M. G. (2006) Protection by sucrose against heat-induced lethal and sublethal injury of *Lactococcus lactis*: an FT-IR study. *Biochimica et Biophysica Acta - Proteins and Proteomics,* **1764**, 1188–1197.

Kim, H. S., Kamara, B. J., Good, I. C., and Enders, G. L. (1988) Method for the preparations of stable microencapsulated lactic-acid bacteria. *Journal of Industrial Microbiology*, **3**(4), 253-257.

Kim, S. S., Bhowmik, S. R. (1990) Survival of lactic acid bacteria during spray drying of plain yogurt. *J. Food Sci.*, **55**(4), 1008–1011.

Kim, H. J., Camilleri, M., McKinzie, S., Lempke, M. B., Burton, D. D., Thomforde, G. M., *et al*. (2003) A randomized controlled trial of a probiotic, VSL#3, on gut transit and symptoms in diarrhoea-predominant irritable bowel syndrome. *Alimentary Pharmacology & Therapeutics,* **17**(7), 895-904.

Ko, R., Smith, L. T. and Smith, G. M. (1994) Glycine betaine confers enhanced osmotolerance and cryotolerance on *Listeria monocytogenes*. *Journal of Bacteriology,* **176**(2), 426-431.

Kong, J*., & Yu, S. (2007). Fourier transform infrared spectroscopic analysis of protein secondary structures. *Acta Biochimica et Biophysica Sinica,* **39***, 549–559.

Korobkina, G. S., Brents, M., Kalinana, N. N., Vorob'Eva, V. M., and Shamanova, G. P. (1982) Development of dried acidophilus milk formulae for infant feeding. *Dairy Sci. Abstr.*, **44**(6), 396.

Kos, B., Suskovic, J., Goreta, J., and Matosic, S. (2000) Effect of protectors on the viability of *Lactobacillus acidophilus* M92 in simulated gastrointestinal conditions. *Food Technology and Biotechnology,* **38**(2), 121-127.

Kosanke, J. W., Osburn, R. M., Shuppe, G. I., and Smith, R. S. (1992) Slow rehydration improves the recovery of dried bacterial-populations. *Canadian Journal of Microbiology,* **38**(6), 520-525.

Kotikalapudi, B. L., Low, N. H., Nickerson, M. T., and Korber, D. R. (2010) *In vitro* characterization of probiotic survival, adherence and antimicrobial resistance: candidate selection for encapsulation in a pea protein isolatealginate delivery system. *International Journal of Probiotics and Prebiotics*, **5**(1), 1-12.

Krimm, S., & Bandekar, J. (1986) Vibrational spectroscopy and conformation of peptides, polypeptides, and proteins. *Advances in Protein Chemistry*, **38**, 181–364.

Kudra, T., & Mujumdar, S. (1988) Special drying techniques and novel dryers. Handbook of Industrial Drying. URL: http://203.158.253.140/media/eBook/Engineer/Process/Handbook%20of%20Ind ustrial%20Drying/DK4102_C020.pdf

Kullen, M. J. & Klaenhammer, T. R. (1999) Identification of the pH-inducible, proton-translocating F1F0-ATPase (atpBEFHAGDC) operon of *Lactobacillus acidophilus* by differential display: gene structure, cloning and characterization. *Molecular Microbiology,* **33**(6), 1152-1161.

Kullisaar, T., Zilmer, M., Mikelsaar, M., Vihalemm, T., Annuk, H., Kairane, C., *et al*. (2002) Two antioxidative lactobacilli strains as promising probiotics. *International Journal of Food Microbiology,* **72**(3), 215-224.

Kumar, S. K., Jayaprakasha, H. M., Paik, H. D., Kim, S. K., Han, S. E., Jeong, A. R., *et al*. (2010). Production of ready-to-reconstitute functional beverages by utilizing whey protein hydrolysates and probiotics. *Korean Journal for Food Science of Animal Resources,* **30**(4), 575-581.

Kurtmann, L., Carlsen, C. U., Risbo, J., and Skibsted, L. H. (2009) Storage stability of freeze-dried *Lactobacillus acidophilus* (La-5) in relation to water activity and presence of oxygen and ascorbate. *Cryobiology*, **58**(2), 175-180.

Lankaputhra, W. E. V., & Shah, N. P. (1996) A simple method for selective enumeration of *Lactobacillus acidophilus* in yogurt supplemented with *L-acidophilus* and bifidobacterium spp. *Milchwissenschaft-Milk Science International,* **51**(8), 446-451.

Lankaputhra, W. E. V., Shah, N. P., and Britz, M. L. (1996) Survival of bifidobacteria during refrigerated storage in the presence of acid and hydrogen peroxide. *Milchwissenschaft-Milk Science International,* **51**(2), 65-70.

Lapsiri, W., Bhandari, B., and Wanchaitanawong, P. (2013) Stability and probiotic properties of *Lactobacillus plantarum* spray-dried with protein and other protectants. *Drying Technology,* **31**(13–14), 1723–1733.

Leslie, S. B., Israeli, E., Lighthart, B., Crowe, J. H., and Crowe, L. M. (1995) Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Applied and Environmental Microbiology*, **61**(10), 3592-3597.

Lewis, R., & McElhaney, R. N. (1998) The structure and organization of phospholipid bilayers as revealed by infrared spectroscopy. *Chemistry and Physics of Lipids*, **96**(1-2), 9-21.

Lian, W. C., Hsiao, H. C., and Chou, C. C. (2002) Survival of bifidobacteria after spray-drying. *Int. J. Food Microbiol.*, **74**(1–2), 79–86.

Lievense, L. C., & van 't Riet, K. (1994) Convective drying of bacteria. II. Factors influencing survival. *Advances in biochemical engineering/biotechnology*, **51**, 71-89.

Linders, L. J M., Wolkers, W. F., Hoekstra, F. A., and vantRiet, K., (1997) Effect of added carbohydrates on membrane phase behavior and survival of dried *Lactobacillus plantarum*. *CRYOBIOLOGY*, **35**(1), 31–40.

Linko, P. (1985) Immobilized lactic acid bacteria. *In Enzymes and Immobilized Cells in Biotechnology, ed. A Larson, Minto Park, CA: Benjamin Cummings*, 25-36.

Maathuis, A. J. H., van den Heuvel, E. C., Schoterman, M. H. C., and Venema, K. (2012) Galacto-Oligosaccharides Have Prebiotic Activity in a Dynamic In Vitro Colon Model Using a C-13-Labeling Technique. *Journal of Nutrition,* **142**(7), 1205-1212.

Madene, A., Jacquot, M., Scher, J. and Desobry, S. (2006) Flavour encapsulation and controlled release - a review. *International Journal of Food Science and Technology,* **41**(1), 1-21.

Maity, C., & Gupta, A. K. (2019). A prospective, interventional, randomized, double-blind, placebo-controlled clinical study to evaluate the efficacy and safety of *Bacillus coagulans* LBSC in the treatment of acute diarrhea with abdominal discomfort. *European Journal of Clinical Pharmacology*, **75**(1), 21-31.

References

Maltini, E., Torreggiani, D., Venir, E., and Bertolo, G. (2003) Water activity and the preservation of plant foods. *Food Chemistry,* **82**, 79–86.

Maragkoudaki, M., Chouliaras, G., Moutafi, A., Thomas, A., Orfanakou, A., and Papadopoulou, A. (2018) Efficacy of an Oral Rehydration Solution Enriched with *Lactobacillus reuteri* DSM 17938 and Zinc in the Management of Acute Diarrhoea in Infants: A Randomized, Double-Blind, Placebo-Controlled Trial. *Nutrients*, **10**(9), 11.

Marteau, P., Pochart, P., Bouhnik, Y. and Rambaud, J. C. (1993) The fate and effects of transiting, nonpathogenic microorganisms in the human intestine. *World Review of Nutrition and Dietetics; Intestinal flora, immunity, nutrition and health*, 1-21.

Mary, P., Ochin, D. and Tailliez, R. (1986) Growth status of rhizobia in relation to their tolerance to low water activities and desiccation stresses. *Soil Biology & Biochemistry,* **18**(2), 179-184.

Mary, P., Moschetto, N., and Tailliez, R. (1993) Production and survival during storage of spray-dried *Bradyrhizobium japonicum* cell concentrates, *Journal of Applied Bacteriology*, **74**, 340–344.

Marnett, L. J., Hurd, H. K., Hollstein, M. C., Levin, D. E., Esterbauer, H., and Ames, B. N. (1985) Naturally occurring carbonyl compounds are mutagens in Salmonella tester strain TA 104. *Mutation Research*, **148**, 25–34.

McFarland, L. V., Ship, N., Auclair, J., and Millette, M. (2018) Primary prevention of Clostridium difficile infections with a specific probiotic combining *Lactobacillus acidophilus*, *L. casei*, and *L. rhamnosus* strains: assessing the evidence. *Journal of Hospital Infection*, **99**(4), 443-452.

McLauchlan, G., Fullarton, G. M., Crean, G. P. and McColl, K. E. (1998) Comparison of gastric body and antral pH : a 24 hour ambulatory study in healthy volunteers. *Gut,* **30**, 573-578.

Meng, X. C., Stanton, C., Fitzgerald, G. F., Daly, C. and Ross, R. P. (2008) Anhydrobiotics: The challenges of drying probiotic cultures. *Food Chemistry,* **106**(4), 1406-1416.

Miao, S., Mills, S., Stanton, C., Fitzgerald, G. F., Roos, Y., and Ross, R. P. (2008) Effect of disaccharides on survival during storage of freeze dried probiotics. *Dairy Science & Technology*, **88**, 19–30.

Mille, Y., Obert, J. P., Beney, L. and Gervais, P. (2004) New drying process for lactic bacteria based on their dehydration behavior in liquid medium. *Biotechnology and Bioengineering,* **88**(1), 71-76.

Mills, S., Stanton, C., Fitzgerald, G. F., and Ross, R. P. (2011) Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again. *Microbial Cell Factories*, **10**, 15.

Mokoena, M. P. (2017). Lactic Acid Bacteria and Their Bacteriocins: Classification, Biosynthesis and Applications against Uropathogens: A Mini-Review. *Molecules*, **22**(8), 13.

Morgan, C. A., Herman, N., White, P. A. and Vesey, G. (2006) Preservation of micro-organisms by drying; A review. *Journal of Microbiological Methods,* **66**(2), 183-193.

Mortazavian, A., Razavi, S. H., Ehsani, M. R. and Sohrabvandi, S. (2007) Principle's and methods of microencapsulation of probiotic microorganisms. *Iranian Journal of Biotechnology,* **5**(1), 1-18.

References

Motlagh, A. M., Johnson, M. C. and Ray, B. (1991) Viability loss of foodborne pathogens by starter culture metabolites. *Journal of Food Protection,* **54**(11), 873-&.

Mugnier, J. & Jung, G. (1985) Survival of Bacteria and Fungi in Relation to Water Activity and the Solvent Properties of Water in Biopolymer Gels. *Applied and Environmental Microbiology*, **50**, 108-114.

Muller, H. (2016). Probiotics for prevention and therapy of traveler's diarrhea - Study results shows efficacy of a new developed synbiotic. *Flugmedizin Tropenmedizin Reisemedizin*, **23**(5), 232-236.

Munoz-Atienza, E., Araujo, C., Lluch, N., Hernandez, P. E., Herranz, C., Cintas, L. M., and Magadan, S. (2015) Different impact of heat-inactivated and viable lactic acid bacteria of aquatic origin on turbot (*Scophthalmus maximus L*.) head-kidney leucocytes. *Fish & Shellfish Immunology*, **44**(1), 214-223.

Murray, B. S., Dickinson, E., Du, Z. P., Ettelaie, R., Kostakis, T., and Vallet, J. (2005) Disproportionation kinetics of air bubbles stabilized by food proteins and nanoparticles. *In E. Dickinson (Ed.), Food Colloids: Interactions, Microstructure and Processing,* 259-272, Cambridge: Royal Soc Chemistry.

Muthukumarasamy, P., Allan-Wojtas, P. and Holley, R. A. (2006) Stability of *Lactobacillus reuteri* in different types of microcapsules. *Journal of Food Science,* **71**(1), M20-M24.

Naidu, A. S. & Clemens, R. A. (2000) Probiotics. *Natural Food Antimicrobial Systems (Ed. A. S. Naidu),* CRC Press LLC., 432-462.

Nag, A., Han, K. S., and Singh, H. (2011) Microencapsulation of probiotic bacteria using pH-induced gelation of sodium caseinate and gellan gum. *International Dairy Journal*, **21**(4), 247-253.

Nag, A., & Das, S. (2013) Improving ambient temperature stability of probiotics with stress adaptation and fluidized bed drying. *Journal of Functional Foods*, **5**(1), 170-177.

Nazzaro, F., Fratianni, F., Nicolaus, B., Poli, A., and Orlando, P. (2012) The prebiotic source influences the growth, biochemical features and survival under simulated gastrointestinal conditions of the probiotic *Lactobacillus acidophilus*. *Anaerobe*, **18**(3), 280-285.

Oldenhof, H., Wolkers, W. F., Fonseca, F., Passot, S. P., and Marin, M. (2005) Effect of sucrose and maltodextrin on the physical properties and survival of air-dried *Lactobacillus bulgaricus*: an *in situ* Fourier transform infrared spectroscopy study. *Biotechnology Progress,* **21**, 885–892.

O'Grady. B. & Gibson. R. G. (2005) Microbiota of the human gut. *Probiotic Dairy Products (ed. Adnan Tamine),* Blackwell Publishing Limited, 1-15.

O'May, G. A. & Macfarlane, G.T. (2005) Health claims associated with probiotics. *Probiotic Dairy Products (ed. Adnan Tamine),* Blackwell Publishing Limited, 138-166.

O'Riordan, K., Andrews, D., Buckle, K., and Conway, P. (2001) Evaluation of microencapsulation of a Bifidobacterium strain with starch as an approach to prolonging viability during storage. *Journal of Applied Microbiology,* **91**, 1059–1066.

References

O'Sullivan, M. A. & O'Morain, C. A. (2000) Bacterial supplementation in the irritable bowel syndrome: A randomised double-blind placebo-controlled crossover study. *Digestive and Liver Disease,* **32**(4), 294-301.

Oksanen, P. J., Salminen, S., Saxelin, M., Hamalainen, P., Ihantolavormisto, A., Muurasniemiisoviita, L., *et al*. (1990) Prevention of travelers diarrhea by lactobacillus GG. *Annals of Medicine,* **22**(1), 53-56.

Orndorff, G. R. and Mackenzi.Ap (1973) Function of suspending medium during freeze-drying preservation of *Escherichia coli. Cryobiology,* **10**(6), 475-487.

Pace, F., Pace, M., and Quartarone, G. (2015) Probiotics in digestive diseases: focus on *Lactobacillus GG*. *Minerva Gastroenterologica E Dietologica*, **61**(4), 273-292.

Park, H. J., Lee, G. H., Jun, J., Son, M., and Kang, M. J. (2016) Multiple-unit tablet of probiotic bacteria for improved storage stability, acid tolerability, and in vivo intestinal protective effect. *Drug Design Development and Therapy*, **10**, 1355-1364.

Pehkonen, K. S., Roos, Y. H., Miao, S., Ross, R. P. and Stanton, C. (2008) State transitions and physicochemical aspects of cryoprotection and stabilization in freeze-drying of *Lactobacillus rhamnosus* GG (LGG). *Journal of Applied Microbiology,* **104**(6), 1732-1743.

Perez, V. M., Gonzalez, C. N. O., and Torres, G. Z. (2019) Impact of the Association of fermented milk intake and hypertension: a systematic review. *Poblacion Y Salud En Mesoamerica*, **16**(2), 18.

Pichereau, V., Hartke, A., and Auffray, Y. (2000) Starvation and osmotic stress induced multiresistances influence of extracellular compounds. *International Journal of Food Microbiology*, **55**(1-3), 19-25.

Plummer, S., Weaver, M. A., Harris, J. C., Dee, P. and Hunter, J. (2004) Clostridium difficile pilot study: effects of probiotic supplementation on the incidence of C-difficile diarrhoea. *International Microbiology,* **7**(1), 59-62.

Poddar, D., Das, S., Jones, G., Palmer, J., Jameson, G. B., Haverkamp, R. G., and Singh, H. (2014) Stability of probiotic *Lactobacillus paracasei* during storage as affected by the drying method. *International Dairy Journal,* **39**, 1–7.

Poolman, B. & Glaasker, E. (1998) Regulation of compatible solute accumulation in bacteria. *Molecular Microbiology,* **29**(2), 397-407.

Potts, M., Slaughter, S. M., Hunneke, F., Garst, J. F., and Helm, R. F. (2005) Desiccation Tolerance of Prokaryotes: Application of Principles to Human Cells. *Integr. Comp. Biol.* **45**, 800–809.

Pramanick, R., Parab, S., Mayadeo, N., Warke, H., and Aranha, C. (2018) Cross sectional analysis of vaginal Lactobacillus in asymptomatic women of reproductive age in Mumbai, India. *Journal of Infection in Developing Countries*, **12**(12), 1096-1104.

Prasad, J., McJarrow, P., and Gopal, P. (2003) Heat and osmotic stress responses of probiotic *Lactobacillus rhamnosus* HN001 (DR20) in relation to viability after drying. *Applied and Environmental Microbiology*, **69**(2), 917-925.

Quigley, E. M. M. (2019) Prebiotics and Probiotics in Digestive Health. *Clinical Gastroenterology and Hepatology*, **17**(2), 333-344.

Rajam, R., Karthik, P., Parthasarathi, S., Joseph, G. S., and Anandharamakrishnan, C. (2012) Effect of whey protein-alginate wall systems on survival of microencapsulated *Lactobacillus plantarum* in simulated gastrointestinal conditions. *Journal of Functional Foods*, **4**(4), 891-898.

References

Ranadheera, R., Baines, S. K., and Adams, M. C. (2010) Importance of food in probiotic efficacy. *Food Research International*, **43**(1), 1-7.

Rao, A. V., Shiwnarain, N. and Maharaj, I. (1989) Survival of microencapsulated *Bifidobacterium pseudolongum* in simulated gastric and intestinal juices. *Canadian Institute of Food Science and Technology Journal,* **22**(4), 345-349.

Rebecchi, L., Altiero, T., and Guidetti, R. (2007) Anhydrobiosis: the extreme limit of desiccation tolerance. *Invertebr. Surv. J*, **4**, 65–81.

Rees, C. E. D., Dodd, C. E. R., Gibson, P. T., Booth, I. R. and Stewart, G. (1995) The significance of bacteria in stationary phase to food microbiology. *International Journal of Food Microbiology,* **28**(2), 263-275.

Remmele, R. L., Stushnoff, C., and Carpenter, J. F. (1997) Real-time in situ monitoring of lysozyme during lyophilization using infrared spectroscopy: Dehydration stress in the presence of sucrose. *Pharmaceutical Research*, **14**(11), 1548-1555.

Reuter, G. (1997) Present and future of probiotics in Germany and in Central Europe. *Biosci. Microflora*, **16**, 43-51.

Ricker, J. V., Tsvetkova, N. M., Wolkers, W. F., Leidy, C., Tablin, F., Longo, M., and Crowe, J. H. (2003) Trehalose maintains phase separation in an air-dried binary lipid mixture. *Biophysical Journal*, **84**(5), 3045-3051.

Rieppo, L., Saarakkala, S., Narhi, T., Helminen, H. J., Jurvelin, J. S., and Rieppo, J. (2012) Application of second derivative spectroscopy for increasing molecular specificity of fourier transform infrared spectroscopic imaging of articular cartilage. *Osteoarthritis and Cartilage*, **20**(5), 451–459.

Ringer, D. U., & Mujumdar, A. S. (1984) Analysis of aerodynamics and heat-transfer in vibro-fluidized beds. *Drying Technology,* **2**(4), 449-470.

Roelans, E., & Taeymans, D. (1990) Effect of drying conditions on survival and enzyme activity of microorganims. *In Engineering and Food Volume 3 Advanced Processes; Spiess, W. E. L., Schubert, H., Eds.*; Elsevier Applied Science: London, New York, 559–569.

Roos, Y. H. (2010) Glass Transition Temperature and Its Relevance in Food Processing. In *M. P. Doyle & T. R. Klaenhammer (Eds.), Annual Review of Food Science and Technology*, **1**, 469-496, Palo Alto: Annual Reviews.

Rosenberg, M., Gutnick, D., and Rosenberg, E. (1980) Adherance of bacteria to hydrocarbons – A simple method for measuring cell-surface hydrophobicity. *Fems Microbiology Letters*, **9**(1), 29-33.

Rosenberg, M. & Sheu, T. Y. (1996) Microencapsulation of volatiles by spray-drying in whey protein-based wall systems. *International Dairy Journal,* **6**(3)**,** 273-284.

Rosenfeldt, V., Michaelsen, K. F., Jakobsen, M., Larsen, C. N., Moller, P. L., Pedersen, P., *et al*. (2002) Effect of probiotic lactobacillus strains in young children hospitalized with acute diarrhea. *Pediatric Infectious Disease Journal,* **21**(5), 411-416.

Saarela, M., Mogensen, G., Fonden, R., Matto, J., and Mattila-Sandholm, T. (2000) Probiotic bacteria: safety, functional and technological properties. *Journal of Biotechnology*, **84**(3), 197-215.

Saarela, M., Virkajarvi, I., Alakomi, H. L., Mattila-Sandholm, T., Vaari, A., Suomalainen, T., *et al*. (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. *Journal of Applied Microbiology,* **99**(6), 1330-1339.

Salminen, S., Isolauri, E. and Salminen, E. (1996) Clinical uses of probiotics for stabilizing the gut mucosal barrier: Successful strains and future challenges. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology,* **70**(2-4), 347-358.

Salminen, S., Bouley, C., Boutron-Ruault, M. C., Cummings, J. H., Franck, A., Gibson, G. R., *et al.* (1998) Functional food science and gastrointestinal physiology and function. *British Journal of Nutrition,* **80**, S147-S171.

Santivarangkna, C., Wenning, M., Foerst, P., & Kulozik, U. (2007). Damage of cell envelope of *Lactobacillus helveticus* during vacuum drying. *Journal of Applied Microbiology*, **102**(3), 748-756.

Santivarangkna, C., Kulozik, U., and Foerst, P. (2006) Effect of carbohydrates on the survival of *Lactobacillus helveticus* during vacuum drying. *Letters in Applied Microbiology*, **42**, 271–276.

Santivarangkna, C., Kulozik, U., and Foerst, P. (2007) Alternative drying processes for the industrial preservation of lactic acid starter cultures, *Biotechnology Progress*, **23**, 302–315.

Santivarangkna, C., Higl, B., and Foerst, P. (2008) Protection mechanisms of sugars during different stages of preparation process of dried lactic acid starter cultures. *Food Microbiology,* **25**(3), 429-441.

Santivarangkna, C., Naumann, B., Kulozik, U., and Foerst, P. (2010) Protective effects of sorbitol during the vacuum drying of *Lactobacillus helveticus*: an FT-IR study, *Ann Microbiol,* **60**, 235–242.

Sarkar, S. (2010) Approaches for enhancing the viability of probiotics: a review. *British Food Journal*, **112**(4), 329-349.

Sarkar, S. (2013) Potential of probiotics as pharmaceutical agent: a review. *British Food Journal*, **115**(11), 1658-1687.

Savic, D., Jokovic, N., and Topisirovic, L. (2008) Multivariate statistical methods for discrimination of lactobacilli based on their FTIR spectra. *Dairy Science & Technology*, **88**(3), 273-290.

Savini, M., Cecchini, C., Verdenelli, M. C., Silvi, S., Orpianesi, C., and Cresci, A. (2010) Pilot-scale production and viability analysis of freeze-dried probiotic bacteria using different protective agents. *Nutrients,* **2**, 330–339.

Savitzky, A., & Golay, M. J. E. (1964) Smoothing + differentiation of data by simplified least squares procedures. *Analytical Chemistry*, **36**(8), 1627-&.

Saxelin, M., Lassig, A., Karjalainen, H., Tynkkynen, S., Surakka, A., Vapaatalo, H., *et al*. (2010) Persistence of probiotic strains in the gastrointestinal tract when administered as capsules, yoghurt, or cheese. *International Journal of Food Microbiology,* **144**(2), 293-300.

Scarpato, E., Quitadamo, P., Roman, E., Jojkic-Pavkov, D., Kolacek, S., Papadopoulou, A., and Staiano, A. (2017) Functional Gastrointestinal Disorders in Children: A Survey on Clinical Approach in the Mediterranean Area. *Journal of Pediatric Gastroenterology and Nutrition*, **64**(6), E142-E146.

Schillinger, U., Guigas, C., and Holzapfel, W. H. (2005) *In vitro* adherence and other properties of lactobacilli used in probiotic yoghurt-like products. *International Dairy Journal*, **15**(12), 1289-1297.

References

Schule, S., Friess, W., Bechtold-Peters, K., and Garidel, P. (2007) Conformational analysis of protein secondary structure during spray-drying of antibody/mannitol formulations. *European Journal of Pharmaceutics and Biopharmaceutics*, **65**(1), 1-9.

Seki, H., Shiohara, M., Matsumura, T., Miyagawa, N., Tanaka, M., Komiyama, A., *et al*. (2003) Prevention of antibiotic-associated diarrhea in children by *Clostridium butyricum* MIYAIRI. *Pediatrics International,* **45**(1), 86-90.

Selmer-Olsen, E., Sorhaug, T., Birkeland, S. E. and Pehrson, R. (1999) Survival of *Lactobacillus helveticus* entrapped in Ca-alginate in relation to water content, storage and rehydration. *Journal of Industrial Microbiology & Biotechnology,* **23**(2), 79-85.

Semyonov, D., Ramon, O., Kovacs, A., Friedlander, L., and Shimoni, E. (2012) Air-suspension fluidized-bed microencapsulation of probiotics. *Drying Technology*, **30**(16), 1918-1930.

Sen, S., Mullan, M. M., Parker, T. J., Woolner, J. T., Tarry, S. A. and Hunter, J. O. (2002) Effect of *Lactobacillus plantarum* 299v on colonic fermentation and symptoms of irritable bowel syndrome. *Digestive Diseases and Sciences,* **47**(11), 2615-2620.

Shah, N. P. (2000) Probiotic bacteria: Selective enumeration and survival in dairy foods. *Journal of Dairy Science,* **83**(4), 894-907.

Shrestha, A. K., Howes, T., Adhikari, B. P., Wood, B. J., and Bhandari, B. R. (2007) Effect of protein concentration on the surface composition, water sorption and glass transition temperature of spray-dried skim milk powders. *Food Chemistry*, **104**(4), 1436-1444.

Sikkema, J., Debont, J. A. M., and Poolman, B. (1994) Interactions of cyclic hydrocarbons with biological-membranes. *Journal of Biological Chemistry*, **269**(11), 8022-8028.

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

Simo, G., Fernandez-Fernandez, E., Vila-Crespo, J., Ruiperez, V., and Rodriguez-Nogales, J. M. (2017) Research progress in coating techniques of alginate gel polymer for cell encapsulation. *Carbohydrate Polymers*, **170**, 1-14.

Simon, G. L. & Gorbach, S. L. (1982) Intestinal microflora. *Ned. Clin. North Am.* **66**, 557-574.

Simpson, P. J., Stanton, P., Fitzgerald, G. F., and Ross R. P. (2005) Intrinsic tolerance of Bifidobacterium species to heat and oxygen and survival following spray-drying and storage, *Journal of Applied Microbiology*, **99**, 493–501.

Singh, T. P., Kaur, G., Malik, R. K., Schillinger, U., Claudia, G. and Kapila, S. (2012) Characterization of intestinal *Lactobacillus reuteri* strains as potential probiotics, *Probiotics & Antimicro. Prot*., **4**, 47–58.

Singh, T. P., Malik, R. K., and Kaur, G. (2014) Safety assessment and evaluation of probiotic potential of *Lactobacillus reuteri* strains under *in vitro* conditions, *International Journal of Current Microbiology and Applied Sciences*, **3**(2), 335–348.

Singh, T. P., Malik, R. K., Snehal, K. G. and Kaur, G. (2015) Hypocholesterolemic effects of *Lactobacillus reuteri* LR6 in rats fed on high-cholesterol diet, *International Journal of Food Sciences and Nutrition*, **66**(1), 71-75.

References

Smith, L. J., Fiebig, K. M., Schwalbe, H., and Dobson, C. M. (1996) The concept of a random coil - Residual structure in peptides and denatured proteins. *Folding & Design*, **1**(5), R95-R106.

Song, S. H., Cho, Y. H., and Park, J. (2003) Microencapsulation of *Lactobacillus casei* YIT 9018 using a microporous glass membrane emulsification system. *Journal of Food Science,* **68**(1), 195-200.

Stanton, C., Desmond, C., Coakley, M., Collins, J. K., Fitzgerald, G. and Ross, R. P. (2003) Challenges facing development of probiotic-containing functional foods. *Handbook of Fermented Functional Foods*, 27-58.

Streeter, J. G. (2003) Effect of trehalose on survival of *Bradyrhizobium japonicum* during desiccation. *Journal of Applied Microbiology*, **95**, 484–491.

Stummer, S., Toegel, S., Rabenreither, M. C., Unger, F. M., Wirth, M., Viernstein, H., *et al*. (2012) Fluidized-bed drying as a feasible method for dehydration of Enterococcus faecium M74. *Journal of Food Engineering*, **111**(1), 156-165.

Suharja, A. A. S., Henriksson, A., and Liu, S. Q. (2014) Impact of *Saccharomyces cerevisiae* on viability of probiotic *Lactobacillus rhamnosus* in fermented milk under ambient conditions. *Journal of Food Processing and Preservation*, **38**(1), 326-337.

Sun, W. Q., & Leopold, A. C. (1997) Cytoplasmic vitrification acid survival of anhydrobiotic organisms. *Comparative Biochemistry and Physiology a-Physiology*, **117**(3), 327-333.

Susi, H., & Byler, D. M. (1986) Resolution-enhanced FT-infrared spectra of proteins with parallel-beta-chains. *Biophysical Journal*, **49**(2), A295-A295.

Tannock, G. W., Munro, K., Harmsen, H. J. M., Welling, G. W., Smart, J. and Gopal, P. K. (2000) Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. *Applied and Environmental Microbiology,* **66**(6), 2578-2588.

Tannock, G. W. (2003) Probiotics: time for a dose of realism. [Research Support, Non-U.S. Gov't; Review]. *Curr Issues Intest Microbiol,* **4**(2), 33-42.

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

Teixeira, P., Castro, H., and Kirby, R. (1996) Evidence of membrane lipid oxidation of spray-dried *Lactobacillus bulgaricus* during storage, *Letters in Applied Microbiology*, **22**, 34–38.

Teixeira, P., Castro, H., MohacsiFarkas, C., and Kirby, R. (1997) Identification of sites of injury in *Lactobacillus bulgaricus* during heat stress. *Journal of Applied Microbiology*, **83**(2), 219-226.

Thomas, M. R., Litin, S. C., Osmon, D. R., Corr, A. P., Weaver, A. L. and Lohse, C. M. (2001) Lack of effect of *Lactobacillus* GG on antibiotic-associated diarrhea: A randomized, placebo-controlled trial. *Mayo Clinic Proceedings,* **76**(9), 883-889.

Tiscornia, I., Sanchez-Martins, V., Hernandez, A., and Bollati-Fogolin, M. (2012) Human monocyte-derived dendritic cells from leukoreduction system chambers after plateletpheresis are functional in an in vitro co-culture assay with intestinal epithelial cells. *Journal of Immunological Methods*, **384**(1-2), 164-170.

To, B. C. S. & Etzel, M. R. (1997) Spray drying, freeze drying, or freezing of three different lactic acid bacteria species. *Journal of Food Science,* **62**(3), 576-&.

References

Tripathi, M. K., & Giri, S. K. (2014) Probiotic functional foods: Survival of probiotics during processing and storage. *Journal of Functional Foods*, **9**, 225-241.

Tsvetkov, T., & Brankova, R. (1983) Viability of micrococci and lactobacilli upon freezing and freeze-drying in the presence of different cryoprotectants. *Cryobiology,* **20**(3), 318-323.

Tuomola, E. M., & Salminen, S. J. (1998) Adhesion of some probiotic and dairy Lactobacillus strains to Caco-2 cell cultures. *International Journal of Food Microbiology*, **41**(1), 45-51.

Tuomola, E., Crittenden, R., Playne, M., Isolauri, E., and Salminen, S. (2001) Quality assurance criteria for probiotic bacteria. *American Journal of Clinical Nutrition*, **73**(2), 393S-398S.

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**, 187–216.

Vandenplas, Y., Huys, G., & Daube, G. (2015) Probiotics: an update. *Jornal De Pediatria*, **91**(1), 6-21.

Vega, C., & Roos, Y. H. (2006) Invited review: Spray-dried dairy and dairy-like - emulsions compositional considerations. *Journal of Dairy Science*, **89**(2), 383-401.

Venema, K. (2018) Modulation of the gut microbiota by probiotics and prebiotics. *Febs Open Bio*, **8**, 83-83.

Venugopalan, V., Shriner, K. A., and Wong-Beringer, A. (2010) Regulatory oversight and safety of probiotic use. *Emerging Infectious Diseases*, **16**(11), 1661-1665.

Viernstein, H., Raffalt, J., and Polheim, D. (2005) Stabilisation of probiotic microorganisms - An overview of the techniques and some commercially available products. *Applications of Cell Immobilisation Biotechnology,* **8B**, 439-453.

Vinusha, K. S., Deepika, K., Johnson, T. S., Agrawal, G. K., and Rakwal, R. (2018) Proteomic studies on lactic acid bacteria: A review. *Biochemistry and biophysics reports*, **14**, 140-148.

Vittadini, E., Dickinson, L.C. and Chinachoti, P. (2002) NMR water mobility in xanthan and locust bean gum mixtures: possible explanation of microbial response. *Carbohydate Polymers*, **49**, 261-269.

Vittadini, E., Chinachoti1, P., Lavoie, J. P., and Pham, X. (2005) Correlation of microbial response in model food systems with physico-chemical and "mobility" descriptors of the media. *Innovative Food Science and Emerging Technologies*, **6**, 21– 28.

Wang, Y. C., Yu, R. C., and Chou, C. C. (2004) Viability of lactic acid bacteria and bifidobacteria in fermented soymilk after drying, subsequent rehydration and storage, *International Journal of Food Microbiology*, **93**, 209–217.

Williams, N. T. (2010) Probiotics. *American Journal of Health-System Pharmacy*, **67**(6), 449-458.

Wyber, J. A., Andrews, J., and Gilbert, P. (1994) Loss of salt-tolerance and transformation efficiency in *Escherichia coli* associated with sublethal injury by centrifugation. *Letters in Applied Microbiology,* **19**, 312–316.

References

Ye, Q. Y., Georges, N., and Selomulya, C. (2018) Microencapsulation of active ingredients in functional foods: From research stage to commercial food products. *Trends in Food Science & Technology*, **78**, 167-179.

Yildirim, S., Konrad, D., Calvez, S., Drider, D., Prevost, H., and Lacroix, C. (2007) Production of recombinant bacteriocin divercin V41 by high cell density *Escherichia coli* batch and fed-batch cultures. *Applied Microbiology and Biotechnology*, **77**(3), 525-531.

Yonekura, L., Sun, H., Soukoulis, C., and Fisk, I. (2014) Microencapsulation of *Lactobacillus acidophilus* NCIMB 701748 in matrices containing soluble fibre by spray drying: Technological characterization, storage stability and survival after *in vitro* digestion. *Journal of Functional Foods*, **6**, 205-214.

Zhao, M., Wang, Y., Huang, X., Gaenzle, M., Wu, Z. J., Nishinari, K., and Fang, Y. P. (2018) Ambient storage of microencapsulated *Lactobacillus plantarum* ST-III by complex coacervation of type-A gelatin and gum arabic. *Food & Function*, **9**(2), 1000-1008.