Impact of cryoprotectants during freeze drying on *Lactobacillus plantarum* viability and their role in enhancing probiotic storage stability

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Abstract

The human microbiome has recently garnered the interest of scientists and biopharma industries as studies have revealed the potential use of live bacteria known as probiotics as potential therapeutics for restoring and maintaining human health. These probiotic-biopharma formulations must contain the right strain(s) in sufficient numbers when administered to confer the desired health benefit. Cell dehydration is used to keep the probiotic microbes in an inactivated form during storage, thereby ensuring that there are enough viable cells still present when the probiotic is taken. However, the drying process itself is detrimental to the probiotic cells and can result in reduced viability and stability of cells over storage.

In this study, various cryoprotectants were assessed for their ability to maintain cell integrity and improve yield during the freeze drying dehydration of *Lactobacillus plantarum* towards a potential topical pharmabiotic formulation. Inulin, sucrose, maltodextrin, and skimmed milk at 10% m/v concentration of the drying media were tested for their ability to protect bacterial cells during freeze drying and over a storage period of 12 weeks at 4°C and room temperature. Furthermore glucose, inulin, sucrose, and maltodextrin as sole carbon substrate were investigated as prebiotics in concentrations of 0.5% m/v, 2% m/v, and 4% m/v of the fermentation media by *in vitro* fermentation of *L. plantarum* in glucose-free MRS-free media. The influence of these cryoprotectants and prebiotics on *L. plantarum* was measured against cell viability, growth kinetic parameters (growth rate, lag phase, and maximum cell density), and pH reduction potential of *L. plantarum*.

Improved survival of *L. plantarum* during freeze drying and over 12-weeks of storage was observed with all cryoprotectants. Skimmed milk demonstrated the highest protection after freeze drying, with a survival rate of 91% and viable cell counts of 9.1×10^8 $(\frac{\text{CFU}}{\text{ml}})$ from an initial cell count prior to drying of 1.0×10^9 $(\frac{\text{CFU}}{\text{ml}})$. Inulin demonstrated high protective efficiency, with 85% viability maintained during freeze drying which resulted in final cell counts of 1.1×10^9 $(\frac{\text{CFU}}{\text{ml}})$ from an initial cell count of 1.3×10^9 $(\frac{\text{CFU}}{\text{ml}})$. However, inulin provided the least protection over the 12 week storage period compared to cells dried in the presence of maltodextrin, sucrose, and skimmed milk, with cell counts of only 1.2×10^6 $(\frac{\text{CFU}}{\text{ml}})$ at 4°C and 6.3×10^3 $(\frac{\text{CFU}}{\text{ml}})$ at room temperature recorded at the end of the period. Following skimmed milk, which also demonstrated the highest stability of cells over storage, sucrose performed second best in maintaining the stability of cells at 4°C at the end of the 12 weeks storage, with viability of 33% which resulted in final cell counts of 3.4×10^8 $(\frac{CFU}{ml})$.

Overall, the presence of cryoprotectants and prebiotics demonstrated a significant influence on propagation and viability. The presence of each of the various prebiotics as the sole carbon substrate in the fermentation media promoted proliferation of *L. plantarum*. An increase in cryoprotectant concentrations led to increased biomass yield but with no significant change in the growth rate and lag phase. Cells showed improved stability when stored at 4^{o}C compared to room temperature. A delay in propagation up to 10 hours was observed upon rehydration of stored probiotic cells across all cases except for skimmed milk that resulted in a maximum delay in propagation of 2 hours at both storage temperatures.

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Glossary of terms

Ave	average
BV	bacterial vaginosis
CeBER	Center for Bioprocess Engineering Research
P_c	chamber vapour pressure
T_c	collapse temperature
CFU	colony-forming units
$\frac{CFU}{capsule}$	colony-forming units per capsule
$\frac{CFU}{g}$	colony-forming units per gram
$\frac{CFU}{ml}$	colony-forming units per millilitre
MRS	De Man-Rogosa-Sharpe
EFSA	European Food Safety Authority
T_g'	glass temperature at frozen state
T_g	glass temperature at non frozen state
g/100ml	gram per hundred millilitres
g/L	gram per litre
LAB	lactic acid bacteria
t_{lag}	lag time
Log	logarithm
m/v	mass per volume
OD_{max}	maximum cell density
T_m	melting point temperature
µl	microlitre
ml/L	millilitre per litre
mTorr	milli torr
M_w	moisture content
M	molar mass
ln	natural logarithm
N_o	number of cells initially present
N_t	number of cells present at time t
OD	optical density
OD_{660}	optical density at 660 nm
Pa	pascal

R_p	resistance of dry product layer
R_s	resistance of drying stopper
RT	room temperature
SEM	scanning electron microscopic
μ_{max}	specific growth rate
SD	standard deviation
SEM	standard error
P_{ice}	sublimation front vapour pressure
TEM	transmission electron microscopic
T_r	triple point temperature
USA FDA	United States of America Food and Drug Authority
UCT	University of Cape Town
v/v	volume per volume
wt	weight
W_{db}	weight of dry biomass
W_{fs}	weight of freeze dried sample
W_{os}	weight of sample
W_{wb}	weight of wet biomass

1 INTRODUCTION

1.1 Background of study

The global health crisis of antimicrobial resistance is growing as pathogenic microorganisms are acquiring resistance to currently available antimicrobials and antibiotics. The frequent use as well as misuse of antimicrobials are prominent causes of the antimicrobial resistance. As a result, infections caused by pathogenic bacteria are becoming more difficult to treat. This crisis is said to have a devastating cost on human society as pathogenic disease infections increase, such as pneumonia, tuberculosis, and gonorrhea (Michael, Dominey-Howes and Labbate, 2014). The ineffectiveness of antimicrobials leads to longer hospital stays, increased medical cost, and mortality rate (WHO, 2017). This means that there is an urgent need for other effective alternative therapies to already existing antimicrobials.

The symbiotic relationship between the human body and its microbiome, overlooked in the past decades, has recently gathered growing interest amongst researchers. This developing body of research is identifying ways the human microbiome can be used to prevent and treat various medical conditions. These conditions include eczema, acne, inflammatory bowel disease, dental cavities, cardiovascular diseases, bacterial vaginosis, asthma, allergies, to name a few (Imperial and Ibana, 2016; Broeckx *et al.*, 2017). There are extensive studies on the relationship between the human microbiome and the gastrointestinal tract, but there is a relative paucity of studies on vaginal infections, the skin, and cardiovascular health (Reid and Bruce, 2003; Rastogi *et al.*, 2011; Thushara *et al.*, 2016). More research is therefore needed in these areas. This should include the integration and collaboration of various fields spanning from molecular biology and health science in identifying effective strains, to bioprocessing in realizing the potential of large-scale production and commercialization of identified strains.

The work in this thesis is in support of the development of topical probiotics for the treatment of bacterial vaginosis and bacterial-associated acne vulgaris. These are direct pathogen-induced infections. Bacterial vaginosis (BV) is a condition that results from the disruption of the vaginal flora, leading to a reduced population of commensal bacteria (mainly *Lactobacillus* species) and an increase in pathogenic bacteria, including *Gardnerella vaginalis, Prevotella* species, *Atopobium vaginae* and *Mobiluncus* species (Happel *et al.*, 2020). Acne vulgaris (AV) affects the outer layer of the skin, is a chronic skin disorder and is multifactorial. *Propionibacterium acnes* within the follicle is a triggering factor associated with inflammation in acne (Lebeer *et al.*, 2018). The current primary treatments for both infections have been antibiotics. These include nitroimidazole, clindamycin, secnidazole, and metronidazole for BV and doxycycline, minocycline, and clindamycin for AV (Lebeer *et al.*, 2018; Javed, Parvaiz and Manzoor, 2019). However, an increase

in the prevalence of antibiotic-resistant strains of microorganisms associated with these infections have been observed and have been linked to the prevailing reoccurrences in affected patients (Lebeer *et al.*, 2018; Javed, Parvaiz and Manzoor, 2019).

In addressing the problem of antibiotic resistance, the use of probiotics for treating diseases has in recent years sparked increasing interest amongst researchers. Probiotics are defined as live microorganisms that when administered in the adequate amount confer health benefits on the host (Hill *et al.*, 2014).

Numerous studies have shown that instead of killing pathogenic microbes using antibiotics, the establishment of commensal and sometimes mutualistic microbes may hinder the growth of disease-causing microbes found in the same host microbial environment and additionally promote the population of beneficial bacteria. These beneficial microbes eliminate the disease-causing bacteria through antagonistic mechanisms such as competitive adhesion advantage to host cells, production of lactic acid, production of hydrogen peroxide, and production of bacteriocins. The bioproducts lower the pH, making the environment unsuitable for pathogens to survive (Reid and Bruce, 2003; Goldin, 2019; Plaza-Diaz *et al.*, 2019).

Since the vaginal microbiota of women with BV have been found to contain a reduced number of *Lactobacilli* in comparison with healthy women, various *Lactobacillus* strains administered orally or intra-vaginally have been tested for their effectiveness in treating BV (Falagas, Betsi and Athanasiou, 2007). Scientist have highlighted and proposed a natural alternative therapy to antimicrobial which is the application of live *Lactobacillus* to eliminate pathogenic bacteria by competitive adhesive mechanism and the production of bacteriocins and lactic acid which restores the pathogen antagonistic acidity of the vaginal microbiome (Reid and Bruce, 2003; Sreeja and Prajapati, 2013). Even though little research has been done to verify the effectiveness of *Lactobacillus* strains to promote skin health, Sanders *et al*. (2018) have proposed that strains within the same taxa share similar antagonistic mechanisms.

For probiotic treatments to be effective, it is important that strains in a probiotic formulation have been clinically proven to confer the desired benefits and they must be present in the sufficient number upon administration, as the definition implies. The functionality and efficiency of probiotics can be compromised due to loss of viability during production and over storage periods (Broeckx *et al.*, 2017). This is because various environmental stresses are imposed on probiotic cells during processing and storage. Moreover, the presence of moisture within the cell membrane is detrimental to bacterial cells as water promotes biomolecular interactions within cells. These interactions release metabolites that are detrimental to cells over a period, drastically reducing bacterial stability during storage as a result (Aschenbrenner, Foerst and Kulozik, 2015). Hence the

removal of moisture (dehydration) is an important step in the production process of probiotics. Long term delivery of probiotics can be optimally achieved by preserving and protecting them in a dry form before further necessary formulation. However, drying processes – spray drying, freeze drying, vacuum drying and fluidized bed drying – contribute to stress induced on the probiotic species, hence compromising probiotic viability and stability (Otero, Espeche and Nader-Macías, 2007; Broeckx *et al.*, 2016).

Freeze drying is a frequently used and preferred method for drying *Lactobacillus* cultures because it is the most suited for long term preservation of the bacteria. This is due to its low operating temperature and pressure. At these conditions, the native structure, biochemical properties, and activities of bacterial cells are retained. However, freezing subjects the bacterial cultures to low temperature stress and mechanical stress by ice crystals, and can thereby cause loss of viability and acidification activity of the bacteria. In addition, low product yield, subzero temperatures and batch processing method increases the final cost of frozen cultures (Reddy *et al.*, 2009). To commercialize probiotics, timesaving, and cost-effective methods to increase bacterial cell yield during the production process are necessary.

Several protection strategies have been reported and have been found to enhance probiotic viability. These include addition of protective agents, controlling the fermentation process parameters and pre-stressing the probiotics prior to drying (Derzelle *et al.*, 2003; Chávez and Ledeboer, 2007; Savini *et al.*, 2010; Mills *et al.*, 2011). Protective agents (commonly referred to as cryoprotectants) such as skim milk, whey proteins, sugars, or other bio-polymers have been studied to protect cells from damage (Reddy *et al.*, 2009). Additionally, the possible protective role of certain substrates during freeze drying has been demonstrated. These substrates are known as prebiotics and are selectively utilized by probiotic bacteria. This indicates that prebiotics could be used for the combined purposes of (i) stimulating proliferation and activity of probiotic bacteria upon administration and (ii) as protective agents against environmental stresses during production (Succi *et al.*, 2017).

1.2 Scope of study

Lactobacillus plantarum was isolated in a study by Happel (2018), as part of a Ph.D. thesis by the Faculty of Health Science, University of Cape Town that forms the background of this study. The focus of that thesis was the isolation, characterization and ranking of potential probiotic strains, with the motivation of the development of commercial probiotics to potentially treat bacterial vaginosis amongst South African women. The upscale feasibility studies around the bioprocess engineering aspect of this project are still in progress within the Center for Bioprocess Engineering

Research (CeBER), Department of Chemical Engineering, University of Cape Town (UCT), through which this study forms a part. Furthermore, the potential for this strain to additionally treat bacterial associated acne vulgaris has been identified through a review of literature done in this study. Therefore, this study aims to identify and test cryoprotectant candidates that could also act as a prebiotic upon administration. This is a step towards the development of an efficient live biotherapeutic for the treatment of bacterial vaginosis and bacterial associated acne vulgaris using *L. plantarum* as a model strain.

This thesis focused on the inclusion of saccharides in the drying media during the downstream processing of probiotic bacteria and elucidated their potential to enhance the stability of its concentrates after freeze drying dehydration and over shelf life at different storage temperatures. Furthermore, the impact of various saccharides on the growth and functionality of *L. plantarum* upon re-growth was investigated. The various saccharides investigated were glucose, inulin, lactose present in skimmed milk, maltodextrin, and sucrose. The performance of the saccharides towards the role as a cryoprotectant was evaluated by the viability of cells after freeze drying and at different time points over a storage period of 12 weeks. The performance of these cryoprotectants as potential prebiotics was evaluated by the growth stimulation of *L. plantarum* by these various saccharides respectively present as the sole carbon substrate nutrient in the growth media. Prior to this, the possible inhibiting effect of these excipients on the propagation of *L. plantarum* was investigated.

The outline of this dissertation is in the proceeding order of the following chapters.

- Review of literature
- Definition of project
- Experimental details
- Results and discussion
- Conclusion
- Recommendations

2 REVIEW OF LITERATURE

2.1 Human microbiome

The human body contains millions of microorganisms collectively referred to as "the human microbiome". The human microbiome is said to be made up of bacteria, archaea, eukaryotic viruses, bacteriophages, and eukaryotic microbes such as fungi and protozoa. They are found on the surfaces of every human epithelial tissue, with the largest population found in the human gut (Falony *et al.*, 2019). Their presence is essential and contributes to maintaining good human health. This collection of microbes is unique to each individual and is a function of its environment. It is constantly evolving with time (Proctor *et al.*, 2019). They interact with one another and with host cells, creating what is considered a commensal and mutualistic environment. These interactions have developed today to what is called the modern human microbiome (Proctor, 2016).

Over the past decade, this field of science has garnered questions around how the microbiome affects human evolution, the roles they play in infectious diseases, and promote human health and wellbeing. The quest to find answers to these questions led to the launch of the Human Microbiome Project in 2007 (Turnbaugh *et al.*, 2007).

An imbalance (dysbiosis) of the human microbiome renders the human body prone to invasion by foreign and harmful bacterial species called pathogens. Consequently, imbalances may cause diseases or increase the risks of the occurrence of diseases. The reasons for these imbalances are not fully known (Cribby, Taylor and Reid, 2008). However, human activities including modern practices may have contributed to this distortion, eliminating beneficial bacteria (Proctor *et al.*, 2019).

2.1.1 Skin microbiome

The skin is the largest human organ (Yousef and Sharma, 2017). Generally, it is considered as a cool, acidic, and low moisture environment (Lebeer *et al.*, 2018). It is made up of a microbiome with a collection of microorganisms that are either resident or transient. These microorganisms include bacteria, fungi, viruses. Species that are classified as resident remain on the skin and play beneficial roles to inhibit pathogenic microorganisms and metabolize proteins and fatty acids, a metabolism necessary to maintain healthy skin. Transient species are temporary inhabitants and may cause harm to the skin (Bustamante *et al.*, 2020).

Staphylococcus spp, *Corynebacterium spp*, *Propionibacterium spp* and *Streptococcus spp* are species found to be predominantly present on the skin (Lebeer *et al.*, 2018; Bustamante *et al.*,

2020). *Propionibacterium acnes,* now called *Cutibacterium acne* which have been isolated from skin of healthy persons, is associated with triggered inflammations that lead to acne vulgaris.

While *Lactobacillus* species are considered as underestimated commensal species in the skin microbiome, compared to its predominant presence in the human vagina, clinical studies have revealed the modulation of the skin microbiome and improved skin health when topical formulations containing *Lactobacillus* species were applied (Lebeer *et al.*, 2018). These health benefits were attributed to the ability of *Lactobacillus* to restore skin acidity and adhere competitively to the epithelial tissues. However, there are still few studies around the application of *Lactobacillus* species to improve skin health.

2.1.2 Bacteria associated acne vulgaris

Acne vulgaris is a chronic inflammation of the sebaceous follicles (Lebeer *et al.*, 2018). It is characterized by the formation of comedones, cysts papules, postules or nodules. It is one of the most common skin issues affecting persons who are in their adolescent and youth age. The cause of acne is multifactorial. This includes increased sebum production, hyperkeratinisation, and microbial colonization by *C. acnes* (Lebeer *et al.*, 2018).

Antibiotics have been the primary treatment for bacterial associated acne vulgaris. These include doxycycline, minocycline, and clindamycin. However, there has been an increase in antibiotic resistance resulting to inefficient treatment of the pathogenesis (Lebeer *et al.*, 2018). Formulations containing *Lactobacillus* species are now being researched for their potential in delivering effective treatments (Lebeer *et al.*, 2018).

2.1.3 Vaginal microbiome

The natural human vaginal flora is made up of a wide variety of species. It is dominated by the facultative microaerophilic anaerobic *Lactobacillus* species (Redondo-lopez, Cook and Sobel, 1990). Over fifty *Lactobacillus* strains have been isolated from the vaginal flora. Some of the most isolated species are *L. iners, L. crispatus, L. gasseri* and *L. jenesenii*, followed by *L. acidophilus, L. fermentum, L. plantarum, L. brevis, L. casei, L. vaginalis, L. delbrueckii, L. salivarius, L. reuteri, and L. rhamnosus* (Zhou *et al.*, 2007; Cribby, Taylor and Reid, 2008; Happel *et al.*, 2018). A population of women with predominant species other than *Lactobacillus* is yet to be found (Cribby, Taylor and Reid, 2008).

Many factors can affect the vaginal microbiome, including vaginal epithelium and secretions, and the presence of *Lactobacilli* (Redondo-lopez, Cook and Sobel, 1990). However, the presence of

Lactobacillus is controlled by its ability to adhere to epithelial cells (Pingitore *et al.*, 2015). This ability of *Lactobacillus* to adhere competitively and its ability to produce acidic metabolites such as lactic acid and hydrogen peroxide, naturally inhibits the presence of pathogens (Reid and Burton, 2002).

When the healthy vaginal microbiome is distorted, including the case of a reduced number of beneficial bacteria, pathogenic bacteria can become prevalent. Some of these pathogens include *Gardnerella vaginalis, Candida albicans* and *Escherichia coli*. Urogenital infections, including sexually and non-sexually transmitted infections, caused by such pathogens have affected millions of women globally (Cribby, Taylor and Reid, 2008).

2.1.4 Bacterial vaginosis

Bacterial vaginosis (BV) is an infection that occurs due to an imbalance within the normal vaginal flora. The standard diagnosis of BV includes a vaginal pH equal to or greater than 4.7, release of a fishy odour upon addition of potassium hydroxide to the vaginal discharge and a visible white discharge. BV cases may either be symptomatic or asymptomatic (Reid and Bruce, 2003).

Women with cases of BV have higher chances of contracting sexually transmitted diseases (STD), developing complications during pregnancy, and having pre-term deliveries or underweight babies. Furthermore, it has also been traced to pelvic inflammatory diseases in women (Keane *et al.*, 2006). There is no known direct cause of bacterial vaginosis. It is currently treated with antibiotics such as nitroimidazole, clindamycin, secnidazole, and metronidazole (Javed, Parvaiz and Manzoor, 2019). However, a large percentage of women have a recurrence of the infection after antibiotic treatments (Cribby, Taylor and Reid, 2008). Clinical studies have shown that women with cases of BV can be treated with probiotics with reduced to no cases of recurrence (Reid, 2017).

2.2 Lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) are a group of bacteria which utilize carbohydrates to produce lactic acid as one of the major products of fermentation. LAB can either be homofermentative or heterofermentative. Homofermentative LAB produce only lactic acid as the key catabolite while heterofermentative LAB produce lactic acid amongst other metabolites such as ethanol, acetic acid, carbon dioxide, and acetic acid as by products. LAB are gram-positive, non-sporulating, anaerobic or facultative aerobic bacteria and exist in either rod or cocci form (König and Fröhlich, 2017; Ayivi *et al.*, 2020).

LAB are commonly used in fermented foods due to their ability to prolong food storage, ferment the food, provide favourable taste and texture to the food and because they are considered safe for consumption (Li and Cui, 2010; Hernandez *et al.*, 2012; Behera, Ray and Zdolec, 2018; Ayivi *et al.*, 2020). They have also been exploited in the renewable resources industry for fermenting carbohydrates to produce ethanol and lactic acid (Li and Cui, 2010; Sharma *et al.*, 2020). LAB are made up of four main genera. These are *Lactobacillus, Streptococcus, Leuconostoc* and *Pediococcus* (Ayivi *et al.*, 2020).

2.2.1 *Lactobacillus*

Lactobacilli have been found to be the predominant species isolated from the human body. They have been demonstrated to survive and grow in environment of a wide pH range. They play key roles in parts of the human microbiome such as the urogenital tract, oral cavity and the gastrointestinal tract for maintaining good health (Reid and Bruce, 2003; Rastogi *et al.*, 2011; Thushara *et al.*, 2016). Although they appear to be naturally present in few numbers on the skin, they have been demonstrated to improve skin health through *in vivo* studies (Lebeer *et al.*, 2018; Kim *et al.*, 2021). Their beneficial potential is currently being exploited in different ways. Up to 57 vaginal *Lactobacillus* strains have been isolated from the cervical fluid of South African women (Happel, 2018; Happel et al., 2020). Some of the most dominant species identified included *L. crispastus, L. jensenii, L. gasseri*, *L. mucosae* and *L. vaginalis*.

2.2.2 *Lactobacillus plantarum*

Lactobacillus plantarum is a member of the LAB group and it is one of the most extensively studied bacteria for its application in food and as a starter culture (Behera, Ray and Zdolec, 2018). It is considered the most versatile strain from the *Lactobacillus* genus (Behera, Ray and Zdolec, 2018). *L. plantarum* being isolated from the human gastrointestinal tract has been extensively studied for its antagonism against intestinal pathogens (Hernandez *et al.*, 2012). Although this strain is not commonly reported as vaginal isolates (Goldstein, Tyrrell and Citron, 2015; Happel 2018; Happel *et al.*, 2020), compared to its recurrence in studies as gastrointestinal isolates (Hernandez *et al.*, 2012), studies have reported improved vaginal health by the administration of formulations containing *L. plantarum*. A study by Vicariotto, Mogna and Piano (2014) demonstrated that a slow-release vaginal tablet formulation containing *L. plantarum* was effective in treating and improving symptoms of BV related *Gardnerella* infection in a placebo clinical trial. Another report by Cianci *et al.* (2018) showed that there was a lower risk of BV recurrence within 4 months of treatment with antibiotics alongside formulations containing *L. plantarum* compared to untreated women. However, the result was considered not very significant due to a lower-than-expected rate of

infection in the population investigated. *L. plantarum* has also been demonstrated to improve skin health when formulations containing this microorganism were applied (Kim *et al.*, 2021). These studies are amongst the emerging studies and clinical trials that reveal the potential of the strain to be included in formulations that treat pathogen induced skin and vaginal infections.

2.2.3 Microbial growth of *L. plantarum*

L. plantarum can utilize carbohydrates such as glucose in a homofermentative metabolism to produce lactic acid as the main fermentation products (Hernandez *et al.*, 2012). The metabolites produced play significant roles in protecting the body from pathogens (Reid and Burton, 2002). The metabolic pathway by *L. plantarum* towards the production of lactic acid is illustrated in Figure 2-1 (Sharma *et al.*, 2020).

Lactobacillus can be cultured in reactors ranging from small 50-100ml serum bottles or in large scale reactors (in Litres) containing nutrient rich media (Maier, 2000). De Man-Rogosa-Sharpe (MRS) media is a common selective media that contains favourable nutrient to support the growth of *Lactobacillus* (Lee and Lee, 2008; Schillinger *et al.*, 2012; Menon *et al.*, 2013).

Microbial growth in a closed system where the limiting substrate nutrient is sufficient typically goes through three different phases of growth as illustrated in Figure 2-2. These are the lag phase (t_{lag}) which is characterized by zero growth, followed by the exponential phase where cells begin to grow by cellular division in an exponential manner through the catabolic break down and utilization of nutrients present in its controlled environment (Zwietering *et al.*, 1990; Maier, 2000).

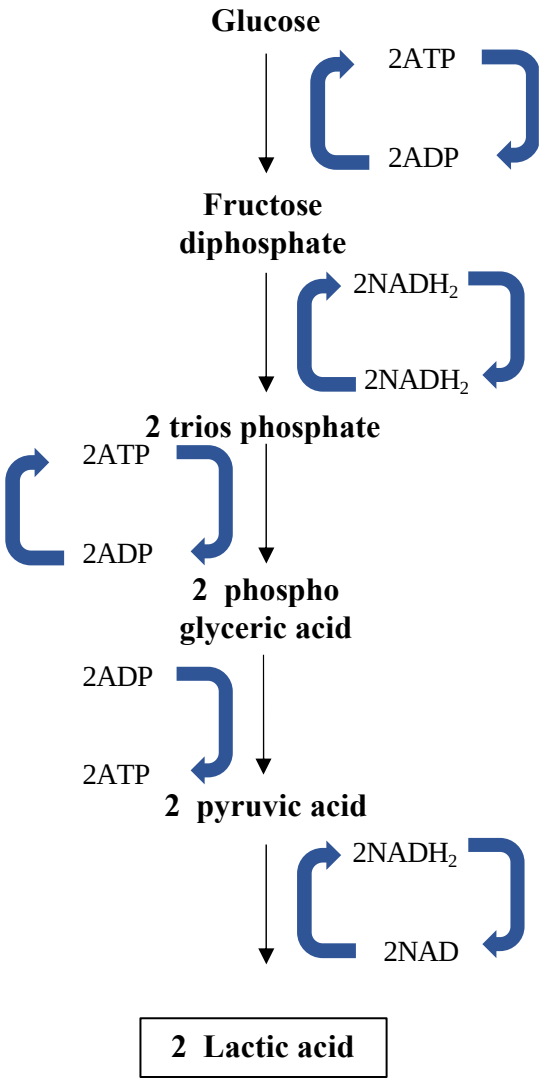

Figure 2-1 Metabolic pathway for the conversion of glucose substrate to lactic acid by *Lactobacillus* (Sharma *et al.*, 2020)

The exponential phase is followed by the stationary phase, at this stage, the depletion of substrate nutrients contained in the media leads to a zero growth. Cells can then begin to produce inhibiting substances that cause death of some cells, which leads to the death phase (Zwietering *et al.*, 1990; Maier, 2000).

The cell growth in the exponential phase can be mathematically described by the differential equation in Equation 2-1 which represents the rate of change in the amount of cells, where X is the amount of cells (mass/volume) at time, t and μ_{max} (1/time) is the specific growth constant (Maier, 2000). Integrating Equation 2-1 gives Equation 2-2, where X_o is the initial amount of cells (mass/volume) at time t_o.

$$\frac{dX}{dt} = \mu_{max}X$$ Equation 2-1

$$lnX = \mu_{max}t + lnX_O$$ Equation 2-2

The growth of *Lactobacillus* in the culture can be measured in different ways. Three commonly used methods include enumeration of the colony forming units of bacteria cells on agar media, measurement of the cell dry weights or by measurement of the turbidity of the culture through the optical density as growth progresses (Maier, 2000; Pla *et al.*, 2015).

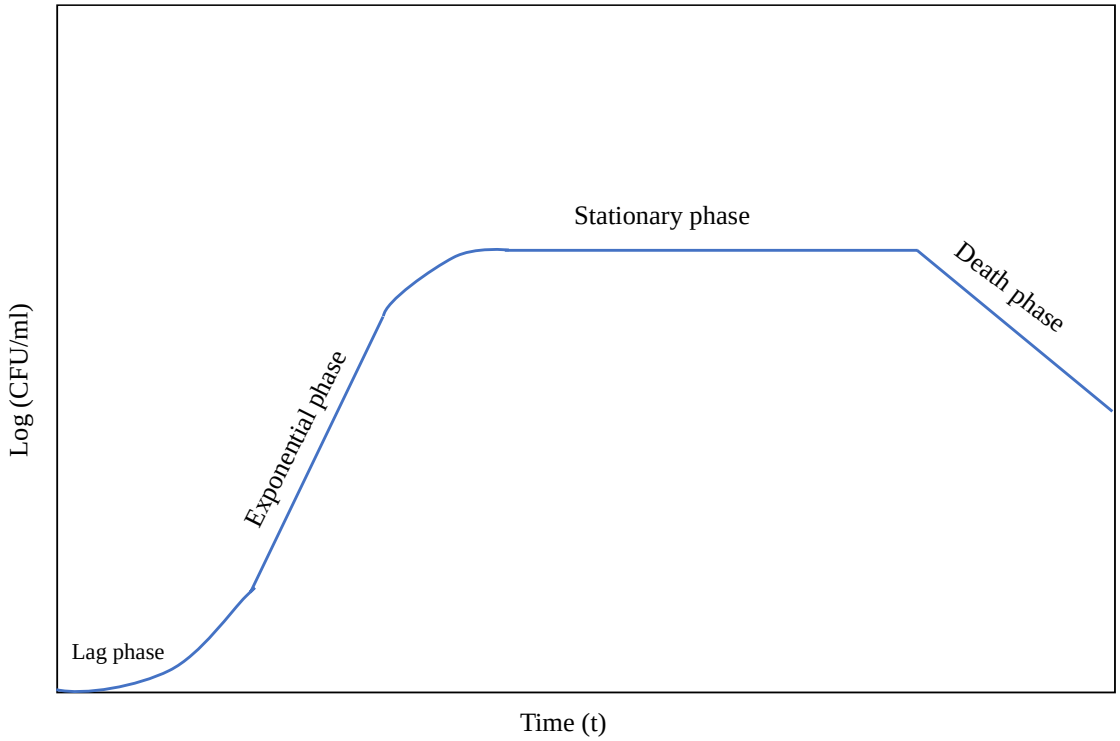

Figure 2-2 Microbial growth curve for a bacterial population in a closed batch system

2.3 Probiotics

2.3.1 Brief history

The utilization of microbes to confer health benefits dates from centuries ago when humans noticed the beneficial effects of eating fermented foods and has since been a practice to date. At that time, there was no scientific explanation or proof for the beneficial effects. The discovery of fermentation was a case of serendipity that resulted in the preserved foods and varied food tastes as well as an improvement to digestive health when consumed (Gogineni, 2013).

In 1875, Louis Pasteur scientifically concluded that lactic acid fermentation was due to the presence of microorganisms that initiated the fermentation. Years after, Pasteur's investigation set a foundation for studies by more scientists in this field. Henry Tisser recommended bifidobacteria should be administered by infants with diarrhoea when he found out that bifidobacteria was a predominant component of the intestinal flora in 1899. Ilya Ilyich Metchnikoff, who took over from Pasteur as director of the Pasteur Institute in 1895, later went on to receive the Nobel prize award in medicine in 1905 for his contributions to immunology, a large part of which constituted of his work on ingestion of microorganisms. He was the first to scientifically describe the health benefits of consuming microorganisms, now known as the probiotic principle (Gogineni, 2013).

2.3.2 Modern use of probiotics

The following early studies have led to the current modern use of beneficial bacteria and exploitation of the human microbiome to confer health benefits today, based on formal research. Puebla-Barragan and Reid (2019) in a review, classified the modern use of probiotics based on two factors: (1) the ability of live bacteria to replenish the microbiome, and (2) their ability to eliminate pathogens by the metabolites they produce.

According to Hill *et al*. (2014), probiotics are defined as:

> *"Living microorganisms that, when administered in adequate amounts, confer a health benefit on the host".*

Until recent years, the use of beneficial microbes targeted towards human health has been focused on gastrointestinal health benefits. However, new findings have emerged and are attracting the interest of researchers, on the presence and beneficial impact of the microbiome in other parts of the human body. These include the urogenital tract, the skin, the mouth, and the heart (Reid and Bruce, 2003; Rastogi *et al.*, 2011; Thushara *et al.*, 2016).

Based on the mechanistic action of the natural human microbiome against pathogens, scientists have proposed that instead of killing pathogenic microorganisms with antibiotics (which could also wipe out the beneficial bacteria) establishing or introducing an increased number of beneficial microbes could naturally fight pathogens in the body (Reid and Bruce, 2003; Sreeja and Prajapati, 2013). There has also been an interest by the pharmaceutical industry to include these live biotherapeutics to treat diseases in formulations known as pharmabiotics (Broeckx *et al.*, 2017).

2.3.3 Health benefits of probiotics

For decades, probiotics have been perceived to improve digestive health, showing positive results after consumption of fermented foods. Modern-day consumption of probiotics has evolved as *in vitro* and *in vivo* clinical trials have produced positive results and proved the efficacy of probiotics in treating diseases (Reid, 2017). Human clinical trials show that bacterial vaginosis, recurrent urinary tract infections in women, acne vulgaris, diarrheal diseases, inflammatory bowel diseases, and serious intestinal conditions can be treated and prevented by the intake of probiotics. Additional health benefits of probiotics include ameliorating digestive disorders, preventing allergies or eczema in infants, reduction of bowel inflammation in adults, use as adjuvants in the treatment of obesity, liver disease and type 2 diabetes (Goldin, 2019).

No evidence exists which shows adverse effects on healthy persons who consumed probiotics; however, it is warned that probiotics may lead to adverse effects in certain health compromised patients. Therefore, caution should be taken during the administration of probiotics (Reid, Zalai and Gardiner, 2010; Sreeja and Prajapati, 2013; Sanders *et al.*, 2018).

2.3.4 Probiotic mechanisms

The mechanistic action of probiotic bacteria is diverse, heterogeneous, and strain-specific. Depending on the bacterial strain(s) and health need, probiotics may exhibit one or more of the following mechanisms of action: antagonism towards pathogenic microorganisms, competitive cell adhesion advantage by the production of mucins, normalization of perturbed microbial communities (microbiome), competitive exclusion of pathogens, production of bacteriocins, enzymatic activity and production of fatty acids, interaction with brain axis by regulation of endocrine and neurologic functions, modulation of the immune system, and reduction of pH by the production of lactic acid or hydrogen peroxide (Plaza-Diaz *et al.*, 2019).

2.3.5 Limitations in the use of probiotics

Probiotics are still largely considered dietary/food supplements in many countries and are not accepted to confer certain pharmaceutical health benefits due to insufficient or low-quality data to back the health claims. Saxelin (2008) reported that organizations such as the European Food Safety Authority (EFSA) and the United States of America Food and Drug Authority (USA FDA) had not approved probiotic administration as a drug for the following reasons: (i) insufficient characterization, (ii) undefined claims, (iii) non-beneficial claims, (iv) lack of relevant human studies, (v) lack of measurable outcomes that reflect benefits for humans, (vi) quality of presented studies. Some of these concerns are still prevalent and are discussed in the following paragraphs.

Puebla-Barragan and Reid (2019) in a review, highlight the attitude of scientists who dismiss the beneficial effects of probiotics and consider them not safe as a critical challenge of acceptance of probiotic therapy. This review implies that this critique is not valid because it contradicts results from clinical studies that prove otherwise. However, it can be understandable why the use of probiotics as a medical therapy struggles to be generally accepted because in the same review, Puebla-Barragan and Reid (2019) presented around 10% out of 20000 published papers on probiotics in Pub Med in the last forty-five years. This could be an indicator that there is still a gap of sufficient quality of the study on probiotics for human health to prove probiotic health claims. Rather than dismissing the critique, these findings could be a source of motivation to push towards more studies to clinically investigate probiotic claims. Another reason for the struggle for acceptance is the fact that this school of science is relatively new as with other medical therapies that encountered similar challenges at early stages of development.

Furthermore, many studies lack adequate insight into the mechanistic action of probiotics. Also, manufacturers and marketers have exploited the term "probiotics" as many products that claim to be probiotic do not necessarily contain live microorganisms, rather, they contain metabolites from probiotic bacteria. Some of these products do not indicate the strains contained and some of them have adapted names to rename strains to keep their product formula discrete and confidential. Additionally, the probiotic formulations show discrepancies between the actual probiotic contents in colony-forming units (CFU) and the amount stated on the label within the shelf-life period. All these lead to decreased confidence in the use of probiotics as a biological treatment option or drug by physicians (Sreeja and Prajapati, 2013; Mehdi-Alamdarloo *et al.*, 2016). Therefore, tighter regulations around probiotics need to be implemented.

2.3.6 Advances in the use of probiotics

Advances in the field of probiotics have led to a proposal for its use as a drug. According to LeBegue, Love and Wyatt (2020):

> *"A pharmabiotic is proposed to be a live microbial therapeutic with an indication that has been validated by clinical trials with measurable end points of efficacy and safety".*

Probiotics for intended use as drugs must meet this definition before approval by the USA FDA. As it stands, clinical trials have been insufficient to meet these requirements. However there has been an increase in clinical studies on Pub Med revealing potential for development of biotherapeutics in the not-so-distant future (LeBegue, Love and Wyatt, 2020).

2.4 Process development and design of pharmabiotics

The design of pharmabiotics encompasses various key disciplines. This spans from the biological and health sciences, primarily involved in the genetic and phenotypic identification, characterization of strains and *in vivo* and *in vitro* testing for safety evaluation, to bioprocess engineering sciences, primarily involved in the technological characterisation. Figure 2-3 illustrates the key steps required for the development of an effective probiotic therapy, involving a collective team made up of these research fields (Nader-Macías and Tomás, 2015).

One of the key challenges surrounding the design of probiotics is achieving an acceptable amount of viable and active bacterial cells, high enough to survive manufacturing conditions and through the product's shelf life. As the probiotics definition highlights, it is important that the probiotics administered contain the right strain(s) of bacteria and that the bacteria cells are present in the adequate amount. The exact amount required can vary between strains and the desired health benefit (Broeckx *et al.*, 2016; Sanders *et al.*, 2018). However, a minimum of 10^6 to 10^9 ($\frac{CFU}{ml}$) is generally accepted (Hernandez-Hernandez *et al.*, 2012; Sreeja and Prajapati, 2013). Therefore, the development of suitable process strategies to ensure that the viability and functionality of bacterial cells are enhanced and prolonged is fundamental in the design of pharmabiotics.

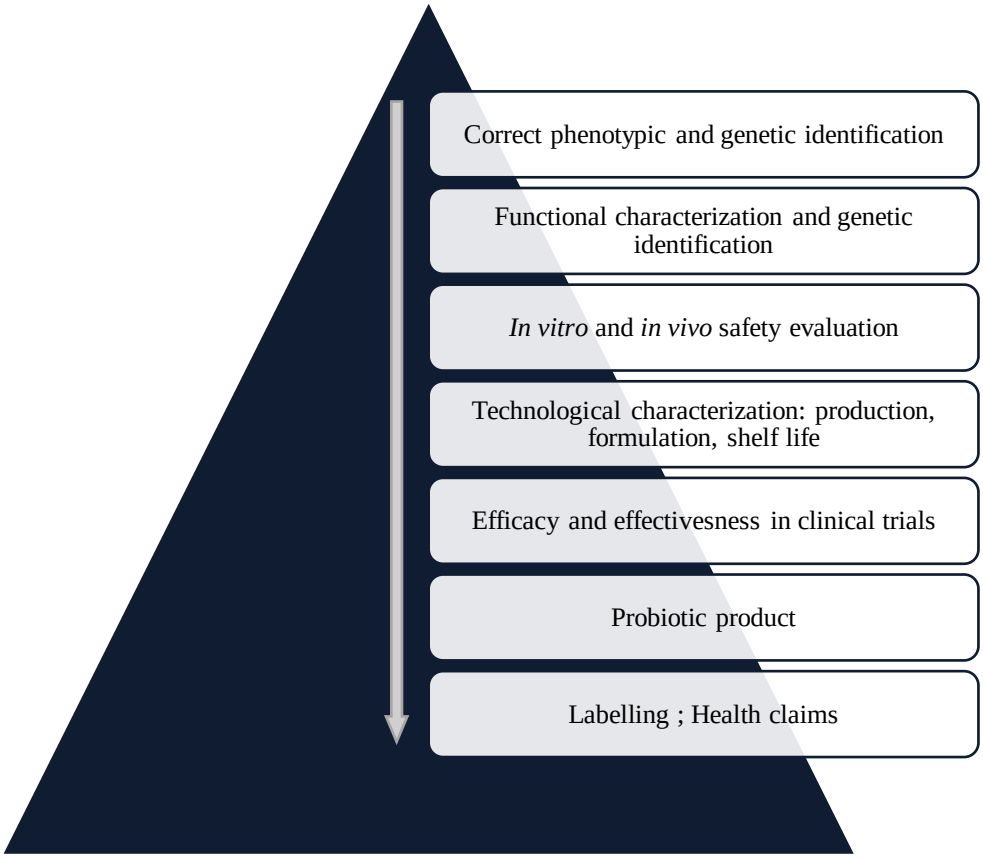

Figure 2-3 Key steps towards the development of pharmabiotics, adapted from Nader-Macías and Tomás (2015)

2.5 Drying of probiotics

The processing of probiotics involves fermentation (upstream process), separation, dehydration, and encapsulation (downstream processes) as shown in Figure 2-4. Drying is a key step in the processing of probiotics. This is because the presence of moisture is detrimental to bacterial cells over storage. Moisture promotes undesired biochemical activities within the cells resulting in a release of cell-damaging metabolites (Iaconelli *et al.*, 2015; Broeckx *et al.*, 2016). Moreover, keeping cells in their dry form promotes ease of handling during processing and therefore reduces transportation costs.

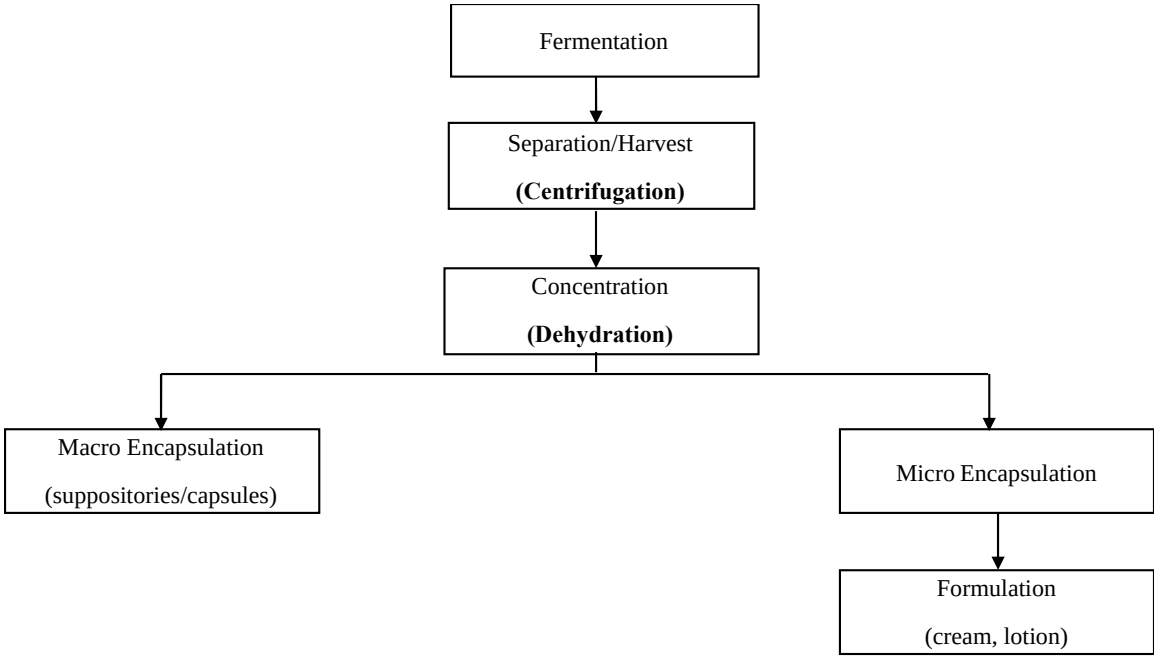

Figure 2-4 Bioprocessing steps in the production of probiotics

Various probiotic drying methods have been studied. These include spray drying (Leja *et al.*, 2009; Riveros, Ferrer and Bórquez, 2009; Broeckx *et al.*, 2017; Moayyedi *et al.*, 2018), freeze drying (Otero, Espeche and Nader-Macías, 2007; Martin-Dejardin *et al.*, 2013; Perdana *et al.*, 2013; Broeckx *et al.*, 2016), electro-spraying (Moayyedi *et al.*, 2018), vacuum drying (Foerst *et al.*, 2012), explosion puffing drying (Cui et al., 2018), and single droplet drying (Schutyser, Perdana and Boom, 2012; Perdana *et al.*, 2013; Khem, 2015). These methods have been successfully demonstrated to dehydrate bacterial cells. However, the success of any method depends on the operating conditions at which they are carried out. Often, to ensure effective dehydration, bacterial cells are subjected to extreme conditions or stresses (either temperature, shear, mechanical, etc.). These conditions are detrimental to the cell membranes. The severity of these conditions depends on the drying method applied. They cause cell death or reduced cell viability and stability over time. Many studies in the literature have focused on improving the survival of *Lactobacillus* during dehydration but few studies evaluate their stability over storage (Tomás *et al.*, 2009).

Furthermore, most available literature has focused on drying common probiotic strains, particularly gastrointestinal isolates. There is a lack of literature available that shows, for example, the behaviour of recently isolated vaginal *Lactobacillus* strains during dehydration. Therefore, more knowledge about these strains is needed.

Table 2-1 shows common drying methods, the advantages, and disadvantages around them. Spray drying and freeze drying are the most commonly used drying methods for the commercial production of probiotics (Riveros, Ferrer and Bórquez, 2009; Savini *et al.*, 2010). Spray drying is

more cost-effective and flexible for drying biomaterials on large scale in contrast to freeze drying which involves a slower and more expensive batch process (Riveros, Ferrer and Bórquez, 2009). However, freeze drying is usually preferred for low heat resistant biomaterials like bacteria because the desired biological properties are retained after drying (Reddy *et al.*, 2009).

Table 2-1 Advantages and disadvantages of various drying methods

Drying Methods	Advantages	Disadvantages	References
Spray drying	• Well-known and described process. • Suitable for large scale production. • Cheaper than freeze drying (6 times). • Easily adjustable process parameters to fit requirements of the desired product. • Ease of control on particle characteristics.	• High operating temperature. • Poor survival of microorganisms. • Loss of native state of heat-sensitive biomaterial. • Not suitable for low heat resistant bacteria.	(Riveros, Ferrer and Bórquez, 2009; Thorat and Joshi, 2011; Broeckx *et al.*, 2016)
Freeze drying	• Well-known and described process. • Low processing temperature and pressure conditions. • Native state of biomaterials is retained.	• High cost compared to other drying methods. • Time and energy consuming. • Limited control of particle characteristics. • Further step necessary to break cake product into loose particles.	(Thorat and Joshi, 2011; Aschenbrenner, Foerst and Kulozik, 2015; Broeckx *et al.*, 2016)
Fluidized bed drying	• Uniform temperature distribution. • Optimal heat and mass transport conditions. • Less time and energy consumption compared to freeze drying.	• Limited available knowledge and experience about the process. • Granulate material is necessary.	(Thorat and Joshi, 2011; Broeckx *et al.*, 2016)
Vacuum drying	• Mild operating conditions. • Relatively cheaper than freeze drying method (2 times).	• Limited available knowledge and experience about the process. • Long drying times. • Further step necessary to break up dried cake product.	(Strasser *et al.*, 2009; Broeckx *et al.*, 2016)

2.5.1 Damage due to dehydration

During the inactivation of probiotic cells via dehydration, the removal of water molecules is detrimental to the cell membranes. This is because the bi-phospholipid layers within the cell membrane which are responsible for retaining the native structure of cells and are bonded and held in place by water molecules, lose their structure and constituents. These include deoxyribonucleic acid (DNA) and proteins essential for cell function (Aschenbrenner, Foerst and Kulozik, 2015). Figure 2-5 shows an example of structural damage seen on *Lactobacillus helveticus*, adapted from (Aschenbrenner, Foerst and Kulozik, 2015). The defects observed on cell membranes were due to dehydration after being vacuum dried.

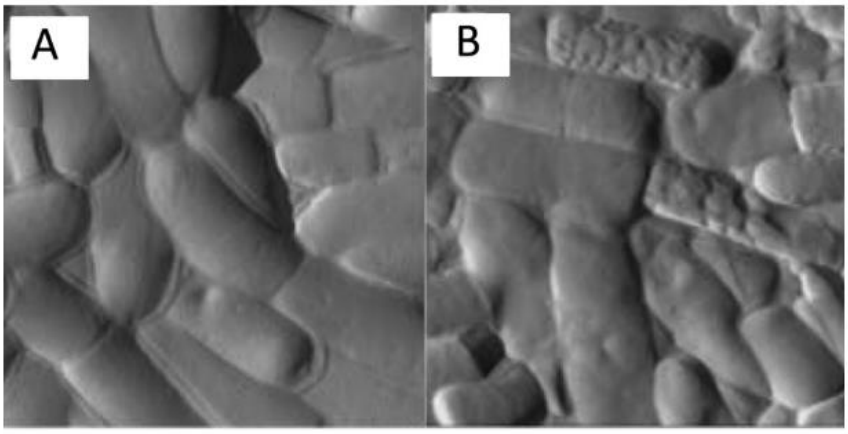

Figure 2-5 Atomic Force Microscopic images of *Lactobacillus helveticus* before drying (A) and after vacuum drying (B), showing cell membrane damage, adapted from Aschenbrenner, Foerst and Kulozik (2015)

Moayyedi *et al*. (2018), investigated the effects of different drying methods on *L. rhamnosus*: electro spraying, spray drying, and freeze drying. Cell membrane damage, measured by the dried microorganism's sensitivity to NaCl, bile, Penicillin G and lysozyme, was observed after the drying process for all methods applied. The least reduction in viability of 26.98% was observed in freeze dried cells due to their sensitivity against Penicillin G, while cells dried by electro spraying showed the highest reduction in viability of 53.3% due to their sensitivity against NaCl 4%. Spray dried cells demonstrated similar extent of cell damage to the freeze dried cells.

These reports reflect the detrimental effect caused by the drying process on probiotic cells.

2.5.2 Survival of probiotic cells during dehydration

Various factors can influence the survival of probiotic cells during dehydration. These include the intrinsic factors (strain-dependent), growth conditions (phase of harvest, media composition),

sublethal treatments, inclusion of excipients (cryoprotectants), and drying process conditions (temperature, pressure, drying rate). These factors are further expanded on in the following subsections.

Intrinsic factors

The survival of bacteria during dehydration is strain dependent. The genetic make-up that controls certain properties of cells such as morphology, environmental resistance, and protein production, differs between strains, which influences each strain's survival during drying (Carvalho *et al.*, 2004). For example, *Enterococci* demonstrated more resistance during freeze drying compared to larger-sized rod-like *Lactobacillus* (Fonseca, Béal and Corrieu, 2000; Carvalho *et al.*, 2004). Fonseca, Béal and Corrieu (2000) suggest that this behaviour could be linked to the smaller extent of cellular membrane damage owing to its smaller surface area in contrast with that of *Lactobacillus.*

Growth conditions

The harvest time also influences the survival rate of probiotic cells during dehydration. Investigations by Corcoran *et al.* (2004) showed that *L. rhamnosus* harvested at different growth phases (lag phase, exponential phase, and stationary phase) and spray dried in 20% reconstituted skimmed milk exhibited varying survival rates, with the highest rate obtained from cells harvested in the stationary phase (> 50%), followed by cells harvested in the early log phase (14%). Cells harvested in the lag phase were the most susceptible to the dehydration process with survival rates of barely 2%.

Sublethal stress exposure

It has been well investigated and accepted that pre-stressing probiotic cells before they are subjected to compromising environments can improve robustness and cell survival through technological processing or journey through the gastrointestinal tract (Mills *et al.*, 2011). An example of this is the over production of three cold shock proteins (CspC, CspP and CspL) by *L. plantarum* after being exposed to cold shock (8^{o}C) in the early exponential growth phase (Derzelle *et al.*, 2003). This sublethal cold shock exposure of *L. plantarum* resulted in an enhanced capacity to survive the freeze drying process.

Inclusion of excipients in the drying media

The inclusion of excipients during dehydration of probiotics is a strategy that has been proven to influence the survival of bacteria during drying (de Urraza and de Antoni, 1997). Carbohydrates,

sugar alcohols, salts and some proteinaceous substances provide protection to cells that enable them to survive the drying process.

Drying process conditions

Different drying methods have varying technological factors that impose stress on bacteria. For example, effective spray drying involves elevated temperature. However, measures can be taken to reduce these stresses. Chávez and Ledeboer (2007) attempted spray drying *Bifidobacterium* immersed in Soy Protein Isolate and Maltodextrin at a low outlet temperature of 49°C. Although a decent number of cells survived the process, this condition was insufficient to obtain moisture contents low enough (<5%) to sustain stability through storage. Additional application of a second vacuum drying step at 45°C under reduced pressure of 10 bar for 24 hours, showed a shift in moisture content from 7.2% to 3% and a resulting increase in cell survival by 5% over 2 months of storage.

2.6 Freeze drying of probiotics

Freeze drying is the focus of this thesis and is thus now expanded on in more detail.

2.6.1 Freeze drying process

The freeze drying process is commonly used for the drying of probiotics. Moisture content as high as 95% can be removed from the biomaterial through this method (Perdana *et al.*, 2013; Aschenbrenner, Foerst and Kulozik, 2015). It is most preferable for dehydration of probiotics because of its low temperature and pressure operating conditions, as low as -80°C and 100 mTorr respectively (Shamekhi Fatemeh, 2011; Perdana *et al.*, 2013) At these conditions, the native structure of the biomaterial can be retained, compared to other drying processes. The resulting product is usually highly native in structure, biological activity, and in chemical and physical composition (Aschenbrenner, Foerst and Kulozik, 2015). However, the freeze drying process results in low product yield, mainly due to the freezing step involved in the process. Moreover, it is usually configured as a batch process, making it relatively more time-consuming and expensive to operate. These are the major challenges around this drying method. The cost of freeze drying can be reduced by concentrating the cells through centrifugation or ultrafiltration before the process. This results in shorter drying times and reduced energy consumption. Additionally, to reduce drying time, the combination of freeze drying with other drying methods can be considered. Cui *et al.* (2018) tried this by a combination of microwave drying with freeze drying and achieved the same moisture content as freeze drying done alone, but within a shorter drying time of 18.5 h compared 36 h for only freeze-drying.

The inclusion of excipients known as cryoprotectants has been demonstrated to improve the yield of viable probiotic cells after freeze drying. Reddy *et al*. (2009) investigated the survival of freeze dried LAB strains *L. plantarum*, *L. salivarius*, and *P. acidilactici*, and obtained as high as 83% survival rate in the presence of maltodextrin. The resulting freeze dried powder showed better stability when stored at 4°C compared to being stored at room temperature. This indicates that the storage temperature is also a key factor in processing probiotics.

The main components of the freeze dryer are illustrated in Figure 2-6, within which the freeze-drying process takes place. It generally consists of three main steps as illustrated in Figure 2-7. These are: freezing, primary drying, and secondary drying. During freezing, the solvent (usually water) crystallizes under atmospheric conditions and separates from the residual sample. Consequently, the unfrozen fraction concentrates. In the subsequent primary drying step, the frozen solvent is removed by transferring it directly from the solid state to the gaseous state (sublimation) with concomitant avoidance of the liquid aggregate state. In the final secondary drying step, the additional non-frozen solvent is removed from the sample via desorption and the sample reaches its final water content (Aschenbrenner, Foerst and Kulozik, 2015).

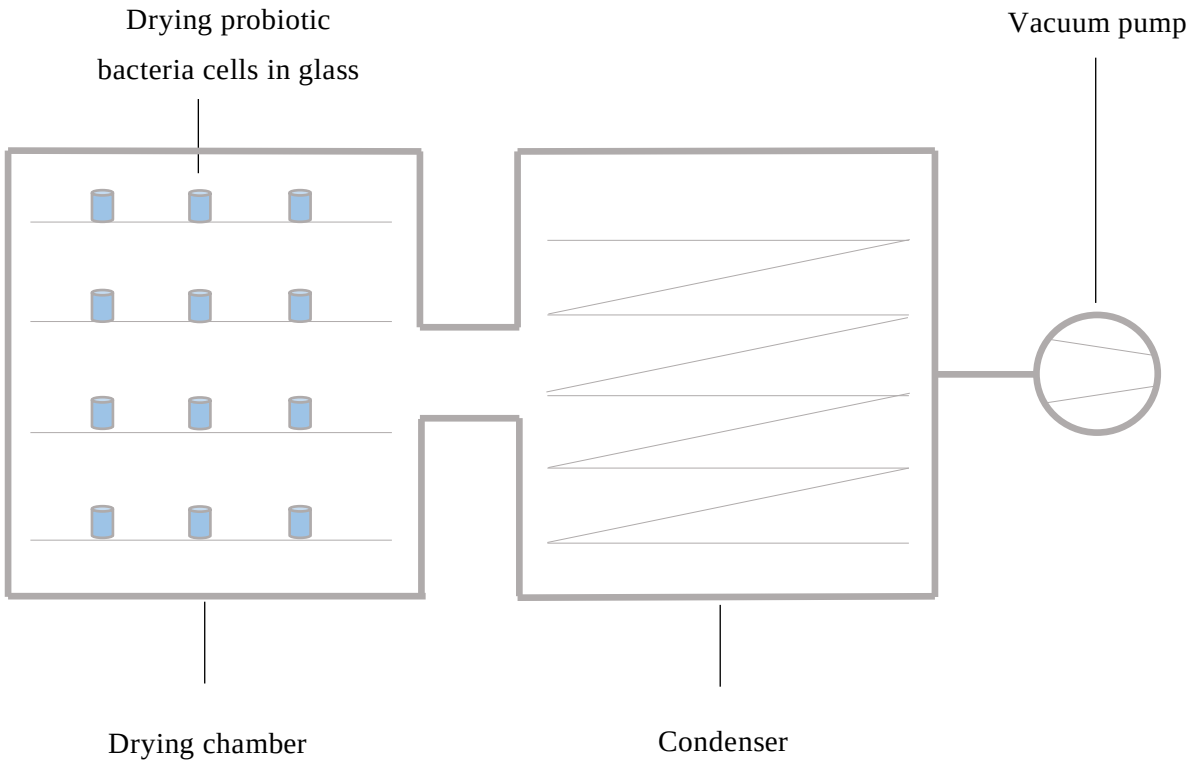

Figure 2-6 Components of a freeze dryer, adapted from Aschenbrenner, Foerst and Kulozik (2015)

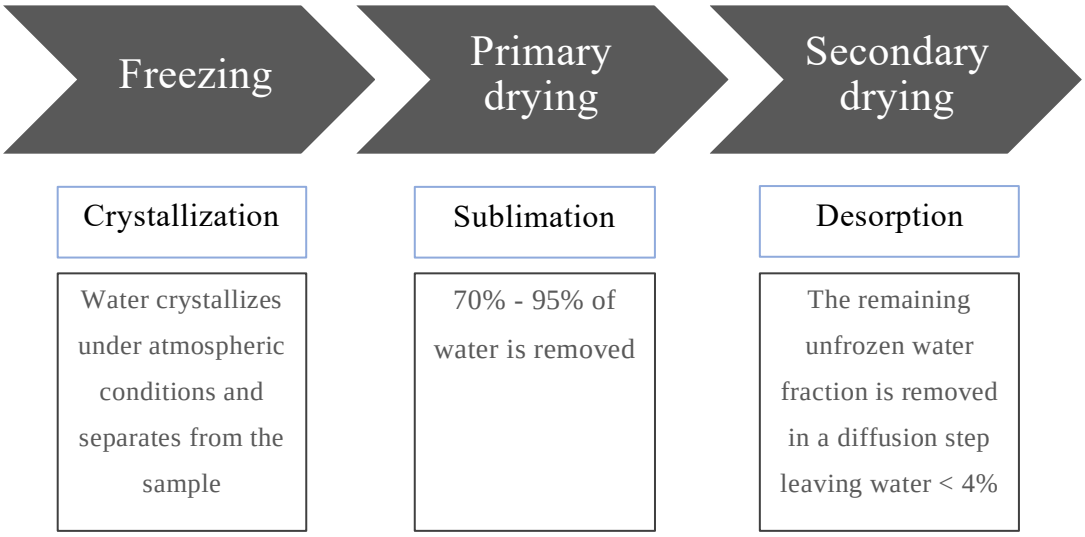

Figure 2-7 Freeze-drying process, adapted from Aschenbrenner, Foerst and Kulozik (2015)

Results from De Giulio *et al.* (2005) show that across all cells freeze dried, (*Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus salivarius* subsp. *Thermophilus* and *Lactobacillus acidophilus*), there was a reduction in the number of viable cells after the freezing step and a further decline in survival after subsequent dehydration steps (primary and secondary drying). These results agree with (Aschenbrenner, Foerst and Kulozik, 2015) that both nucleation and removal of water molecules independently can be detrimental to cell membranes and cause cell death.

The three steps are now expanded on:

Freezing

The freezing step is the most crucial part of the entire process (Nowak and Jakubczyk, 2020). It can either be done outside or within the freeze dryer. The suspension to be frozen is usually a mixture of water and either sugars, biomaterials or salts, placed in either large plates or in low volume plates, ampoules or vials (in the 10's of ml range), arranged in multiple numbers on large trays for industrial purposes (Aschenbrenner, Foerst and Kulozik, 2015; Ward and Matejtschuk, 2019). During freezing, the behaviour of the drying mixture usually follows the pattern as illustrated in Figure 2-8. As the suspension is cooled, it reaches the freezing line at a temperature of T_m where nucleation begins. As cooling continues, the mixture goes through an ice + rubbery state until it attains the ice + glassy state at the frozen glass transition temperature $T_{g'}$. At this temperature, the sample reaches its maximally frozen concentration $C_{g'}$ where about 75% of water is in its solid frozen state, leaving the solute in a glassy matrix surrounded by ice and the remaining unfrozen water (Singh and Roos, 2007; Roos, 2021). Differential scanning calorimetry is a commonly used pharmaceutical analytical method in determining the T_g' values of frozen samples

(Ward and Matejtschuk, 2019). It is important that during the freezing step, the temperature is below the T_g' of the sample (Aschenbrenner, Foerst and Kulozik, 2015).

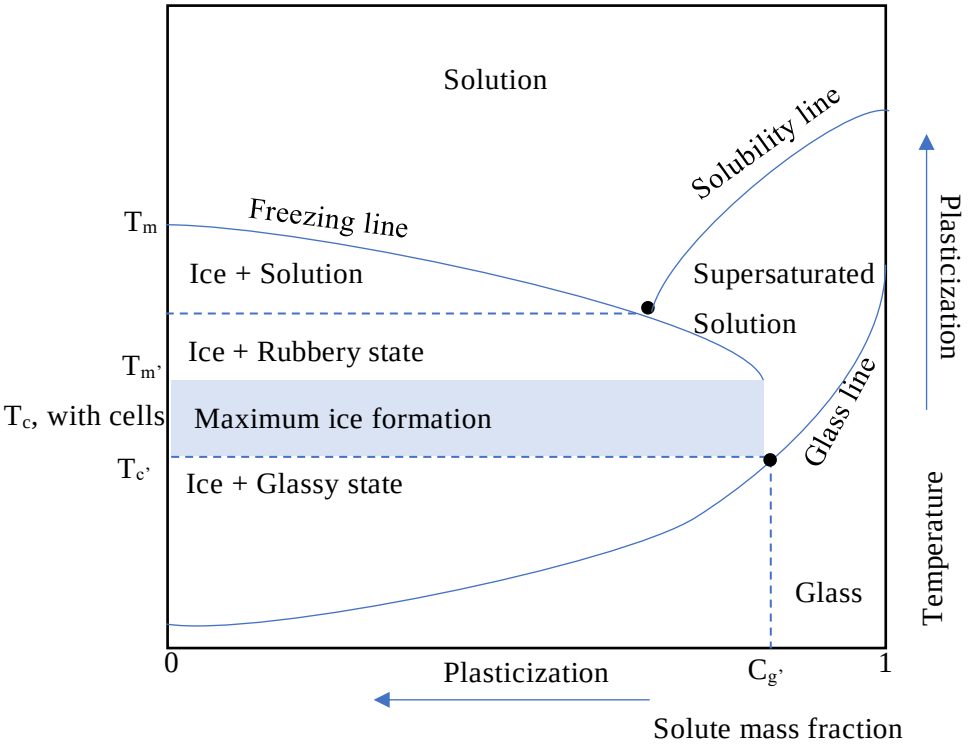

Figure 2-8 Behaviour of sample states during freezing, adapted from Aschenbrenner, Foerst and Kulozik (2015)

During this step, a drop in temperature below the freezing point results in the formation of ice crystals. The solvent then becomes concentrated depending on the degree of freezing. The rate of formation of ice crystals and their size is dependent on the freezing rate. Larger crystals are formed at lower freezing rates while smaller ice crystals are formed at higher freezing rates. The formation of larger crystals results in faster sublimation at the subsequent primary drying step and *vice versa*. However, the formation of larger crystals results in more damage caused to the cell membrane during this step. Furthermore, the size of ice crystals formed determines the nature and behaviour of the dried product in terms of porosity and dissolution (Aschenbrenner, Foerst and Kulozik, 2015). Therefore, optimization of the freeze drying process is necessary to implement the best operating conditions towards achieving the desired product.

Primary drying

During this step, about 70-95% of the moisture is removed through a sublimation step at a very low temperature of about 100 mTorr and low temperature under vacuum below the triple point (T_r) in a phase shift illustrated in Figure 2-9 (Aschenbrenner, Foerst and Kulozik, 2015). Compared to pure water, T_r values for mixtures are usually higher. The driving force for the sublimation process is

the difference in pressure between the sublimation front and chamber pressure. The condenser chamber is usually within the freeze dryer or adjacent to the freeze dryer as seen in Figure 2-6. Water vapour condenses in this chamber and forms ice at its surface (Aschenbrenner, Foerst and Kulozik, 2015).

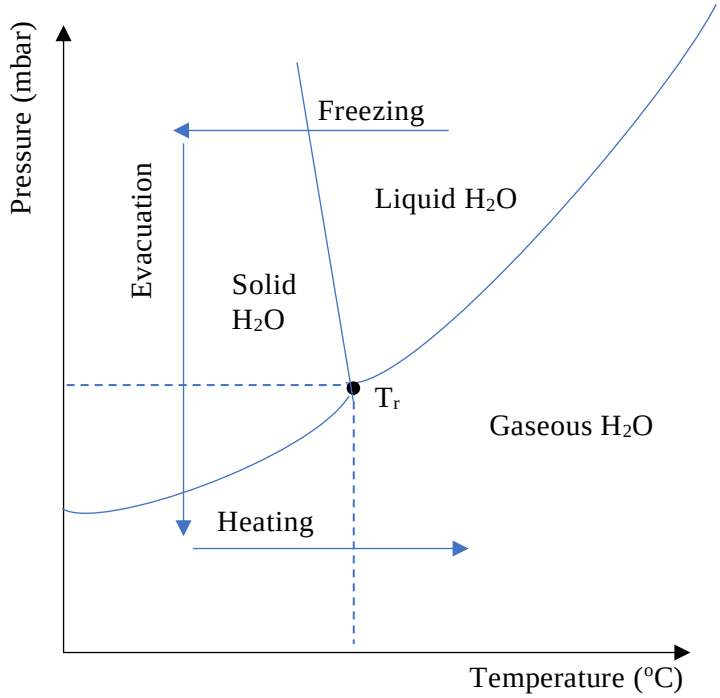

Figure 2-9 Behaviour of sample states during primary drying, adapted from Aschenbrenner, Foerst and Kulozik (2015)

Equation 2-3 shows the rate of sublimation dm/dt as a function of sublimation front and chamber vapor pressures P_{ice} (Pa) and P_c (Pa) respectively, and resistance of the dry product layer and drying stopper, R_p (Pa. g^{-1}.s) and R_s (Pa.g^{-1}.s), respectively. In typical pharmaceutical processes, the drying material is usually within a vial with a rubber stopper that gradually closes during the sublimation process and is completely sealed at the end of the process (Aschenbrenner, Foerst and Kulozik, 2015). The dry product layer increases as the drying process proceeds. Hence resistance due to these factors increases as the drying process progresses.

$$\frac{d_m}{d_t} = \frac{P_{ice} - P_c}{R_p - R_s}$$ Equation 2-3

During the drying process, water vapour moves through the outer dry surface area as shown in Figure 2-10. The value of R_s is usually neglected, due to its absence for drying systems without vial stoppers or its relatively low value for systems with vial stoppers compared to the R_c values.

The value of R_c depends on the nature of the dry material in terms of porosity. The nature of the solute and the freezing step, as mentioned previously, are determinants of this property (Aschenbrenner, Foerst and Kulozik, 2015).

$T_{g'}$ and $T_{m'}$ values are still very useful during the primary drying. The drying temperature must not exceed the collapse temperature $T_{c'}$ during this step. At this temperature, the sample experiences structural changes due to a drop in viscosity. $T_{c'}$ is usually slightly above $T_{g'}$ but below $T_{m'}$.

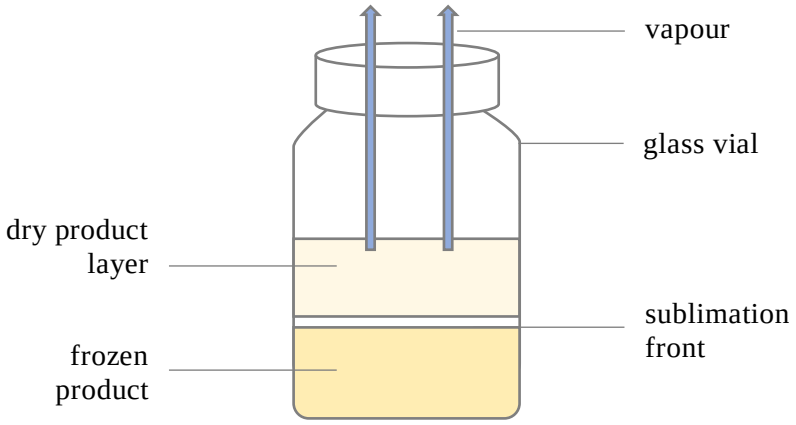

Figure 2-10 Product layers, during primary drying, adapted from Aschenbrenner, Foerst and Kulozik (2015)

Secondary drying

The remaining unfrozen portion of water is removed in a secondary drying diffusion step. At the end of the primary drying process, about 5% to 30% (Aschenbrenner, Foerst and Kulozik, 2015) of water remains bonded to the drying material after the maximally frozen fraction of water is removed (Aschenbrenner, Foerst and Kulozik, 2015). In this step, water molecules diffuse through the solid matrix and evaporate at the solid/vapour front in a desorption process.

2.6.2 Cryoprotectants

To avoid damage and achieve high cell viability yields, protectants known as cryoprotectants are added to the biomaterial before drying. The identification of the right protective agents to enhance cellular survival during storage is the key challenge (Savini *et al.*, 2010). The choice of cryoprotectants by manufacturers has been on a trial-and-error case based on factors such as protection performance, availability, cost and resulting physical characteristics of the final product (Flores-Ram-Rez *et al.*, 2019). Commonly used probiotic cryoprotectants include disaccharides (saccharose, lactose, trehalose), polyols (mannitol, sorbitol), and polymers (maltodextrin, dextran, inulin) (Aschenbrenner, Foerst and Kulozik, 2015). A good cryoprotectant can be easily vitrified

and can protect the embedded bacterial cells throughout the whole freeze drying process and during subsequent storage. Figure 2-11 shows the protective efficiencies of various cryoprotectants. This protective capacity is also dependent on the strain.

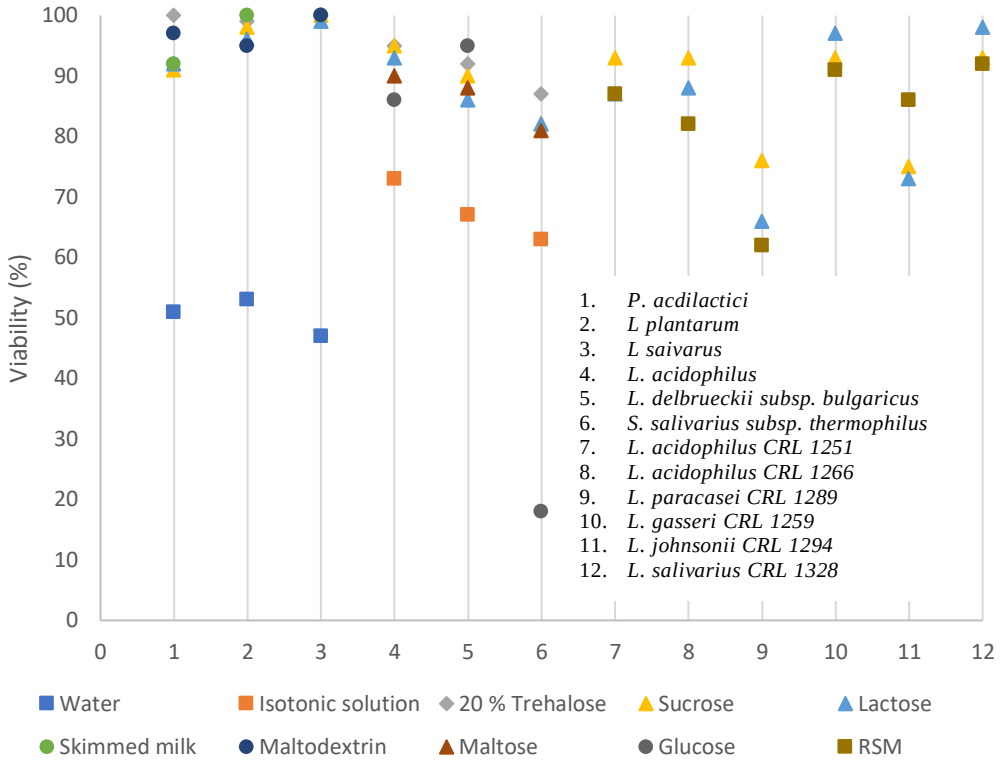

Figure 2-11 Survival rates of various *Lactobacillus* strains after freeze drying in different cryoprotectants (Reddy *et al.*, 2009; Tomás *et al.*, 2009)

Disaccharide sugars and oligomeric sugars are preferred as additives for freeze drying not only because they can be easily vitrified but also because they are small molecular structures that could easily replace the water molecules removed during drying, and thus maintain the cell integrity (Aschenbrenner, Foerst and Kulozik, 2015). Carvalho *et al.* (2004b) suggest that the saccharide present in the growth media should be the first option to consider when selecting a cryoprotectant. The reason for this suggestion is to eliminate possible lag periods for *Lactobacillus* to get familiar with a new substrate for proliferation.

Common cryoprotectant options are now presented in further detail:

Glucose

Glucose, also referred to as dextrose, is a single unit of sugar belonging to the class of carbohydrates called monosaccharides. Glucose can be found in honey, fruits, or present as free circulation sugar in the blood of humans and animals (Shendurse and Khedkar, 2015). It is the main carbon source

contained in standard MRS broth for *Lactobacillus.* Glucose can protect biomaterials as reported by Tomás *et al.* (2009). However, glucose has low a T_g value of –54°C (Flores-Ram-Rez *et al.*, 2019). The makes it less preferred for use as a cryoprotectant compared to other saccharides with higher T_g, values such as maltodextrin or sucrose.

Inulin

Inulin is a plant-derived fructoligosaccharide. It is a fructans polymer with a chemical structure made up of chains with β-(2-1) glycosidic bonds terminated by an α-β-d-(1-2)-glucopyranoside ring group (glucose) glycosylic bonds. It is commonly used in food as dietary fibre, supplements, or as a storage carbohydrate (Franck, 2002; Leyva-Porras *et al.*, 2014). In the European Union inulin is legally considered safe as a food additive (Franck, 2002). Chicory is the main source of inulin used for industrial purposes because about 79% of inulin can be extracted from a dried chicory root material, compared to other sources (Leyva-Porras *et al.*, 2014). Other sources include fruits and vegetables (Franck, 2002). Inulin exists as an amorphous powder at non-equilibrium conditions i.e., when dehydrated or stored below its melting point or T_g (Leyva-Porras *et al.*, 2014).

Inulin is considered a good choice as a prebiotic not only because it can be fermented by LAB, as demonstrated by Oliveira *et al*. (2011), but also because it is an indigestible fibre that reaches the human intestine intact (Oliveira *et al*., 2011).

Inulin has the ability to form a glassy matrix and has a low T_g, value (Rodriguez *et al*., 2013). This makes it a good option as a cryoprotectant. However, over storage, inulin has demonstrated poor protection of LAB. This could be due to the loss of its glassy form over storage because of its high affinity to moisture (Saavedra-Leos *et al.*, 2014).

Sucrose

Sucrose is a disaccharide, made up of 50% glucose and 50% fructose. It is the most available naturally occurring saccharide manufactured industrially from either sugarcane or sugar beet (Queneau *et al.*, 2007). As a result of this abundance, it is a relatively cheap, pure, and stable raw material. Sucrose is a top choice of carbohydrate for the industrial production of ethanol by fermentation (Queneau *et al.*, 2007). As a cryoprotectant, sucrose has demonstrated good protection over LAB during freeze drying and over subsequent storage.

Maltodextrin

Maltodextrins are hydrolysis products of starch. Maltodextrin is commonly used as a plasticizer to improve flowability and storage stability in foods. It is preferred for use as a cryoprotectant because

of its physiochemical properties that favour long-term storage in foods. Furthermore, Saavedra-Leos *et al.* (2015) suggested that in situations or process where the temperature is kept below 90°C, low molecular weight maltodextrins should be considered. Moreover, maltodextrin has a high Tg' of -6.3°C, which makes it a good excipient for freeze drying (Flores-Ram-Rez *et al.*, 2019).

Skimmed milk

Skimmed milk is obtained from the dehydration of pasteurized milk. It is made up of about 49% to 56% disaccharide lactose (Patel, Chen and Kar, 2005; Cavalcanti *et al.*, 2019). The chemical composition of skimmed milk is illustrated in Figure 2-12. Skimmed milk has been known to demonstrate good protection over *Lactobacillus* during various drying methods and over subsequent storage (Reddy *et al.*, 2009).

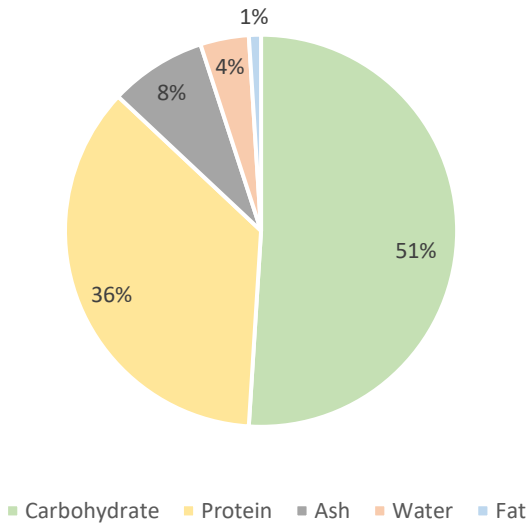

Figure 2-12 Chemical composition of skimmed milk

2.6.3 Protective mechanism of cryoprotectants during freeze drying

Various mechanisms have been proposed for the protection of biomaterials by excipients during dehydration. These include the vitrification hypothesis and water replacement theory. Their contribution to an overall protective effect in systems like bacteria is complex and difficult to determine. The available information is mainly based on investigations with simple model systems (Aschenbrenner, Foerst and Kulozik, 2015).

Vitrification hypothesis

According to the vitrification hypothesis, biomaterial embedded in a glassy sugar matrix shows significantly improved stability due to the intrinsic low mobility that retards detrimental chemical and physical reactions (Aschenbrenner, Foerst and Kulozik, 2015). In a protective glassy matrix, all diffusion-controlled processes that could be detrimental are slowed down, thus enhancing bacterial viability during long-term storage (Broeckx *et al.*, 2017).

Water replacement theory

The water replacement theory suggests that bacterial cell membrane integrity is maintained as water molecules get replaced by the saccharide (cryoprotectant) hydroxyl-groups after evaporation. The hydroxyl-groups can also interact with the polar groups of the phospholipid bilayers of the membrane (Aschenbrenner, Foerst and Kulozik, 2015).

2.6.4 Stability of freeze dried probiotics over storage

The desired health benefits of probiotics are linked to the cells' survival over the product's shelf life (Succi *et al.*, 2017). As mentioned above, the survival of probiotics over storage is influenced by similar factors to that of the dehydration process. Other factors of the storage conditions affect the survival of probiotics during storage, such as temperature, duration of shelf life and the moisture content after drying (Nader-Macías and Tomás, 2015).

The moisture content of freeze dried probiotic bacteria plays a huge role in ensuring stability throughout its shelf life and the viability over storage (Santiago and Gante, 2015). It is generally accepted that probiotic moisture content should be kept below 5% for the stability of *Lactobacillus* (Chávez and Ledeboer, 2007). Thorat and Joshi (2011) observed a correlation between the moisture content and log reduction in CFU counts of probiotic yeast *Saccharomyces boulardi* after heat pump assisted fluidized bed drying. Improved viability was observed with a reduction in moisture content of the dried probiotic yeast. In another experiment by Santiago and Gante (2015), during freeze drying of *Bifidobacterium infantis* and *Lactobacillus*, both strains retained a viable CFU counts of 10^9 ($\frac{CFU}{g}$) after 1 month storage at 30% relative humidity (RH), 22 ± 1°C. In contrast, there was an exponential decline instability at 53% RH, 30 ± 2°C to below 10^7 ($\frac{CFU}{g}$), the minimum required.

Calculation of moisture content

Various methods can be used to calculate the moisture content of dried probiotics. These include the galvanometric or oven drying method, Karl Fischer method, hygrometric or vapor pressure method (Baker, 1955). Most commonly, the presence of water in a biomaterial is either measured by the moisture content or water activity.

The galvanometric method is widely used. The moisture content is calculated by the ratio of relative weights of samples before oven drying and the samples after drying as seen in Equation 2-4, where M_w (%) represents the residual moisture content, W_{wb} (g) is the wet biomass weight and W_{db} (g) is dry biomass weight.

$$M_w = \frac{W_{wb} - W_{db}}{Wwb} \times 100 \qquad \text{Equation 2-4}$$

2.7 Prebiotics and synbiotics

For a while, the generally accepted definitions of prebiotic substances were limited to gastrointestinal health benefits. One was defined by Gibson *et al*. (2004) as:

> "*A selectively fermentable ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health".*

However, progress in the field of probiotics which extends its application beyond the gastrointestinal tract to other parts of the body has prompted a review of the definition. Moreover, the principle holds that the main role of prebiotic substances is to be fermented to improve proliferation of beneficial microorganisms. Therefore, a new definition of the term was established in 2016 by a panel of experts convened by the International Scientific Association for Probiotics and Prebiotics (ISAPP) (Gibson *et al.*, 2017). The prebiotic term has now been updated to:

> "*A substrate that is selectively utilized by host microorganisms conferring a health benefit".*

This new definition covers substrates including non-carbohydrates that promote proliferation for probiotic cells included in new formulations, for example formulations targeted towards topical delivery. With the reviewed definition, substances that meet this criterion, including digestible and non-digestible fibers targeted at regions beyond the gastrointestinal, can classified under this terminology (Gibson *et al.*, 2017).

Non-digestible fibers are most preferred to fit this definition because they remain intact through their journey to the intestine. These include inulin, raffinose, lactulose, amongst other fructooligosaccharides and sugar alcohols, all of which are commonly used and are preferred prebiotics to promote growth stimulation of probiotic bacteria in the gastrointestinal flora (Savini *et al.*, 2010).

The combined use of prebiotics in probiotic formulations known as "synbiotics" has been an important interest in the industrial manufacture of probiotics. For example, prebiotics are contained in probiotic foods such as yogurt that act to promote growth in the bowel when ingested (Savini *et al.*, 2010). According to Swanson *et al*. (2020), the term "synbiotics" is defined by the ISAPP as:

> "*A mixture comprising of live microorganisms and substrate(s) selectively utilized by host microorganisms that confers health benefits on the host".*

The use of prebiotics to supplement the growth of vaginal *Lactobacillus* was first introduced in 1955 by the Lawson Health Research Institute when skimmed milk was tested for its ability to improve vaginal *Lactobacillus* numbers in women with urinary tract infections (Collins *et al.*, 2018). Collins *et al.* (2018) investigated the growth stimulation potential of vaginal *Lactobacillus* strains by various known intestinal prebiotics candidates, including lactitol, lactulose, raffinose and oligofructose. The criteria for performance were the *Lactobacillus* growth rate in media and improved acidity in the growth media at the end of the incubation period. In this work, *Lactobacillus crispatus, Lactobacillus iners, Lactobacillus gasseri, Lactobacillus vaginalis* and *Lactobacillus jensenii* were monocultured in prebiotics supplemented media, using glucose free media as a control. BV-associated pathogens were also included in this experiment. Results showed that lactulose promoted stimulation across all vaginal *Lactobacillus* strains and improved the acidity in growth media compared to the control ($P<0.0001$-0.05). The other prebiotics showed lesser preference for stimulation by the vaginal strains. After establishing that lactulose was the best performing candidate, vaginal swabs were collected from healthy women and incubated with 0.5% (m/v) of prebiotics. The following conclusions were drawn from this work:

- Not all intestinal prebiotics support the growth stimulation of vaginal *Lactobacillus* strains.
- Some prebiotics can be antagonistic towards some vaginal strains.
- Some prebiotics may not promote the growth of vaginal *Lactobacillus* but could potentially improve acidity.
- Some prebiotics could inhibit the growth of BV-associated pathogens.
- The presence of prebiotics improves the dominance of vaginal *Lactobacillus*.

Succi *et al.* (2017) reported that strains belonging to *Lactobacillus rhamnosus* taxa have a genetic makeup that allows them to have a wide range of adaptability and selectivity to various substrates. These strains can grow in environments rich in lactose, inulin, sucrose, trehalose, starch, fructans maltose, cellobiose and raffinose.

Finally, some prebiotics are being considered for their possible protective role on probiotic bacteria (Avila-Reyes *et al.*, 2014; Cheow, Kiew and Hadinoto, 2016; Succi *et al.*, 2017). This approach is of great interest since prebiotics could be used to play a double role both as sugars *"for their historical role by stimulating proliferation and activity of probiotic bacteria in the colon and as protective agents against various environmental stresses"* (Succi *et al.*, 2017).

2.8 Probiotics delivery techniques, formulation and dosage

For decades, probiotics have been consumed in fermented foods. Dairy foods such as yogurt, milk and cheese are the most consumed sources of probiotics in Europe, with *Lactobacillus rhamnosus* GG and *Lactobacillus casei* being amongst the most included strains in probiotic products (Sreeja and Prajapati, 2013). Other non-dairy food formulations that contain probiotics include fermented juices and flavored drinks (Sreeja and Prajapati, 2013). The probiotic strains are either added to the food formulation simultaneously during fermentation or added to the mixture after fermentation and stored under low temperature/refrigerated conditions. Dried probiotic forms have a longer shelf life when stored under airtight conditions (Sreeja and Prajapati, 2013).

As with pharmabiotics, probiotics are usually delivered either orally, topically, or intravaginally with the method depending on the target system. Oral forms may either come in tablets, encapsulated in capsules, or powdery forms contained in sachets (Sreeja and Prajapati, 2013). Lebeer *et al.* (2018), formulated topical delivery probiotic cream containing *Lactobacillus rhamnosus* for the skin. The freeze dried probiotic powder was microencapsulated with alginate and formulated in an oil/water base suspension for delivery. According to this work, upon application, the probiotic cells can be released through mechanical abrasion from rubbing. This allows the water in the formulation to rehydrate and reactivate probiotic cells to establish themselves on the surface of the skin.

Intravaginal drug delivery in form of gel systems, suppositories and emulsions have been proposed (Caramella *et al.*, 2015). A similar delivery mechanism can be applied in considering the use of vaginal suppositories for vaginal probiotic administration. These vaginal formulations are designed to melt or dissolve in the vaginal cavity upon direct contact with the vaginal tissues when inserted. The freeze dried powder with or without excipients can be macroencapsulated within the pessary

and reactivated after rehydration by vaginal fluid upon discharge for establishment on the vaginal mucosae tissues (Caramella *et al.*, 2015).

It is still unclear what standard minimum dosage of probiotics is required to achieve the desired health benefit. Generally, doses with a minimum level of more than 10^6 to 10^9 ($\frac{CFU}{g}$) of probiotics are suggested with regular consumption of 100 g per day of food/product (Sreeja and Prajapati, 2013; Amund, 2016; Swain and Rani, 2018). Table 2-2 and Table 2-3 show the clinical guides for vaginal probiotics in the USA (Brown *et al.*, 2021) and formulations of probiotics available in South Africa, respectively. The data on the available probiotics in South Africa was obtained directly from available probiotics in South African health and wellness stores /pharmacy. It can be seen that the minimum dosage as advised across all products contains 1×10^9 CFU claimed per capsule, with the exception of "RepHresh™ Pro-B™ Probiotic" which has a cell count of 0.5×10^9 ($\frac{CFU}{g}$) per dosage. These data on commercially available probiotics in the USA and South Africa both agree with literature on the minimum cell count present per advised dosage. However, it can be drawn that the doses may vary depending on the strain or the required health benefit. It is essential for more research that elucidates on the relationship between the probiotic strain, the dose, and the required health benefits because knowledge in this area is still scarce and not fully understood.

Table 2-2 Clinical guide to oral vaginal probiotics available in the United States (Brown *et al.*, 2021)

	Brand Name	**Strain**	**CFU/Dosage**	**No of doses per day**
Oral Vaginal probiotics	AZO Complete Feminine Balance	*L. crispatus* LbV 88 *L. jensenii* LbV 116 *L. gasseri* LbV 150N *L. rhamnosus* LbV 96	5×10^9 ($\frac{CFU}{\text{capsule}}$)	1 capsule
	Jarro-Dophilus® Women	*L. crispatus* LbV 88 *L. jensenii* LbV 116 *L. gasseri* LbV 150N *L. rhamnosus* LbV 96	5×10^9 ($\frac{CFU}{\text{capsule}}$)	1 capsule
	Jarrow Formulas® Fem-Dophilus	*L. rhamnosus* GR-1 L. *L. reuteri* RC-14	2.5×10^9 ($\frac{CFU}{\text{capsule}}$)	1 capsule
	RepHresh™ Pro-B™ Probiotic	*L. rhamnosus* GR-1 L. *L. reuteri* RC-14	0.5×10^9 ($\frac{CFU}{\text{capsule}}$)	1 capsule
	UltraFlora® Women's	*L. rhamnosus* GR-1 L. *L. reuteri* RC-14	1×10^9 ($\frac{CFU}{\text{capsule}}$)	1 capsule

Table 2-3 Formulations of common probiotics available in South Africa (this data was obtained directly from available probiotics in South African health and wellness stores /pharmacy)

Brand Name	Probiotic application	Strain(s)	Excipient(s)	Dosage form	CFU /dosage	No dosage per day	Storage form	Total no of capsule	Price/pack	Country of origin
Femina (Velobiotics)	Vaginal health	*L. acidophilus, L. plantarum, L. casei, L. reuteri, L. rhamnosus, L. fermentum, L. gasseri, B. lactis, B. bifidum, B. longum*	Cranberry fruit 700mg, inulin (chicory root extract) 200mg	Capsules	5×10^9	1 capsule/day	Room Temperature	30	R175	South Africa
Velo16 (Velobiotics)	Digestive health	*L. acidophilus, L. plantarum, L. casei, L. reuteri, L. rhamnosus, L. fermentum, L. salivarus, L. paracasei, L. bulgaricus, L. helveticus, B. lactis, B. infantis, B. breve, B. bifidum, B. longum, S. thermophiles*	Hydroxypropyl Methylcellulose, Sugar, Maltodextrin, Emulsifying Salt (Magnesium Stearate), Colourants*, Anti-Caking Agent (Silicon Dioxide). *Natural Red Beet Colour, inulin (chicory root extract) 200mg	Capsules	5×10^9	1 capsule/day	Room Temperature			South Africa
Metagenics UltraFlora Spectrum	Digestive and immune health	*S. boulardii, B. lactis, L. plantarum, L. salivarius, L. acidophilus, S. thermophilus, B. lactis*	Hydroxypropyl methylcellulose, sodium copper chlorophyllin), microcrystalline cellulose, silica, sorbitan monostearate, and stearic acid (vegetable). (Capsules are composed of plant-derived ingredients)	Capsules	30×10^9	1 capsule/day	Refrigerated	30	R875	USA
Metagenics UltraFlora balance	Digestive immune and vaginal health	*L. acidophilus, B. lactis*	Microcrystalline cellulose, capsule (hydroxypropyl methylcellulose), magnesium stearate (vegetable), silica	Capsules	15×10^9	1 capsule/day	Refrigerated for best results or up to 28 days at room temperature	30	R295	USA
Metagenics UltraFlora Synergy	Digestive immune and vaginal health (with prebiotics)	*L. acidophilus, B. lactis*	Fructooligosaccharides (FOS), rice maltodextrin	Powder	15×10^9	0.75 g in 120 ml of water, 1-2 times/ day	Refrigerated for best results or up to 28 days at room temperature	30	R295	USA

Brand Name	Probiotic application	Strain(s)	Excipient(s)	Dosage form	CFU /dosage	No dosage per day	Storage form	Total no of capsule	Price/pack	Country of origin
The Real Thing PRO-Probiotic Vegicaps	Digestive health (with prebiotics)	*L. acidophilus, B. longum, B. bifidum, L. casei, L. sporogenes, L. paracasei, L. plantarum, L. rhamnosus, S. thermophiles, S. boulardi*	Inulin (50%), oligosaccharide (50%)	Capsules	20 x 10^9	1 capsule/day	Refrigerated or 2 weeks shelf life at room temperature	30	R290	South Africa
NeoGenesis BlissFlora	Gastrointestinal health	*Lactobacillus paracasei*	PS23* Probiotic Extract (30 billion cells), Inulin (100 mg).	Capsules	30 x 10^9	1 capsule 1-2 times per day	Refrigeration not required but recommended once opened	30	R440	South Africa
Sally Ann Creed Maxi Biotic 20	Immune health	*L. acidophilus, B. bifidum, B. longum, L. rhamnosus, L. plantarum, L. casei, S. thermophilus, L. paracasei, L. sporogenes, S. boulardii*	Gelatin Capsule, Biomix, Magnesium Stearate	Capsules	20 x 10^9	1 capsule 1-3 times per day	Dry cool place at or below 25°C away from direct sunlight	30	R359	South Africa
Good Health Immuno-Biotic	Gastrointestinal and immune health	*B. coagulans*	Yeast fermentate, L-Glutamine, Zinc (as Gluconate)	Capsules	-	1 capsule 1-2 times per day	Cool (<30°C), dry place out of direct sunlight	30	R379	New Zealand
Solgar Advanced Multi-Billion Dophilus	Gastrointestinal health	*L. acidophilus, B. lactis, L. paracasei and L. rhamnosus GG*	Maltodextrin, microcrystalline cellulose, sodium alginates, anti-caking agents (silicon dioxide, vegetable magnesium stearate), vegetable capsule shell (hydroxypropyl methylcellulose)	Capsules	-	2 capsule 1-2 times per day		60	R440	USA

Brand Name	Probiotic application	Strain(s)	Excipient(s)	Dosage form	CFU /dosage	No dosage per day	Storage form	Total no of capsule	Price/pack	Country of origin
Efficient Microbes Health Booster	Gastrointestinal and Immune health	*B. animalis, B. bifidum, B. longum, L. acidophilus, L. buchneri, L. bulgaricus, L. casei, L. delbrueckii, L. fermentum, L. plantarum, Lactococcus diacetylactis, L. lactis, S. thermophilus., B. subtilis, S. cerevisiae*	100% natural fruit juices, kelp, molasses, sodium chloride, purified and	Fluid	-	Adults: 3 tbsp (3 × 15 ml) per day Children: 3 tsp (3 × 5ml) per day	-	1 Litre	R199	
Rawbiotics Gut Repair		*Bifidobacterium animalis, Bi. bifidum, Bi. longum, Lactobacillus acidophilus, L. buchneri, L. bulgaricus, L. casei, L. delbrueckii, L. fermentum, L. plantarum, Lactococcus diacetylactis, L. lactis, Bacillus subtilis, Saccharomyces cerevisiae, Streptococcus thermophilus*	100% Natural Fruit juices, Kelp, Molasses, Sodium Chloride, Herbal extract blend (Slippery Elm, Lemon Balm, Peppermint, Rose Hip		-	30 ml per day (start with 10 ml per day and build up to recommended dosage)		1 Litre	R260	South Africa
Nature's Choice Ultimate Probiotic			Purified Water, Probiotic Lactobacillus Cultures, Organic Sugar Cane Molasses, Mineral Powder, Sea Salt, Rice Bran Liquid Extract, Grape Juice Concentrate, Herbal Extract Mix (Astragalus, Chamomile, Elderberry, Olive Leaf, Siberian Ginseng, Hypericum Perforatum)	Fluid	-	15 ml – 45 ml daily	Cool place, out of direct sunlight	500 ml	239.5	South Africa

3 DEFINING THE PROJECT

3.1 Problem statement

Research has identified promising *Lactobacillus* strains, which if administered as probiotics could treat bacterial vaginosis and bacterial associated acne vulgaris. For the potential of this pharmabiotic treatment to be realized, its probiotic effects need to be retained until the end of its shelf life. This can be achieved by dehydrating cells and keeping them in their dry form. However, the dehydration process is detrimental to cell structure. This causes a compromise in viability and functionality. Additionally, storage conditions lead to further loss of probiotic cells over time. Previous studies have demonstrated enhanced survival of cells during dehydration and subsequent storage by the inclusion of various cryoprotectants. However, few studies address the impact of storage on the functional properties of cells and roles of cryoprotectants in retaining these properties. It is therefore important to investigate the impact of cryoprotectants on the viability and functional properties of probiotic *Lactobacillus*. The result of this work will be of importance in selecting a suitable protective candidate in the formulation of freeze dried *Lactobacillus* concentrates towards the development of pharmabiotics in treating bacterial vaginosis and bacterial-associated acne vulgaris.

3.2 Research aim and objectives

This study herein investigates the impact of various saccharides on the growth and functionality of *L. plantarum* (as a model strain) and their potential to enhance the stability of its concentrates after dehydration and over shelf life. The goal of this work is to identify a cryoprotectant candidate(s) that could play a double role to protect cells and act as a prebiotic to stimulate proliferation upon topical probiotic administration.

The objectives of the study are thus:

- Investigate potential inhibitory effects of selected cryoprotectants on the growth of *Lactobacillus.*
- Investigate the prebiotic potential of selected cryoprotectants on the growth of *Lactobacillus.*
- Investigate the protective effects of selected saccharides on *Lactobacillus* during and after freeze drying.
- Investigate the survival of *Lactobacillus* in the presence of various cryoprotectants over the products' shelf life at different storage conditions.

3.3 Statement of key questions

- To what extent does the presence of the various cryoprotectants selected protect *Lactobacillus* during freeze drying?
- What is the stability of dehydrated *Lactobacillus* over a shelf life of 3 months in the presence of the various cryoprotectants?
- How does the drying process affect *Lactobacillus* growth activity and pH reduction potential?
- Will the presence of sugars affect the growth of *Lactobacillus* when rehydrated upon administration? If so, how?
- What is the influence of the presence of a cryoprotectant on the cell membrane structure?

3.4 Research hypothesis

The ability of disaccharides and oligosaccharides to form a glassy matrix and replace the hydrogen bonds between water molecules and bi-phospholipid layers in the cell membrane of biomaterials has been proven. Furthermore, they are considered as good carbon sources that enhance bacterial growth. Because of these abilities, the inclusion of saccharides in the bacterial suspension before freeze drying will improve the survival of *Lactobacillus* during dehydration, enhance subsequent stability during storage and promote proliferation upon rehydration.

3.5 Implications of research project

This research aims to explore methods to improve the stability of *Lactobacillus* during storage. It is important that cells remain alive and in a stable form in which their structure, biological and biochemical activity are retained upon delivery. This will enable them to deliver the desired probiotic health benefits. Approaches to improve viability and stability of probiotic cells are highly dependent on the strains as performance of these approaches is strain specific. Many studies have widely investigated methods to improve the viability and stability of primarily *Lactobacillus* strains commensal to the human gut as this area of research has been widely studied in the past. Probiotics for vaginal and skin application is an emerging area of study now gaining the interest of researchers. Studies to improve stability of *Lactobacillus* strains suitable for these applications is scarce and therefore warranted. The success of this approach to keep freeze dried *Lactobacillus* concentrates viable and stable after processing and during storage is a step towards the validation of candidate strains for vaginal and skin probiotic delivery as an effective biotherapy for preventing or treating

diseases. This is in line with the Sustainable Development Goal (SDG) 3, towards attaining good health and wellbeing.

4 EXPERIMENTAL DETAILS

This chapter describes in detail the research approach, materials, and methods implemented to address the research questions and to prove the hypothesis defined in the previous chapter.

4.1 Research methodology

The research for this study was approached quantitatively by experimental data collected through the structure illustrated in Figure 4-1 where the work was divided into two main parts. These experimental strands are described in brief below and further expanded on in the subsequent subsections of the chapter.

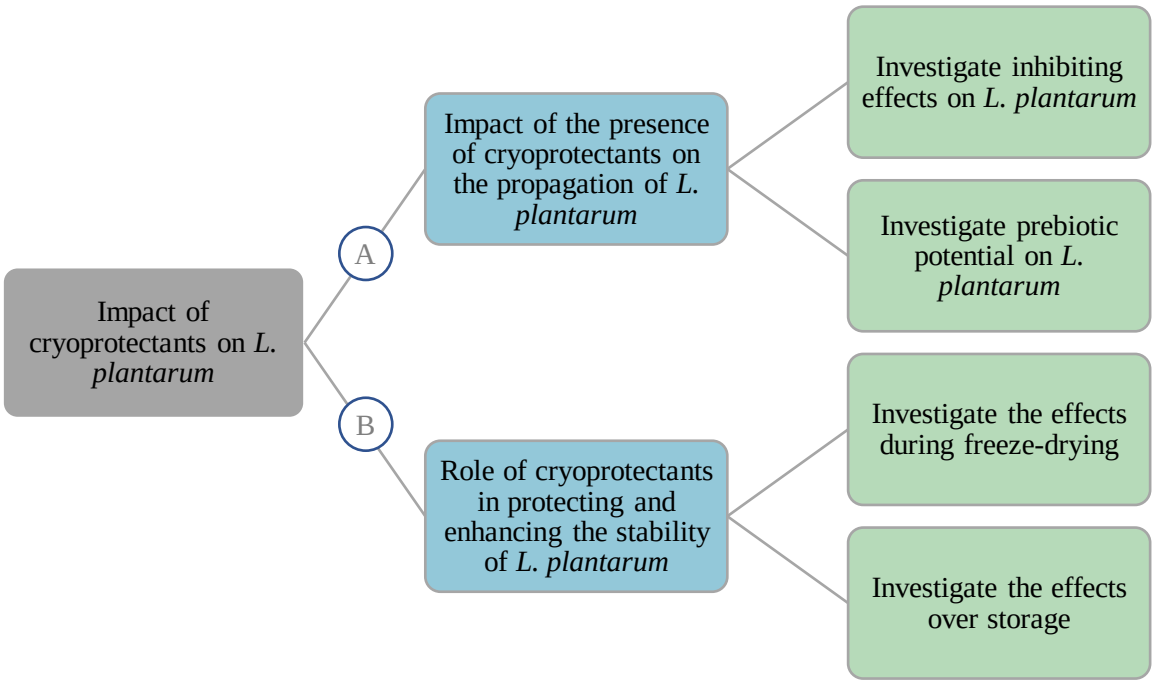

Figure 4-1 Flow diagram showing experimental analysis steps to investigate the (A) prebiotic potential and (B) cryoprotectant potential of various sugars on *L. plantarum*

The motivation for the first approach, A, was to answer questions around the possible effect of incorporated cryoprotectants on rehydrated *L. plantarum* upon administration. It aimed to address the question: would the cryoprotectants inhibit or promote proliferation upon rehydration? In this strand, *L. plantarum* was grown in the presence of the various cryoprotectants for the two different objectives as illustrated in Figure 4-1.

The second approach, B, was carried out to understand the protective effects of various cryoprotectants on freshly grown *L. plantarum* during freeze drying and to assess the microbe's

survival over a shelf-life period of 3 months under two different storage temperature conditions, room temperature (RT) and 4ºC standard refrigeration.

4.2 Materials

4.2.1 Collection and isolation of strain

L. plantarum was obtained from the culture collection developed in the study by Happel *et al.* (2018), which collected, isolated and characterised strains from Softcup® menstrual cup samples of cervicovaginal mucus from BV negative South African women (ages 18-25).

The primary *L. plantarum* isolate was frozen at -80ºC in bacterial glycerol stocks made up of 20% (v/v) *L. plantarum* culture and glycerol solution made up of 60% glycerol content as described in (Happel *et al.*, 2018). 25% (v/v) *L. plantarum* secondary glycerol stocks were made from the primary *L. plantarum* glycerol stocks at the Centre for Bioprocess Engineering Research (CeBER), UCT by thawing and growing the primary stocks in 50 g/l MRS media in a 100 ml working volume at 36ºC, overnight. The culture from this fermentation was stored in 2 ml cryovials at -80ºC in 50% glycerol solution, made up of 25% *L. plantarum* culture for use in this study.

4.2.2 Reagents and cryoprotectants

The following reagents and cryoprotectants were used in this study:

- MRS broth (product number, 69966, 99% purity), skimmed milk for bacteriological purposes (product number 70166, ≥ 50% reducing sugars), D-(+)-glucose (product number G8270, ≥ 99.5% purity) and L-Cysteine (product number 168149, 97% purity), all purchased from Sigma Aldrich, Germany.
- Inulin, purchased from NOW foods, USA.
- Food-grade sucrose, purchased from Pick n Pay stores, South Africa.
- Maltodextrin, purchased from Supplement Factory, South Africa.

Water used for this work was deionized using the Millipore Elix Essential 15 deionizing equipment and was sterilized with the Hirayama; HG50 autoclave at 121ºC for 20 minutes. All reagents were sterilized after preparation before use except for skimmed milk drying media suspensions which were prepared using sterilized water. The skimmed milk powder used for this study was purchased in sterilized in sealed containers and stored under sterile conditions throughout the duration of this study to avoid contamination. Furthermore, its contents were also tested for contamination by spreading a diluted suspension in sterile deionized water on agar plates for 48 hours and not contamination was observed.

Table 4-1 presents the substrate content of MRS media used in this work. Tween 60 was added to every media, prepared in a fraction of 1 ml/L of MRS media before sterilization. L-cysteine was also added in the quantity stated in Table 4-1.

Table 4-1 MRS media content

Substrate	**g/100 ml**
Glucose	2
Peptone	1
Yeast extract	0.4
Meat extract	0.8
Dipotassium hydrogen phosphate	0.2
Sodium acetate trityhydrate	0.5
Triammonium citrate	0.3
Magnesium sulphate heptahydrate	0.02
Manganous sulphate tetrahydrate	0.0005
L-cystrene	0.0072

4.3 Experimental assays

4.3.1 Enumeration of cells

Colony forming units (CFU)

MRS agar plates were prepared with MRS media (50 g/L) supplemented with bacteriological agar (12 g/L) to make up the solution. Sterile agar was achieved by autoclaving using the Hirayama HG50 autoclave, at 121ºC for 20 minutes and left to cool for 2 hours. After cooling down, the agar was poured into plates (25-30 ml/plate) under laminar flow and left to solidify.

100 µl of fresh bacterial suspension in media was diluted serially (× 10^7-10^8 dilution) in sterile deionized water and spread on agar plates in duplicates. The agar plates were incubated at 37ºC for 48 hours and the CFUs were then counted.

Optical density

Bacterial culture was sampled out of the fermentation serum bottles at 1 hour time intervals using a syringe. The OD of this sample was then read at 660nm wavelengths using the Thermo Scientific

Genesys 10S UV-VIS spectrophotometer. For readings above 0.6, the culture was diluted 10 times using fresh MRS media. Fresh MRS media was used as the blank in this experiment.

Cell dry weight

Fresh bacterial suspensions were concentrated in pre-weighed and pre-dried (80°C, 48 hours) Eppendorf microfuge tubes by centrifugation at 8,000 g using the Eppendorf 5418R microfuge for 15 minutes. They were washed in sterile deionized water and centrifuged again. The pellets were dried at 80°C in the Labotec Eco Therm 277 oven for 48 hours and were placed in a desiccator for 2 hours to cool down. The biomass dry weight was determined by weighing with the Radwag AS220.R2 weighing balance.

A standard curve, presented in Figure 4-2, was created. The linear trendline fitted to this plot was used to obtain the dry biomass in grams contained in the fermented culture during further experiments. For OD readings above 0.6, the culture was diluted 10 times using fresh MRS media. Fresh MRS was used as the blank in the spectrophotometer measurements.

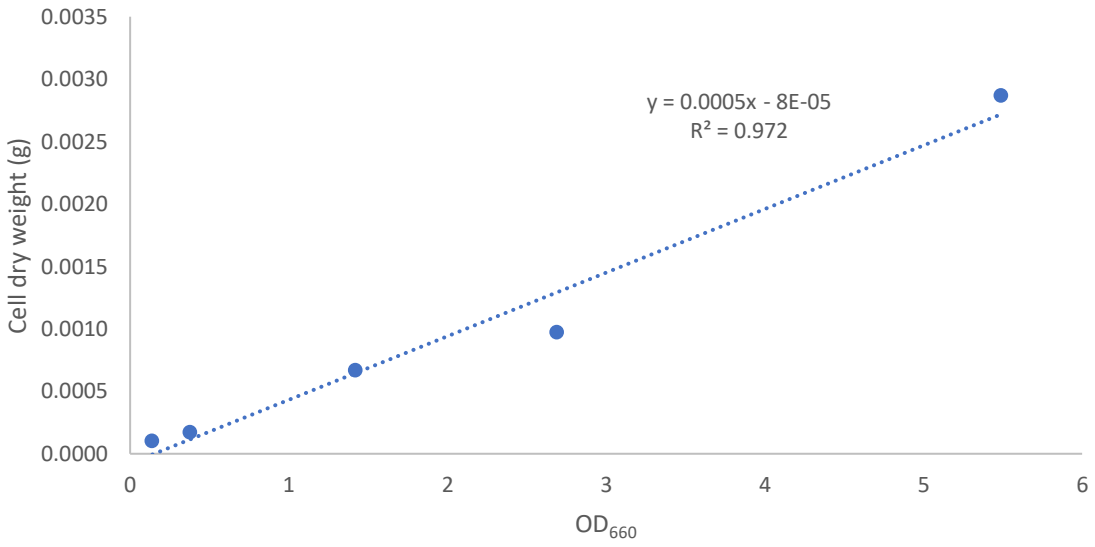

Figure 4-2 Cell dry weight versus absorbance at 660 nm standard curve

4.3.2 pH reduction potential

The drop in pH (caused by lactic acid production by the bacteria) was monitored during all fermentation experiments. The pH was measured using the Lasec pH 50+ DHS pH meter.

4.3.3 Moisture content

The moisture content of freeze dried material was determined by the gravitational method (Shamekhi Fatemeh, 2011). Weighing boats were made from cut aluminium foil sheets in equal sizes, labelled, and oven dried at 80°C for 48 hours using the Labotec Eco Therm 277 oven. The weights of the boats were measured using the Radwag AS220.R2 weighing balance. After freeze drying, samples were transferred on to the boats and weighed immediately (W_{fs}) using the weighing balance. The samples were oven dried at 80°C for 48 hours and placed in the desiccator for 2 hours and then weighed (W_{os}). The moisture content (M_w) was calculated as a percentage of the loss in weight of the samples, as given in Equation 4-1.

$$M_w(\%) = \frac{W_{fs} - W_{os}}{W_{fs}} \times 100 \qquad \text{Equation 4-1}$$

4.3.4 Scanning Electromagnetic Microscopy (SEM) and Transmission Electromagnetic Microscopy (TEM)

SEM and TEM imaging were performed at the Aaron Klug Centre for Imaging and Analysis, UCT. The following chemical fixation protocol was performed for the preparation of the samples for imaging.

For the primary chemical fixation, the samples were immersed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, with a pH of 7.4 for 16 hours. Afterwards, they were washed twice for a duration of 5 minutes each in 0.1 M phosphate buffer with a pH of 7.4. For the secondary chemical fixation, the samples were placed in 1% osmium tetroxide in 0.1 M phosphate buffer, with a pH of 7.4 for 1 hour. Afterwards, they were washed twice for a duration of 5 minutes in 0.1 M phosphate buffer with a pH of 7.4, and washed again, twice with distilled water each with a duration of 5 minutes. The samples were dehydrated for 10 minutes in 50% ethanol, 10 minutes in 70% ethanol, 10 minutes in 90% ethanol, 10 minutes in 95% ethanol, and twice for 10 minutes in 100% ethanol. These samples were ready and sent for SEM.

For TEM analysis, the samples were placed in 50/50 acetone/Spurr's resin overnight, followed by 75:25 resin: acetone for 8 hrs, then 100% resin overnight. This protocol was repeated with 100% resin the following morning. The samples were afterwards left in 100% Spurr's resin for 2 hours. Samples were oriented into mould and resin was added. The mould containing samples was placed in a 60°C oven for 24 hours.

4.3.5 Statistical methods

The students' T-test was performed using Microsoft excel software to determine the significant differences across values obtained.

4.4 Processing of *L. plantarum*

Figure 4-3 presents a process flow of the key process steps applied in this study to obtain freeze dried probiotic *L. plantarum*. Photographic images of the various process steps are presented in Figure 4-4. The process steps are explained in detailed in the following subsections.

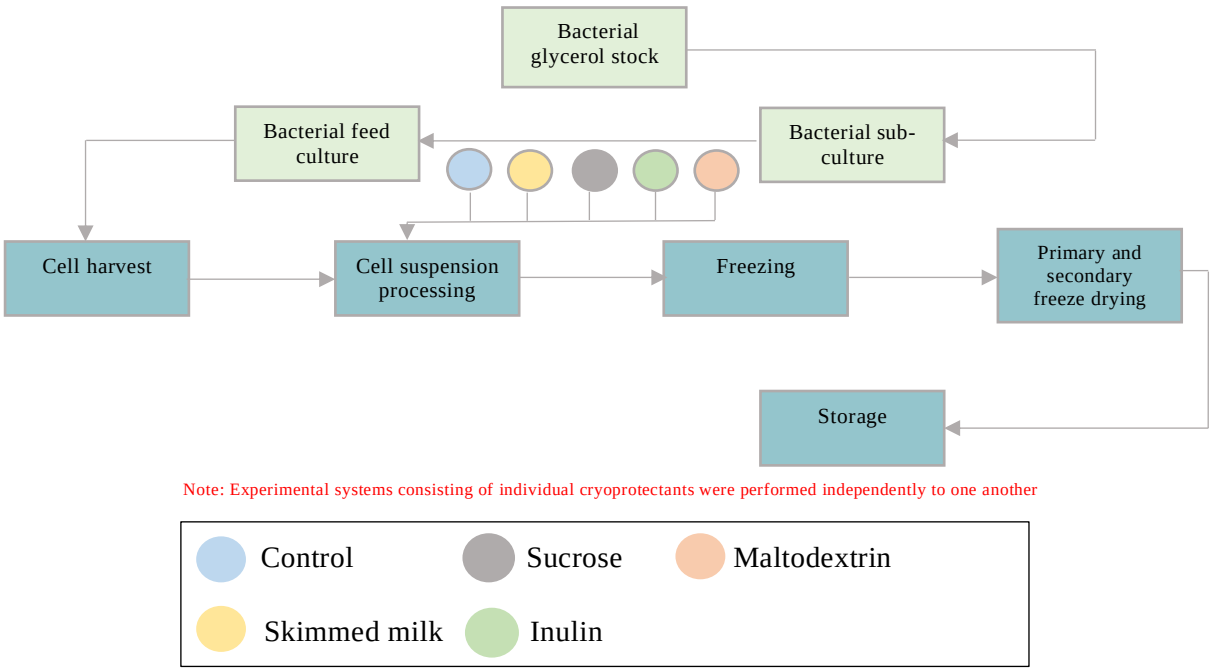

Figure 4-3 Process flow diagram of steps applied to obtain freeze dried probiotic *L. plantarum*

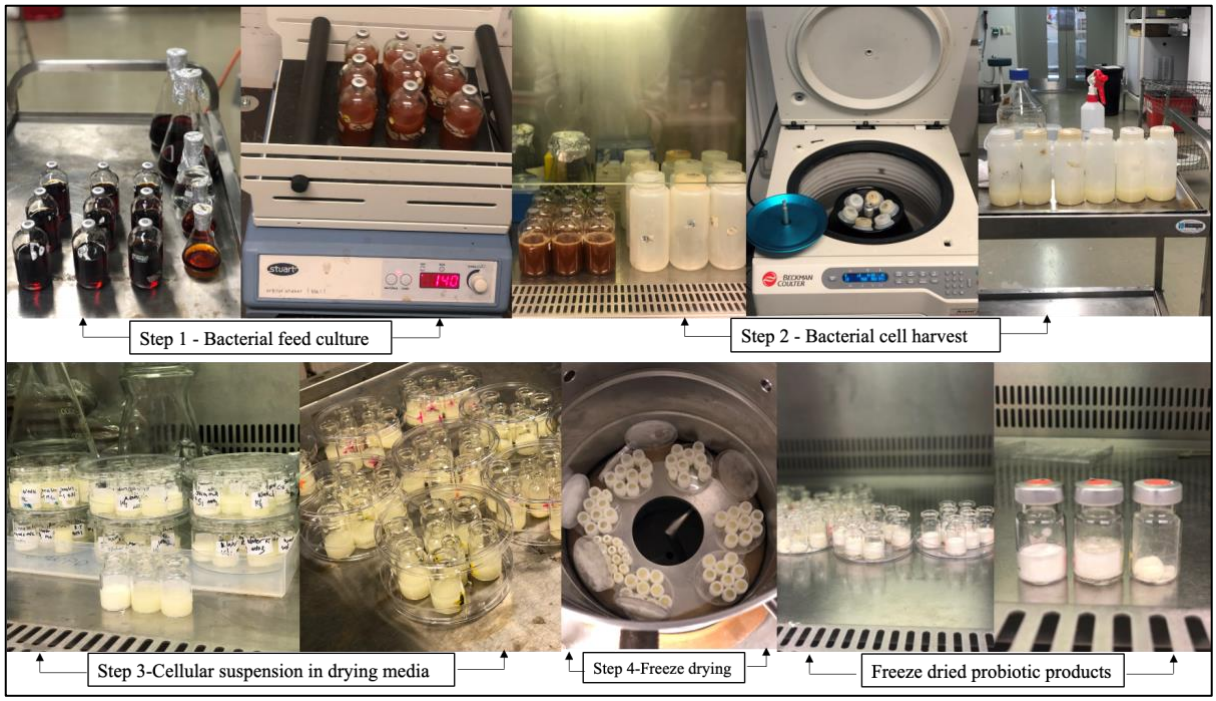

Figure 4-4 Photographic images of process steps applied to obtain freeze dried probiotic *L. plantarum*

4.4.1 Fermentation of *L. plantarum* (Upstream processing)

Preparation of fermentation media

Unless otherwise stated, all standard fermentation media were prepared by dissolving MRS broth in deionized water to make up a solution of 5 g/100 ml concentration. Fermentations were performed in 100 ml serum bottles and sealed with a rubber septum in aluminium caps. The standard media was autoclaved using the Hirayama HG50 autoclave at 121°C for 20 minutes and then cooled to room temperature for use.

Fermentation process

Fresh bacterial sub-culture was prepared from thawed secondary glycerol stocks, discussed in 4.2.1. From the bacterial glycerol stocks, 2 ml was inoculated into 50 ml working volume of sterile MRS media. The fermentation was run overnight at 37°C and 140 rpm using the Stuart SSL1 shaker. At the end of the fermentation, the overnight culture was inoculated into 100 ml fresh and sterile MRS fermentation media to make up an initial OD_{660} reading of 0.1. Fermentation was run under the same conditions as that of the inoculum fermentation until stationary phase was obtained.

4.4.2 Recovery of *L. plantarum* (Downstream processing)

Harvest of cells

When stationary phase was attained under aseptic conditions, the cells were concentrated and separated from the supernatant twice by centrifugation at 8000 g for 15 minutes at 4°C using the Beckman JA-25 and J-A centrifuge. The supernatant was discarded, and the pellet was resuspended by homogenizing at medium speed in sterile deionized water with vortex from Benchmark Scientific BV1000.

Processing of *Lactobacillus* cellular feed suspension

The various cryoprotectants for this experiment were suspended in sterile deionized water to achieve a 10% (m/v) drying media. The different media in conical flasks were then covered with cotton wool and aluminium foil sheets and autoclaved for further processing. Freshly harvested cells from each fermentation system were suspended in 30 ml of the freeze drying media to make up 1% m/v of cells and vortexed at medium speed to achieve a homogenous feed suspension. 1 ml sample was collected from the feed suspension of each fermentation experiment and stored at 4°C for sample analysis before freeze drying. The remainder of the bacterial suspension from each fermentation experiment was separated in 3 ml aliquots into 5 ml sized ampoule vials for the freezing process.

Freeze drying

The multiple vials containing the fresh feed suspensions, as shown in Figure 4-5, were arranged in Petri dishes and transferred to the freezer. Freezing was done at -80°C for 24 hours outside the freeze dryer. After freezing, the frozen samples were transferred to the Instruvac 5KL V freeze dryer in an insulated box. Primary and secondary freeze drying runs were done consecutively and ran at 300 mTorr and -35°C for 24 hours.

(A) (B)

Figure 4-5 Fresh *L. plantarum* prior to freeze drying in: (A) skimmed milk, inulin, and control with only water (left to right); (B) maltodextrin, sucrose, and control with only water (left to right)

4.5 Experimental design

4.5.1 Impact of the presence of cryoprotectants on the propagation of *L. plantarum*

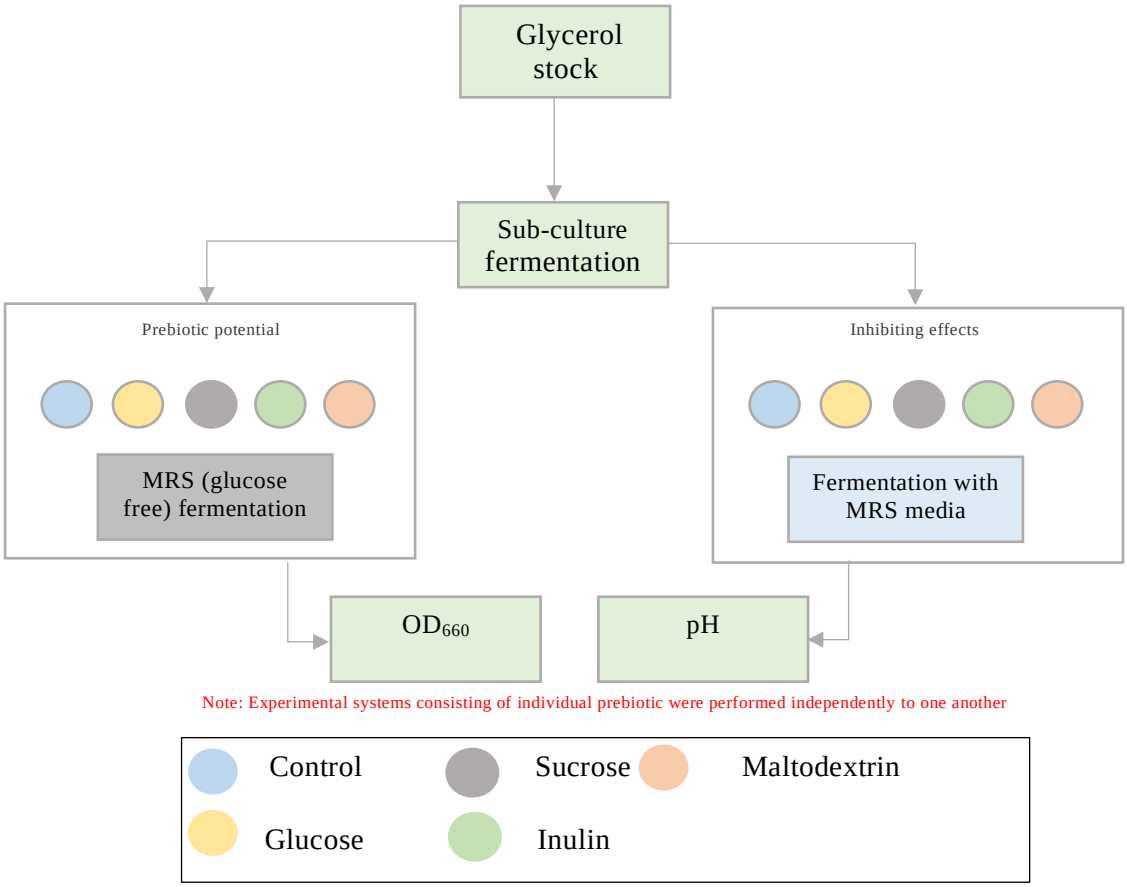

Figure 4-6 Flow diagram showing experimental design to investigate prebiotic potential and inhibiting effects of various sugars on *L. plantarum*

Inhibiting effects

To test for the inhibitory effects of the cryoprotectants on the growth of *L. plantarum* (as illustrated in Figure 4-6), bacterial sub-cultures were fermented in sterilized 100 ml MRS media (constituting of 2% (m/v) glucose) in serum bottles, supplemented with maltodextrin, glucose, skim milk, and inulin. The cryoprotectants were supplemented in different concentrations of 0.5%, 2%, and 4% (m/v) respectively. The various systems and their combinations are presented in Table 4-2, showing the substrate composition in grams per 100 ml added to each system. Fermentation was performed at 37°C and run for 24 hours. Sampling was done hourly to measure the cell density, OD_{660} and pH for determining the growth kinetic parameters and pH reduction potential. This experiment was performed in triplicate under sterile conditions.

Table 4-2 Fermentation broth composition of systems to investigate potential inhibiting effects of cryoprotectants

	Control	Sucrose			Glucose			Maltodextrin			Inulin		
Substrate		0.5%	2%	4%	0.5%	2%	4%	0.5%	2%	4%	0.5%	2%	4%
Glucose (g/100ml)	-	-	-	-	0.5	2	4	-	-	-	-	-	-
Inulin (g/100ml)	-	-	-	-	-	-	-	-	-	-	0.5	2	4
Maltodextrin (g/100ml)	-	-	-	-	-	-	-	0.5	2	4	-	-	-
Sucrose (g/100ml)	-	0.5	2	4	-	-	-	-	-	-	-	-	-
MRS (g/100ml)	5	5	5	5	5	5	5	5	5	5	5	5	5
Total substrate per 100 ml media (g)	**5**	**5.5**	**7**	**9**	**5.5**	**7**	**9**	**5.5**	**7**	**9**	**5.5**	**7**	**9**

Prebiotic potential

To test for the prebiotic potential of the various cryoprotectants on the growth of *L. plantarum* (as illustrated in Table 4-3), bacterial sub-cultures were fermented in sterilized 100 ml *glucose-free* MRS media in serum bottles, with glucose substituted for maltodextrin, glucose, skim milk and inulin. The cryoprotectants were added to make up different concentrations of 0.5%, 2%, and 4% (m/v) respectively, as presented in Table 4-3. Fermentation was performed at 37°C and run for 24 hours. Sampling was done hourly to measure the cell density, OD_{660} and pH for determining the growth kinetic parameters and pH reduction potential. This experiment was performed in triplicate under sterile conditions.

Table 4-3 Fermentation broth composition of systems to investigate prebiotic potential of cryoprotectants

	Control	**Sucrose**			**Glucose**			**Maltodextrin**			**Inulin**		
Substrate		0.5%	2%	4%	0.5%	2%	4%	0.5%	2%	4%	0.5%	2%	4%
Glucose (g/100ml)	-	-	-	-	0.5	2	4	-	-	-	-	-	-
Inulin (g/100ml)	-	-	-	-	-	-	-	-	-	-	0.5	2	4
Maltodextrin (g/100ml)	-	-	-	-	-	-	-	0.5	2	4	-	-	-
Sucrose (g/100ml)	-	0.5	2	4	-	-	-	-	-	-	-	-	-
Glucose-free MRS (g/100ml)	5	3	3	3	3	3	3	3	3	3	3	3	3
Total substrate per 100ml media (g)	**5**	**3.5**	**5**	**7**	**3.5**	**5**	**7**	**3.5**	**5**	**7**	**3.5**	**5**	**7**

4.5.2 Role of cryoprotectants in protecting and enhancing the stability *L. plantarum*

Figure 4-7 illustrates the design of experimental methods applied in this study. Two factors were used as key determinants in evaluating and comparing the influence of different cryoprotectants on freeze dried *L. plantarum* cells. These are the survival and the vitality (upon re-growth) of freeze dried probiotic cells immediately after freeze drying and over storage. An additional factor, the physical structure of freeze dried probiotic cells, was used to evaluate the impact of the freeze drying on the cell membrane and nature of the resulting product. The experimental methods applied were motivated by these factors and are described in the following subsections.

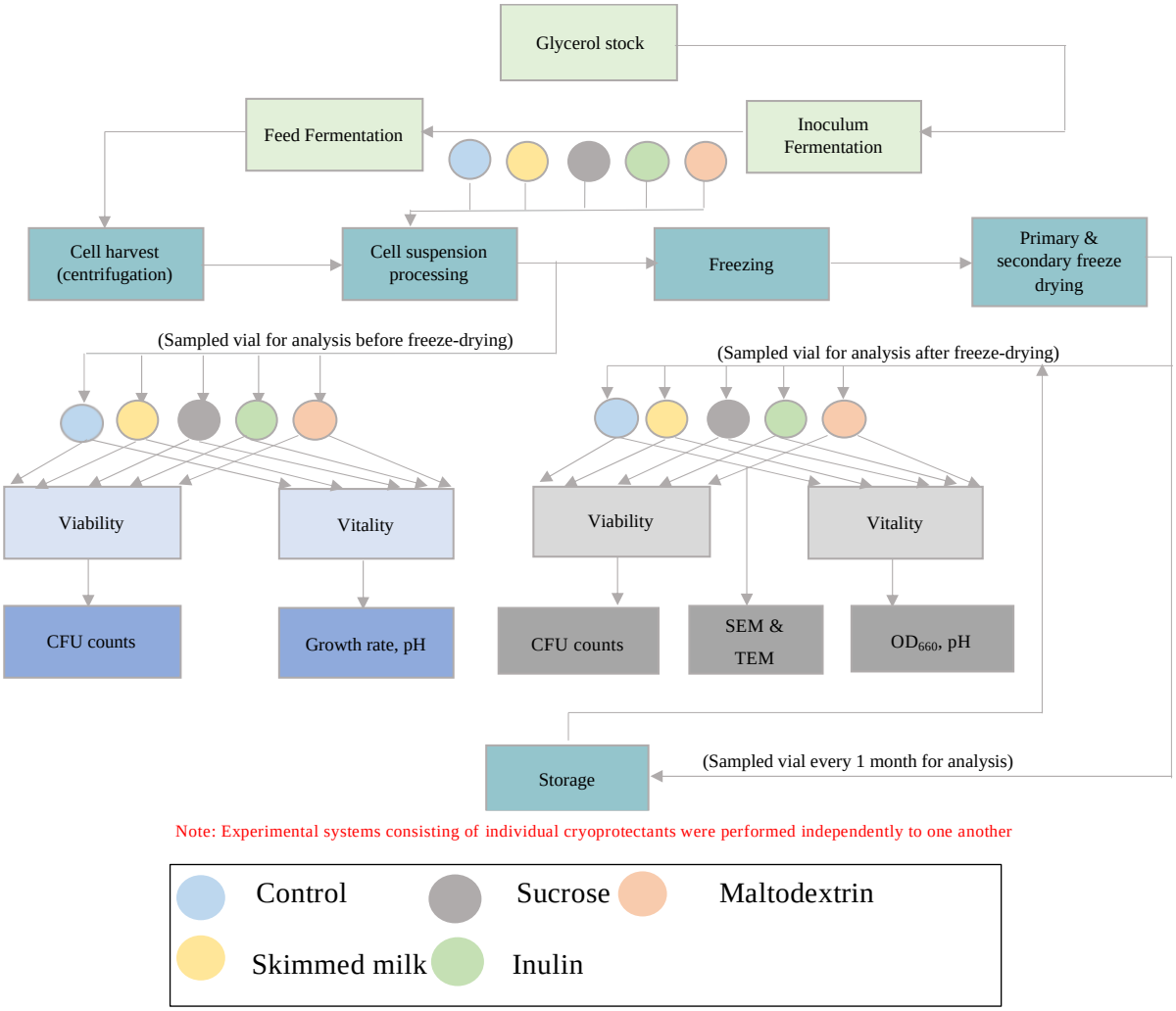

Figure 4-7 Process flow diagram showing the Upstream (fermentation process) and Downstream processes (cell concentration, processing of drying media, dehydration, and analysis steps to investigate the protective effect of various cryoprotectant sugars) on *L. plantarum*

Viability

The survival of probiotic cells was measured in terms of their viability. The viability of cells was calculated using the values of enumerated cells in Colony Forming Unit (CFU) counts before freeze drying and after freeze drying, or after storage. The CFU count assay is described in detail in section 4.3.1. Equation 4-2 shows the formula applied in calculating the viability of cells where N_o represents the number of cell counts before freeze drying, N_t represents the number of cell counts at time t (after freeze drying or after storage). The viability was calculated as a percentage.

$$\text{Viabilty}(\%) = \frac{(N_o - N_t)(\frac{CFU}{ml})}{N_o(\frac{CFU}{ml})} \times 100$$

Equation 4-2

Vitality

The motivation for applying this factor was to understand the *in vitro* performance of cell cultures upon re-growth and the roles the presence of various cryoprotectants play in influencing the propagation after freeze drying and over storage. The vitality of cells was evaluated using two determinants: pH reduction potential and microbial growth kinetic parameters. The assay to determine the pH reduction potential is described in section 4.3.2. The growth kinetic parameters μ_{max}, t_{lag} and OD_{max} evaluated in this study were drawn from the raw data presented in the appendices. μ_{max} was calculated using Equation 2-2 based on the raw data of the OD measurements, t_{lag} and OD_{max} were deduced from the graph plot of OD measurements against time.

5 RESULTS AND DISCUSSION I: INFLUENCE OF THE PRESENCE OF CRYOPROTECTANTS ON THE GROWTH OF *LACTOBACILLUS PLANTARUM*

This chapter presents the results obtained through experimental data extracted based on the methods described in the chapter 4. These findings aim to predict through *in vitro* tests, the possible influence of various cryoprotectants on the candidate strain that could be expected upon administration of a pharmabiotic formulation containing *L. plantarum* with the various cryoprotectants, glucose, inulin, maltodextrin, and sucrose respectively.

In this first part of the study, the possible inhibiting effect of their presence in the pharmabiotic formulation was investigated. Following this, the prebiotic potential of the various cryoprotectants was investigated by assessing their respective ability to stimulate the growth of *L. plantarum* and restore an acidic pH.

5.1 Inhibiting effects of cryoprotectants on *L. plantarum*

To investigate possible inhibiting effects by the cryoprotectants on the fermentation of *L. plantarum*, the microorganism was cultured in the standard (MRS) growth media supplemented with the various cryoprotectants at different levels of concentration: 0.5%, 2%, and 4% (m/v) respectively. Across all the experiments with the respective cryoprotectant, the control was *L. plantarum* fermented in standard MRS media (containing 2% (m/v) of glucose without cryoprotectants). The results from these experimental systems are presented in the following subsections. They elucidate whether the presence of these cryoprotectants will inhibit the ability of *L. plantarum* to proliferate and restore the acidity, which are the essential pharmabiotic outcomes for restoring and maintaining vaginal and skin health.

5.1.1 Inhibiting effects of **glucose**

The growth curves of *L. plantarum* were developed for each system through the measurement of the increase in cell density with time by reading the absorbance (OD_{660nm}) of the fermentation culture containing MRS media supplemented with 0.5%, 2.0%, and 4% (m/v) glucose. These growth curves are presented in Figure 5-1 and are based on the raw data presented in appendix A.

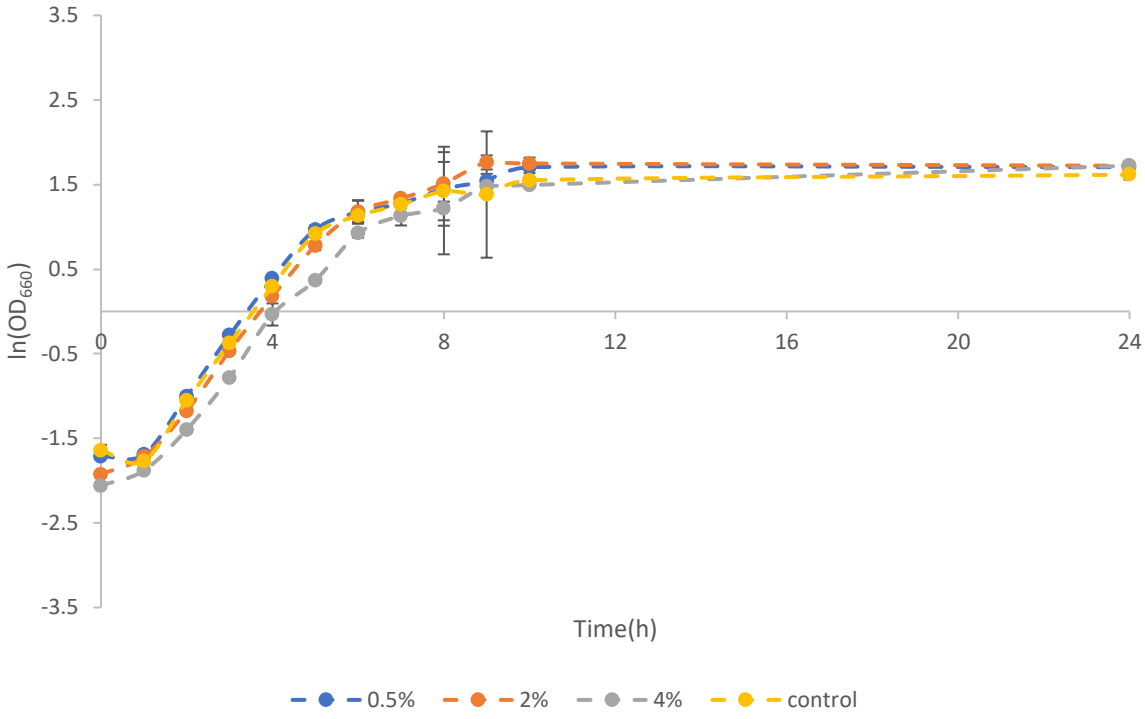

Figure 5-1 Fermentation profiles of *L. plantarum* in the control (MRS media) and MRS media supplemented with 0.5%, 2% and 4% **glucose**. The error bars are representative of the standard deviation between triplicate repeat runs

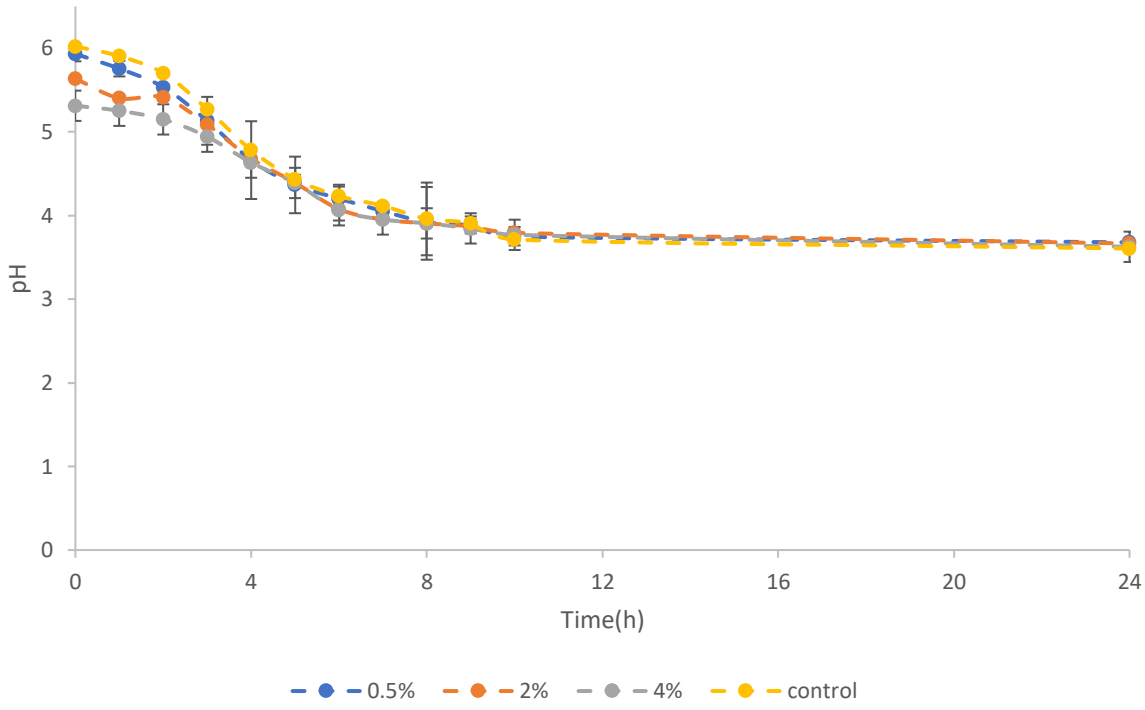

Figure 5-2 pH profiles due to *L. plantarum* lactic acid production in the control (MRS media) and MRS supplemented with 0.5%, 2% and 4% **glucose**. The error bars are representative of the standard deviation between triplicate repeat runs

All fermentation profiles appear to be similar and nearly synchronized with the control. This suggests that the presence of additional glucose supplemented in various concentrations did not inhibit the growth of *Lactobacillus plantarum*.

Table 5-1 presents the $\mu_{max,}$ t_{lag} and OD_{max} obtained from the experimental data on the glucose systems (full data presented in appendix A). Although some small variations were observed in the μ_{max} calculated from the data, there was no significant change between the control and the systems supplemented with 0.5% and 2% (p-value > 0.01) glucose. However, the lowest μ_{max} value of 0.57 ± 0.04 h^{-1} was found at the highest glucose concentration of 4% supplementation. An increase in the OD_{max} was observed when the limiting carbon source, glucose, was increased from the baseline glucose content in MRS media, up until 2% (m/v) of supplemented glucose concentration in the media, with a maximum OD_{max} value of 5.6 with 2% (m/v) of supplemented glucose and a minimum value of 5.03 with the control. However, no increase in the OD_{max} was observed with further increase in the glucose concentration to 4% (m/v) of supplemented glucose substrate. The t_{lag} for all growth profiles remained the same.

In explaining this, the μ_{max} in a closed batch system does not increase when the limiting substrate is in excess (Maier, 2000). This could probably mean that the standard glucose substrate concentration contained in MRS media of 2% (m/v) is sufficient for optimum microbial growth in a batch reactor and beyond that no significant increase in microbial growth rate may be observed. The slight decline in the μ_{max} seen when 4% glucose was supplemented in the MRS media suggests that the growth of *L. plantarum* may have been inhibited at this concentration. This supports that an inhibiting region exists beyond the optimal growth region whereby increasing the substrate nutrient concentration results in a decrease in the growth rate (Hu, 2018).

The fact that the *L. plantarum* had been previously cultured in MRS media with glucose as the carbon substrate, may be the reason that the microorganism did not demonstrate any changes in the lag phase at the various concentrations of glucose. This result aligns literature such as reported by Molina-Ramírez *et al.* (2017) who observed no increase in t_{lag} when *Komagataeibacter medellinesiss* was cultured in media containing various concentrations of glucose, due to the fact the microorganism did not require adjustment to a new substrate. When a new substrate is introduced into a new media, extra time is spent for microorganism to induce specific RNA (mRNA) and synthesize proteins to meet new culture requirements (Maier, 2000).

The pH reduction profiles presented in Figure 5-2 show that there was a reduction in pH of the monoculture owing to the production of acidic metabolites from the fermentation of glucose. There was no significant change in the final pH across all systems and the overall drop in the pH of the

culture was not affected, as seen in Table 5-2. This indicates that the presence of glucose in various concentrations should not inhibit the ability of *L. plantarum* to restore vaginal or skin acidity.

Table 5-1 Inhibiting test μ_{max}, t_{lag}, and OD_{max} values, and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data on *L. plantarum* cultured in various concentrations of **glucose** (0.5%, 2%, 4% (m/v)) supplemented MRS media and the control

Systems (m/v)		μ_{max} (h^{-1})	Ave	SD	SEM	t_{lag} (h)	Ave	SD	SEM	OD_{max}	Ave	SD	SEM
0.5%	Run 1	0.73	0.67	0.07	0.04	1	1	0	0	5.49	5.5	0.01	0.01
	Run 2	0.60				1				5.51			
	Run 3	0.69				1				5.50			
2%	Run 1	0.61	0.63	0.03	0.02	1	1	0	0	5.59	5.60	0.01	0.00
	Run 2	0.63				1				5.60			
	Run 3	0.66				1				5.60			
4%	Run 1	0.62	0.57	0.04	0.03	1	1	0	0	5.56	5.60	0.03	0.02
	Run 2	0.56				1				5.62			
	Run 3	0.54				1				5.62			
Control (contains 2% (m/v) glucose)	Run 1	0.67	0.67	0.00	0.00	1	1	0	0	5.10	5.03	0.06	0.03
	Run 2	0.68				1				5.00			
	Run 3	0.67				1				5.00			

Table 5-2 Inhibiting test starting and final pH values, and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data on *L. plantarum* cultured in various concentrations of **glucose** (0.5%, 2%, 4% (m/v)) supplemented MRS media and the control

Systems (m/v)		Initial pH	Ave	SD	SEM	Final pH	Ave	SD	SEM
0.5%	Run 1	5.94	5.93	0.01	0.00	3.68	3.68	0.01	0.01
	Run 2	5.93				3.69			
	Run 3	5.93				3.67			
2%	Run 1	5.64	5.64	0.01	0.00	3.66	3.67	0.01	0.00
	Run 2	5.63				3.67			
	Run 3	5.64				3.67			
4%	Run 1	5.32	5.31	0.01	0.01	3.62	3.63	0.01	0.00
	Run 2	5.30				3.63			
	Run 3	5.32				3.63			
Control (contains 2% (m/v) glucose)	Run 1	6.04	6.02	0.02	0.01	3.60	3.61	0.01	0.00
	Run 2	6.02				3.61			
	Run 3	6.01				3.61			

5.1.2 Inhibiting effects of **inulin**

The growth curves of *L. plantarum* were developed for each system through the measurement of the increase in cell density with time by reading the absorbance (OD_{660nm}) of the fermentation

culture containing MRS media supplemented with 0.5%, 2.0%, and 4% (m/v) inulin. These growth curves are presented in Figure 5-3 and are based on the raw data presented in appendix A.

All fermentation profiles appear to be similar and nearly synchronized with the control. This suggests that the presence of additional inulin supplemented in various concentrations did not inhibit the growth of *L. plantarum*. These results appear like the results obtained from the glucose systems.

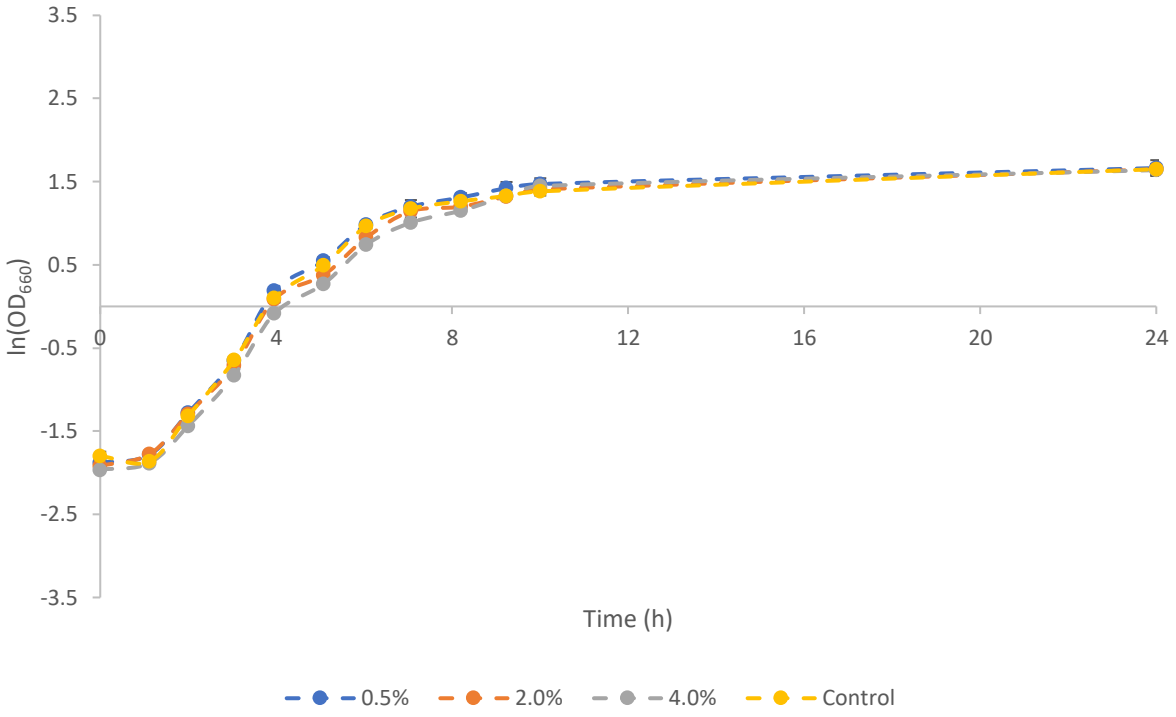

Figure 5-3 Fermentation profiles of *L. plantarum* in the control (MRS media) and MRS media supplemented with 0.5%, 2% and 4% **inulin**. The error bars are representative of the standard deviation between triplicate repeat runs

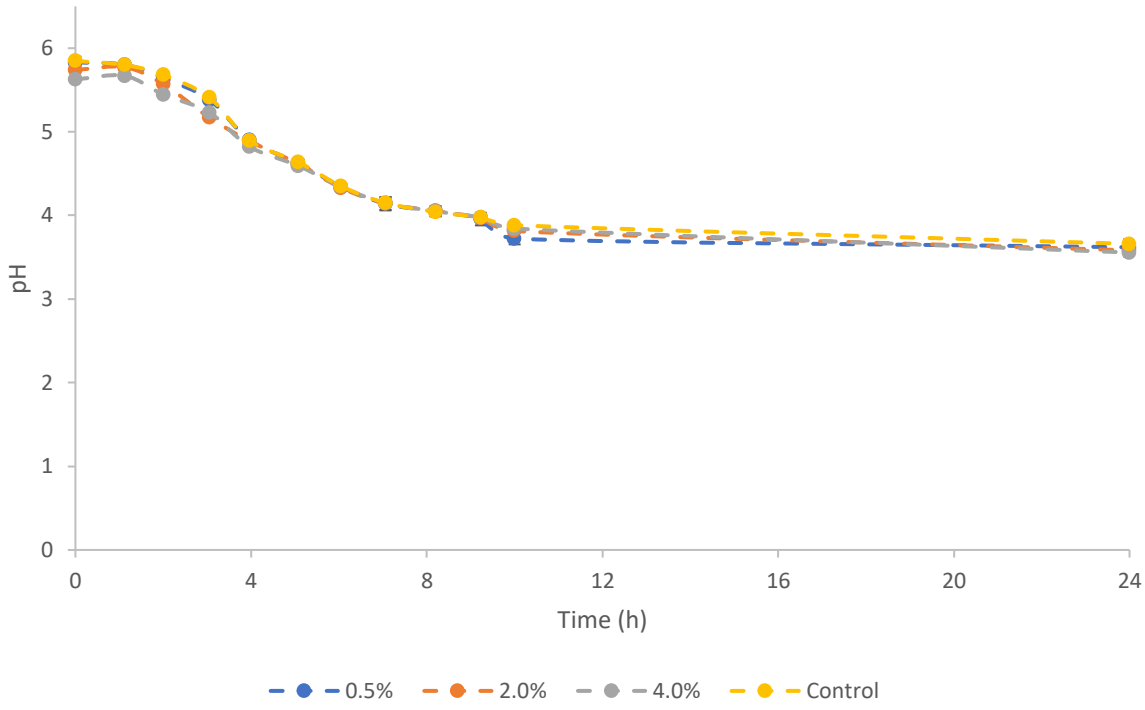

Figure 5-4 pH profiles due to *L. plantarum* lactic acid production in the control (MRS media) and MRS supplemented with 0.5%, 2% and 4% **inulin**. The error bars are representative of the standard deviation between triplicate repeat runs

Table 5-3 presents the μ_{max}, t_{lag} and OD_{max} obtained from the experimental data on the inulin systems. The μ_{max} did not show any significant change across all the systems, (p-value > 0.01). However, like the glucose systems the minimum μ_{max} value of 0.63 ±0.02 h^{-1} was obtained with maximum carbon substrate concentration. There was no significant increase in t_{lag} when inulin was supplemented. However, a slight decline in the OD_{max} was observed when increasing the concentration of inulin.

This means that in the culture, inulin acted as a limiting substrate to a lesser degree compared to that glucose substrate. This may be explained by the microorganism first catabolizing the carbon source from the glucose substrate because it is preferred and well adapted to it (glucose is the carbon substrate in the primary culture of MRS media). Because of this, when inulin was supplemented into the growth media, the μ_{max} nor t_{lag} would not expect to be affected.

A similar pattern to the growth curves can be seen in Figure 5-4 for the pH reduction profiles for inulin. These curves appear to be similar and nearly synchronized, with the pH of the culture reducing as fermentation time increases. As seen in Table 5-4, there was no significant difference in the pH at the end of the fermentation period. This suggests that that the presence of inulin would not inhibit the ability of *L. plantarum* to reduce vaginal or skin pH when the pharmabiotic formulation is administered.

Table 5-3 Inhibiting test kinetic data, μ_{max}, t_{lag}, and OD_{max} values and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data on *L. plantarum* cultured in various concentrations of **inulin** (0.5%, 2%, 4% (m/v)) supplemented MRS media and the control

Systems (m/v)		μ_{max} (h^{-1})	Ave	SD	SEM	t_{lag} (h)	Ave	SD	SEM	OD_{max}	Ave	SD	SEM
0.5%	Run 1	0.64	0.64	0.01	0.01	1	1	0	0	5.29	5.28	0.10	0.06
	Run 2	0.66				1				5.37			
	Run 3	0.63				1				5.18			
2%	Run 1	0.64	0.64	0.01	0.01	1	1	0	0	5.27	5.20	0.07	0.04
	Run 2	0.66				1				5.18			
	Run 3	0.63				1				5.14			
4%	Run 1	0.59	0.63	0.03	0.02	1	1	0	0	5.12	5.15	0.05	0.03
	Run 2	0.66				1				5.21			
	Run 3	0.63				1				5.12			
Control (contains 2% (m/v) glucose)	Run 1	0.68	0.66	0.03	0.02	1	1	0	0	5.26	5.21	0.05	0.03
	Run 2	0.68				1				5.16			
	Run 3	0.63				1				5.20			

Table 5-4 Inhibiting test starting and final pH values, and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data on *L. plantarum* cultured in various concentrations of **inulin** (0.5%, 2%, 4% (m/v)) supplemented MRS media and the control

Systems (m/v)		Starting pH	Ave	SD	SEM	Final pH	Ave	SD	SEM
0.5%	Run 1	5.82	5.83	0.01	0.01	3.63	3.62	0.01	0.01
	Run 2	5.84				3.61			
	Run 3	5.73				3.62			
2%	Run 1	5.73	5.74	0.02	0.01	3.60	3.58	0.02	0.01
	Run 2	5.76				3.58			
	Run 3	5.73				3.57			
4%	Run 1	5.63	5.85	0.02	0.01	3.55	3.56	0.01	0.00
	Run 2	5.64				3.56			
	Run 3	5.61				3.56			
Control (contains 2% (m/v) glucose)	Run 1	5.76	5.85	0.08	0.04	3.68	3.66	0.02	0.01
	Run 2	5.88				3.65			
	Run 3	5.90				3.65			

5.1.3 Inhibiting effects of **maltodextrin**

The growth curves of *L. plantarum* were developed for each system through the measurement of the increase in cell density with time by reading the absorbance (OD_{660nm}) of the fermentation culture containing MRS media supplemented with 0.5%, 2.0%, and 4% (m/v) maltodextrin. These growth curves are presented in Figure 5-5 and are based on the raw data presented in appendix A.

All fermentation profiles appear to be similar and nearly synchronized with the control. This suggests that the presence of additional maltodextrin supplemented in various concentrations did not inhibit the growth of *L. plantarum*. These results appear like the results obtained from the glucose and inulin systems.

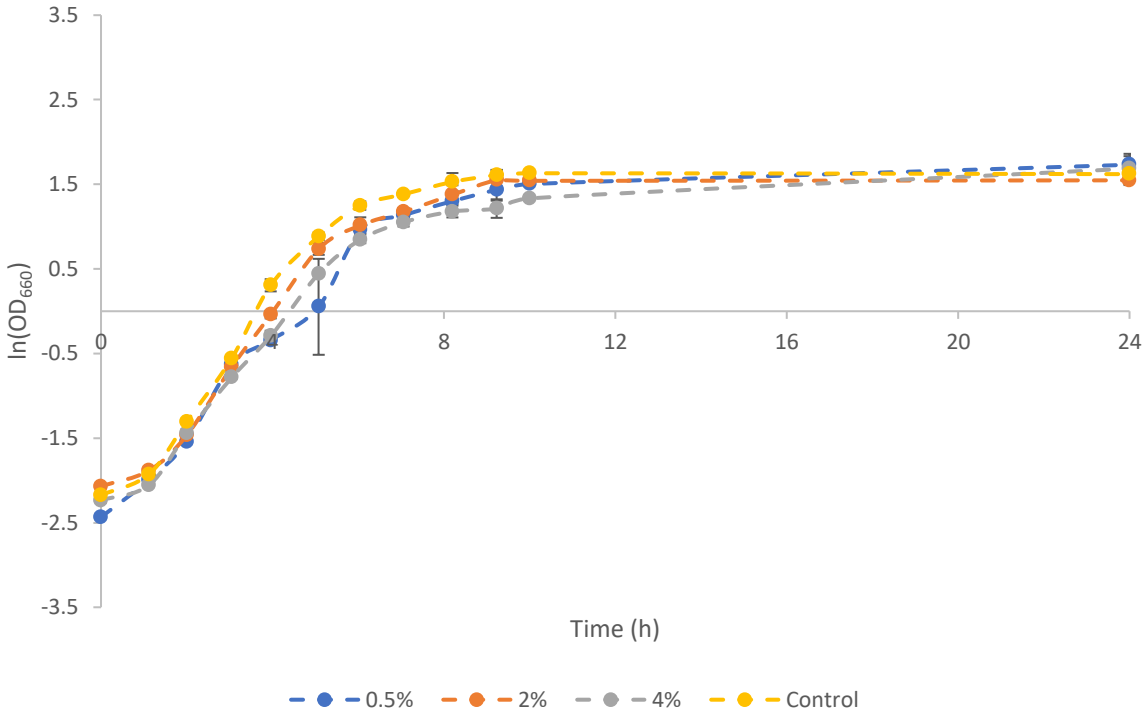

Figure 5-5 Fermentation profiles of *L. plantarum* in the control (MRS media) and MRS media supplemented with 0.5%, 2% and 4% **maltodextrin**. The error bars are representative of the standard deviation between triplicate repeat runs

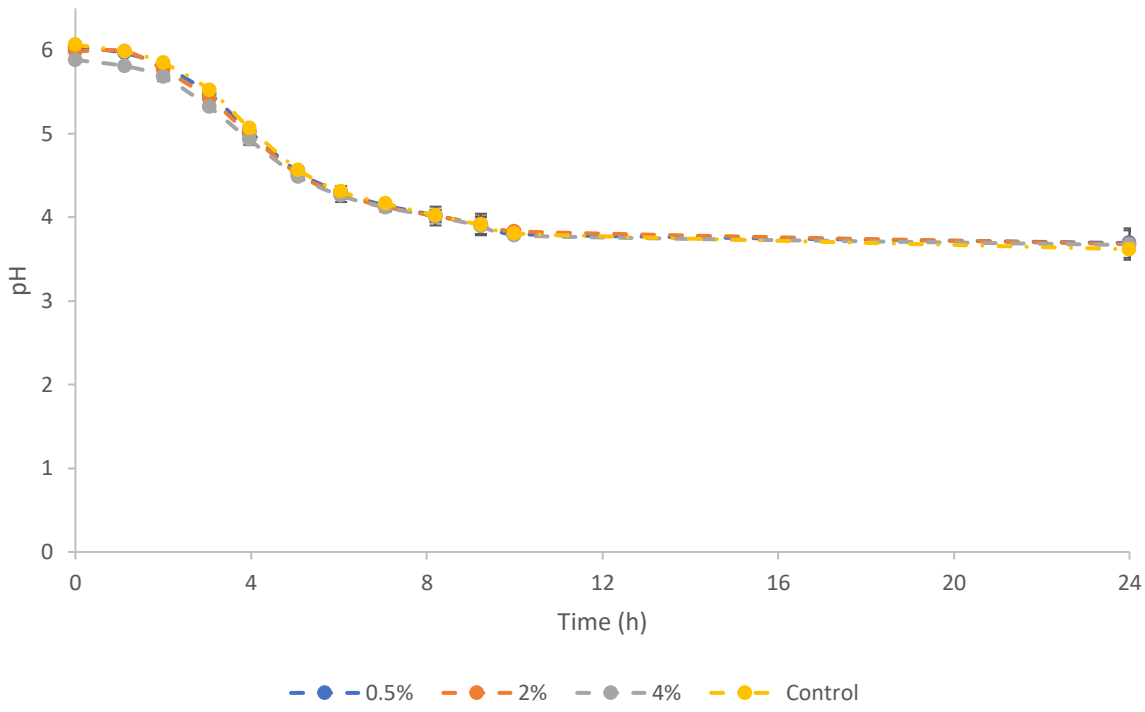

Figure 5-6 pH profiles due to *L. plantarum* lactic acid production in the control (MRS media) and MRS supplemented with 0.5%, 2% and 4% **maltodextrin**. The error bars are representative of the standard deviation between triplicate repeat runs

Table 5-5 presents the μ_{max}, t_{lag} and OD_{max} obtained from the experimental data presented in appendix A on *L. plantarum* cultured in various concentrations of maltodextrin and the control.

The μ_{max} reduced from 0.67 ± 0.01 h^{-1} to a value of 0.57 ± 0.06 h^{-1} when 0.5% (m/v) maltodextrin was supplemented in the MRS media (already containing 2% (m/v) of glucose). This was the same value obtained with maximum carbon substrate concentration in the glucose systems when the growth of *L. plantarum* was suspected to be inhibited. There was no significant increase in t_{lag} when maltodextrin was supplemented. A slight decline in the OD_{max} was observed when increasing the concentration of maltodextrin. However, the fermentation profiles do not suggest a significant inhibiting effect on the growth of the microorganism.

A similar pattern can be seen in Figure 5-6 for the pH reduction potential. Compared to the pH reduction profile of the control, there was no significant change in the profiles of cells grown in MRS media supplemented with maltodextrin. As seen in Table 5-6, the same lowered pH of the culture at the end of the fermentation period was obtained across all systems with no significant difference. This implies that maltodextrin has no inhibiting influence on *L. plantarum* lactic acid production.

Table 5-5 Inhibiting test kinetic data, μ_{max}, t_{lag}, and OD_{max} values and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data on *L. plantarum* cultured in various concentrations of **maltodextrin** (0.5%, 2%,, 4% (m/v)) supplemented MRS media and the control

Systems (m/v)		μ_{max} (h^{-1})	Ave (h^{-1})	SD	SEM	t_{lag} (h)	Ave	SD	SEM	OD_{max}	Ave	SD	SEM
0.5%	Run 1	0.62	0.57	0.06	0.04	1	1	0	0	5.63	5.65	0.10	0.06
	Run 2	0.50				1				5.76			
	Run 3	0.59				1				5.56			
2%	Run 1	0.68	0.68	0.01	0.01	1	1	0	0	4.84	4.73	0.10	0.06
	Run 2	0.69				1				4.71			
	Run 3	0.66				1				4.64			
4%	Run 1	0.62	0.62	0.01	0.01	1	1	0	0	5.25	5.39	0.18	0.10
	Run 2	0.63				1				5.34			
	Run 3	0.62				1				5.59			
Control (contains 2% (m/v) glucose)	Run 1	0.67	0.67	0.01	0.01	1	1	0	0	5.00	5.06	0.05	0.03
	Run 2	0.68				1				5.08			
	Run 3	0.65				1				5.09			

Table 5-6 Inhibiting test starting and final pH values, and the corresponding average (ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data on *L. plantarum* cultured in various concentrations of **maltodextrin** (0.5%, 2%, 4% (m/v)) supplemented MRS media and the control

Systems (m/v)		Starting pH	Ave (h^{-1})	SD	SEM	Final pH	Ave	SD	SEM
0.5%	Run 1	6.05	6.04	0.01	0.01	3.70	3.69	0.00	0.00
	Run 2	6.03				3.60			
	Run 3	6.04				3.69			
2%	Run 1	5.99	5.99	0.01	0.00	3.68	3.68	0.00	0.00
	Run 2	5.98				3.68			
	Run 3	5.99				3.68			
4%	Run 1	5.89	5.88	0.01	0.0	3.67	3.67	0.00	0.00
	Run 2	5.88				3.67			
	Run 3	5.88				3.67			
Control (contains 2% (m/v) glucose)	Run 1	6.06	6.06	0.02	0.01	3.62	3.62	0.00	0.00
	Run 2	6.08				3.62			
	Run 3	6.05				3.62			

5.1.4 Inhibiting effects of **sucrose**

The growth curves of *L. plantarum* were developed for each system through the measurement of the increase in cell density with time by reading the absorbance (OD_{660nm}) of the fermentation

culture containing MRS media supplemented with 0.5%, 2.0%, and 4% (m/v) sucrose. These growth curves are presented in Figure 5-7 and are based on the raw data presented in appendix A.

All fermentation profiles appear to be similar and nearly synchronized with the control. This suggests that the presence of additional sucrose supplemented in various concentrations did not inhibit the growth of *L. plantarum*. These results appear similar to the results obtained from the glucose and inulin systems.

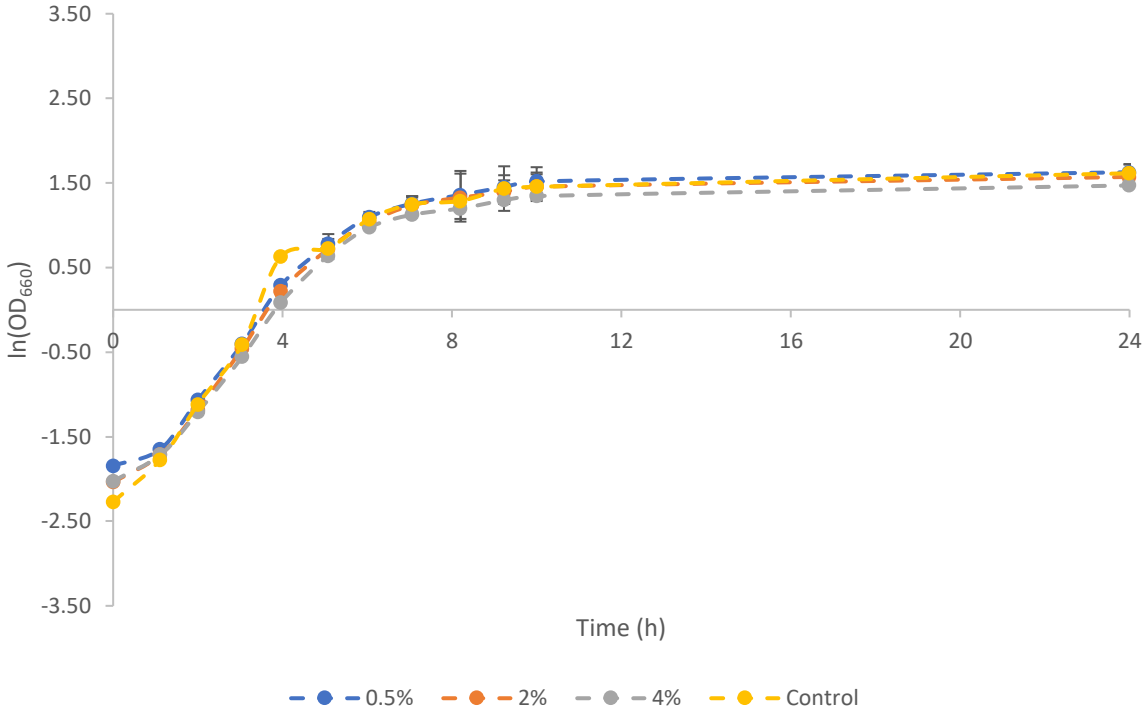

Figure 5-7 Fermentation profiles of *L. plantarum* in the control (MRS media) and MRS media supplemented with 0.5%, 2% and 4% **sucrose**. The error bars are representative of the standard deviation between triplicate repeat runs.

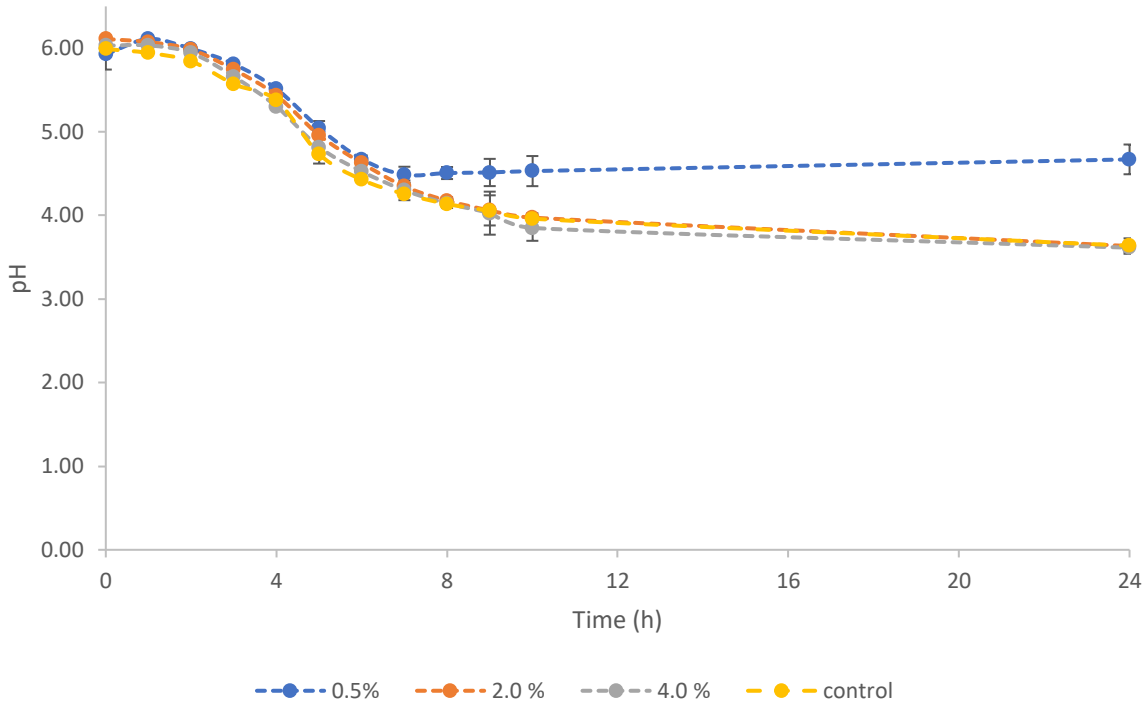

Figure 5-8 pH profiles due to *L. plantarum* lactic acid production in the control (MRS media) and MRS supplemented with 0.5%, 2% and 4% **sucrose**. The error bars are representative of the standard deviation between triplicate repeat runs.

Table 5-7 presents the μ_{max}, t_{lag} and OD_{max} obtained from the experimental data presented in appendix A on *L. plantarum* cultured in various concentrations of sucrose and the control. Across all systems, there was no significant change in the specific growth rates (p-value >0.01). There was also no observed change in the t_{lag} across all systems including the control. However, a decline in the OD_{max} was observed with increasing concentrations of sucrose in the MRS media.

A possible explanation for this decline in OD_{max}, given that there was no observed reduction in the specific growth rates, is that at some point in the glycolytic digestion of glucose, there was a diversion in its anabolism for other uses rather cell mass build-up. This could include the production of other metabolites or for the conversion into energy used in the transportation of cell materials (Hu, 2018). Another possible reason for this explained by Molina-Ramírez et al. (2017), is that fructose which is obtained alongside with glucose by hydrolysis of sucrose can act as an osmotic stressor as the concentration of fructose remains constant throughout the fermentation period.

Figure 5-8 shows that there was no significant change in the profiles of cells grown in MRS media supplemented with sucrose compared to the pH reduction profile of the control. As reported in Table 5-8, the same lowered pH of the culture at the end of the fermentation period was obtained across all systems. This implies that sucrose has no inhibiting influence on *L. plantarum* lactic acid production.

Table 5-7 Inhibiting test kinetic data, μ_{max}, t_{lag}, and OD_{max} values and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data on *L. plantarum* cultured in various concentrations of **sucrose** (0.5%, 2%, 4% (m/v)) supplemented MRS media and the control

Systems (m/v)		μ_{max} (h^{-1})	Ave (h^{-1})	SD	SEM	t_{lag} (h)	Ave	SD	SEM	OD_{max}	Ave	SD	SEM
0.5%	Run 1	0.63	0.63	0.01	0.01	1	1	0	0	4.94	5.09	0.13	0.07
	Run 2	0.61				1				5.15			
	Run 3	0.64				1				5.17			
2%	Run 1	0.66	0.61	0.05	0.03	1	1	0	0	4.88	4.81	0.10	0.06
	Run 2	0.62				1				4.85			
	Run 3	0.56				1				4.70			
4%	Run 1	0.62	0.61	0.09	0.05	1	1	0	0	4.43	4.35	0.23	0.13
	Run 2	0.51				1				4.53			
	Run 3	0.69				1				4.10			
Control (contains 2% (m/v) glucose)	Run 1	0.68	0.68	0.00	0.00	1	1	0	0	4.91	5.03	0.1	0.06
	Run 2	0.68				1				5.10			
	Run 3	0.67				1				5.07			

Table 5-8 Inhibiting test starting and final pH values, and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data on *L. plantarum* cultured in various concentrations of **sucrose** (0.5%, 2%, 4% (m/v)) supplemented MRS media and the control

Systems (m/v)		Starting pH	Ave	SD	SEM	Final pH	Ave	SD	SEM
0.5%	Run 1	6.09	6.15	0.06	0.03	3.67	3.66	0.01	0.01
	Run 2	6.16				3.67			
	Run 3	6.20				3.65			
2%	Run 1	6.21	6.20	0.05	0.03	3.65	3.65	0.00	0.00
	Run 2	6.24				3.65			
	Run 3	6.12				3.65			
4%	Run 1	6.19	6.17	0.04	0.02	3.66	3.66	0.02	0.01
	Run 2	6.19				3.64			
	Run 3	6.12				3.67			
Control (contains 2% (m/v) glucose)	Run 1	6.25	6.25	0.02	0.01	3.62	3.63	0.01	0.01
	Run 2	6.27				3.64			
	Run 3	6.24				3.64			

5.1.5 Summary

Across all cryoprotectants investigated for their potential influence on *L. plantarum* growth and acidifying ability in the immediate growth environment, none of the candidates demonstrated a significant inhibiting effect. This indicates that the inclusion of additional glucose, inulin,

maltodextrin, or sucrose in the pharmabiotic formulation poses no threat to delivering the desired proliferation and restoration of acidity by the production of acidic metabolites, which are the essential desired health outcomes in achieving an efficient probiotherapeutic treatment (Collins *et al.*, 2018).

5.2 Prebiotic potential of cryoprotectants on *L. plantarum*

To determine the prebiotic potential of the selected cryoprotectants on *L. plantarum,* the microorganism was cultured in *glucose-free* MRS media in which the glucose carbon source was substituted with various concentrations of glucose, inulin, maltodextrin, and sucrose. The cryoprotectant concentrations tested were 0.5%, 2% and 4% (m/v) respectively. An exception to this was the glucose experiment where the control of standard MRS media (already containing 2% (m/v) was also counted as the 2% (m/v) run since they both contain the same glucose contents. These experiments were performed to investigate the ability of *L. plantarum* to selectively utilize these carbohydrates, and to enhance proliferation and restore the acidity, which are essential pharmabiotic outcomes for restoring and maintaining vaginal and skin health. The prebiotic potential was quantified by each cryoprotectant's ability to enhance microbial proliferation and reduce the pH of the fermentation system. Across all experiments, the control was standard MRS media (already containing 2% (m/v) of glucose) without cryoprotectants.

5.2.1 Prebiotic potential of **glucose**

The growth curves of *L. plantarum* were developed for each system through the measurement of the increase in cell density with time by reading the absorbance (OD_{660nm}) in the fermentation culture containing glucose-free-MRS media supplemented with 0.5%, 2.0% and 4% (m/v) glucose. These growth curves are presented in Figure 5-9 and are based on the raw data presented in appendix B.

Table 5-9 presents the $\mu_{max,}$ t_{lag} and OD_{max} obtained from the experimental data on the glucose systems. The μ_{max} initially increased slightly as the glucose was increased from 0.5% (m/v) to 2% (m/v), from 0.57 h^{-1} to 0.59 h^{-1}. However, a drop in the μ_{max} value was observed with a further increase in glucose concentration to 4% (m/v) of glucose supplemented glucose-free MRS media. The maximum OD_{max} value of 5.45 was obtained from the 4% (m/v) glucose supplemented glucose-free MRS media system. A minimum OD_{max} value of 3.46 was obtained from the 0.5% (m/v) run. The t_{lag} values remained the same across all runs with a value of 1h.

The pH reduction profiles, presented in Figure 5-10 and Table 5-10, show that there was a reduction in pH of the monoculture owing to the production of acidic metabolites from the fermentation of

glucose in all the cultures. However, the *L. plantarum* propagated in 0.5% glucose resulted in the least pH drop to 4.42 from 5.50. The largest change was seen for the fermentation media containing 4%, for which the pH dropped to 3.45 from 5.22.

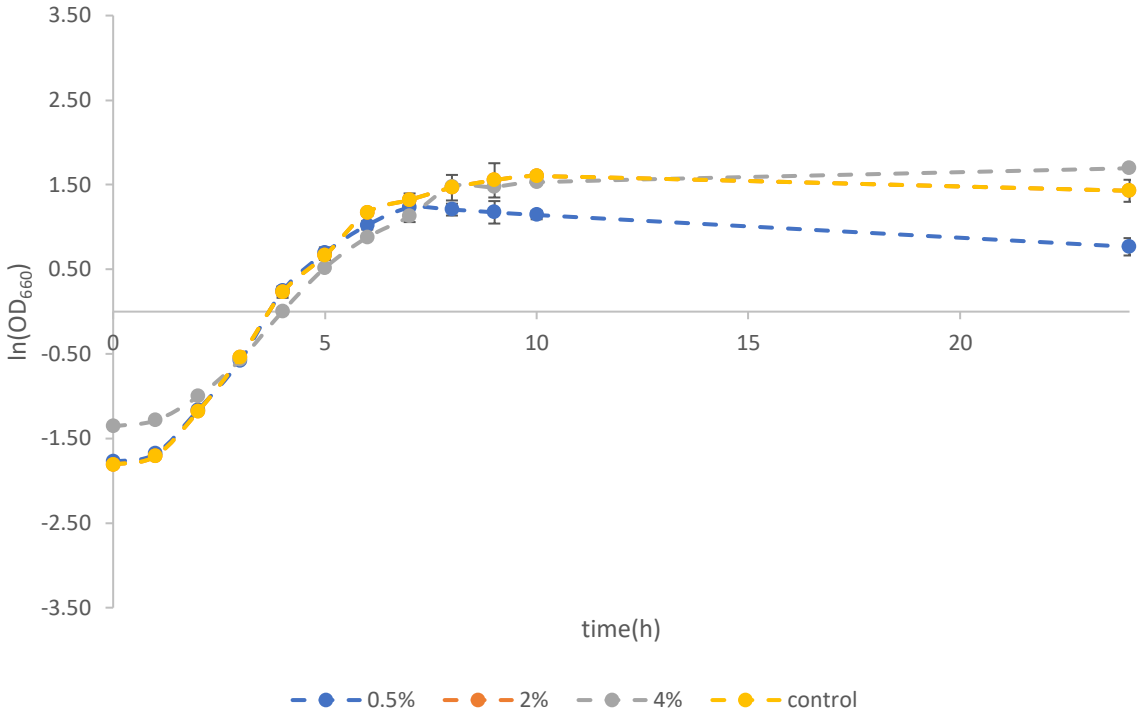

Figure 5-9 Fermentation profiles of *L. plantarum* in the control (MRS media, 2 % glucose) and glucose-free-MRS media supplemented with 0.5%, 2% and 4% **glucose**. The error bars are representative of the standard deviation between triplicate repeat runs[1]

[1] The control of standard MRS media (already containing 2% (m/v) was also counted as the 2% (m/v) and presented as such in Figure 5-9 and Figure 5-10

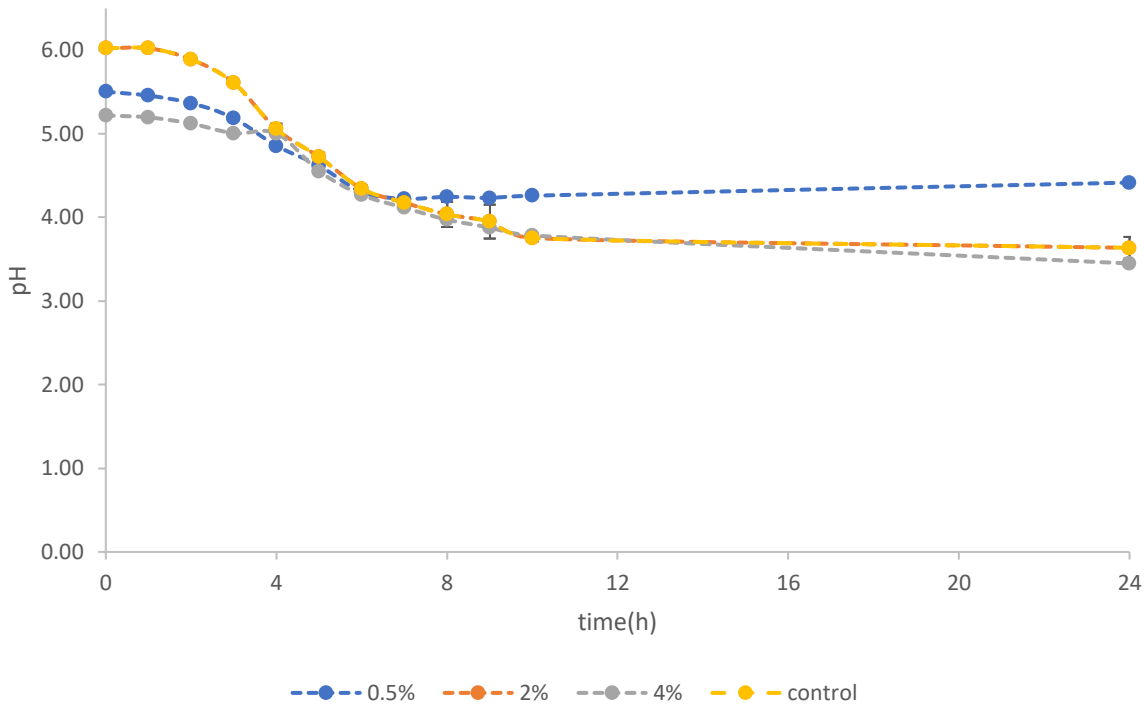

Figure 5-10 pH reduction profiles of *L. plantarum* in the control (MRS media) and glucose-free-MRS media supplemented with 0.5%, 2% and 4% **glucose**. The error bars are representative of the standard deviation between triplicate repeat runs

These results indicate that *L. plantarum* can proliferate and restore the acidity of its growth environment in the presence of glucose being the sole carbon substrate. This is only to be expected, given that *Lactobacillus* is grown in MRS as a standard, the carbon source in which is glucose. Glucose substrate nutrient availability leads to the metabolic conversion by *Lactobacillus* to generate energy for growth and produce metabolites (Hu, 2018). This explains the increase in the OD_{max} values with the increase in the glucose concentrations present in the growth media as more glucose molecules were made available for the utilization of carbon present to increase cell mass. Similar findings were made by Yuksekdag and Aslim (2008). These authors obtained increased biomass and exopolysaccharide production by *Lactobacillus* when the concentration of the carbon substrate source was increased by increasing the glucose concentrations in the range 0.5%, 1.5%, 2%, 2.5%, 3% (m/v). This indicates that the glucose was the limiting reagent for growth.

Furthermore, glucose has been demonstrated to support maximum microbial growth when compared to other substrates as the sole carbon source (Watson *et al.*, 2013; Molina-Ramírez *et al.*, 2017; Toplaghaltsyan, Bazukyan and Trchounian, 2017).

Table 5-9 Prebiotic test μ_{max}, t_{lag}, and OD_{max} values, and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data on *L. plantarum* cultured in various concentrations of **glucose** (0.5%, 2%, 4% (m/v)) supplemented glucose-free- MRS media and the control.

Systems (m/v)		μ_{max} (h^{-1})	Ave	SD	SEM	t_{lag} (h)	Ave	SD	SEM	OD_{max}	Ave	SD	SEM
0.5%	Run 1	0.56	0.57	0.01	0.01	1	1	0	0	3.56	3.46	0.10	0.05
	Run 2	0.58				1				3.46			
	Run 3	0.57				1				3.37			
2%	Run 1	0.59	0.59	0.00	0.00	1	1	0	0	5.00	4.97	0.03	0.02
	Run 2	0.60				1				4.95			
	Run 3	0.59				1				4.95			
4%	Run 1	0.47	0.46	0.02	0,01	1	1	0	0	5.64	5.45	0.34	0.20
	Run 2	0.47				1				5,06			
	Run 3	0.43				1				5.66			
Control (contains 2% (m/v) glucose)	Run 1	0.59	0.59	0.00	0,00	1	1	0	0	5.00	4.97	0.03	0.02
	Run 2	0.60				1				4.95			
	Run 3	0.59				1				4.95			

Table 5-10 Prebiotic test starting and final pH values and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data on *L. plantarum* cultured in various concentrations of **glucose** (0.5%, 2%, 4% (m/v)) supplemented glucose-free- MRS media and the control.

Systems (m/v)		Starting pH	Ave	SD	SEM	Final pH	Ave	SD	SEM
0.5%	Run 1	5.52	5.50	0.02	0.01	4.39	4.42	0.03	0.01
	Run 2	5.49				4.44			
	Run 3	5.50				4.42			
2%	Run 1	6.00	6.02	0.03	0.02	3.65	3.64	0.01	0.01
	Run 2	6.01				3.63			
	Run 3	6.06				3.63			
4%	Run 1	5.24	5.22	0.02	0.01	3.46	3.45	0.01	0.01
	Run 2	5.22				3.44			
	Run 3	5.20				3.45			
Control (contains 2% (m/v) glucose)	Run 1	6.00	6.02	0.03	0.02	3.65	3.64	0.01	0.01
	Run 2	6.01				3.63			
	Run 3	6.06				3.63			

5.2.2 Prebiotic potential of **inulin**

The growth curves of *L. plantarum* were developed for each system through the measurement of the increase in cell density with time by reading the absorbance (OD_{660nm}) in the fermentation culture containing glucose-free-MRS media supplemented with 0.5%, 2.0% and 4% (m/v) inulin.

These growth curves are presented in Figure 5-11 and are based on the raw data presented in appendix B.

Table 5-11 presents the μ_{max}, t_{lag} and OD_{max} obtained from the experimental data on the inulin systems. When 0.5%, 2.0% and 4% (m/v) inulin were substituted in the glucose-free growth media, the μ_{max} was slightly lowered to 0.54 ± 0.06 h^{-1}, 0.59 ± 0.02 h^{-1}, 0.54 ± 0.00 h^{-1} respectively, compared to the μ_{max} value of 0.61 ± 0.03 h^{-1} obtained from the control growth media (containing 2% (m/v) of glucose carbon source substrate). Furthermore, the t_{lag} values remained the same across all systems. A higher concentration of inulin present in the media led to a higher OD_{max} indicating that higher concentrations of inulin yielded higher biomass.

The results presented in Figure 5-12 and Table 5-12 show that *L. plantarum* fermented in all concentrations of inulin and led to a reduction in pH of the growth media. This reduction in pH signifies utilization of inulin to produce acidic metabolites (O'Hanlon, Moench and Cone, 2011). *L. plantarum* fermented in 0.5% inulin showed the least pH drop to 5.39 from 6.11, with an increase in the concentration of inulin resulting in an increased pH drop. This may be explained by the increase in the concentration of inulin increasing the availability of the carbon substrate required for conversion to lactic acid.

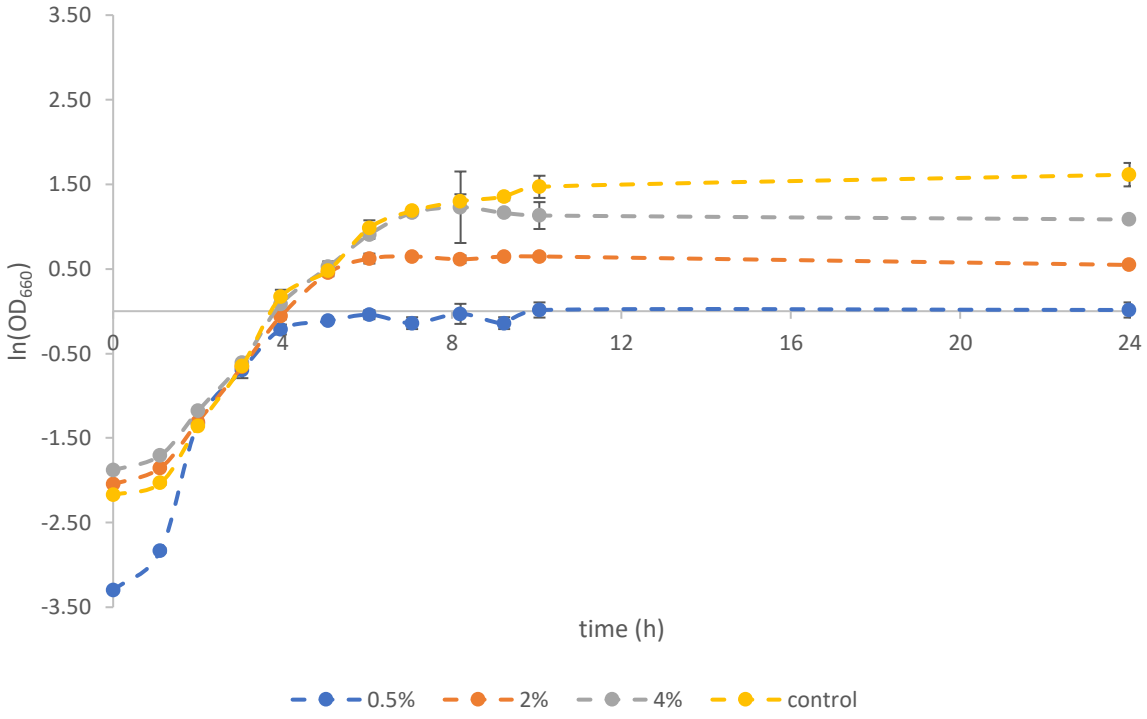

Figure 5-11 Fermentation profiles of *L. plantarum* in the control (MRS media) and glucose-free-MRS media supplemented with 0.5%, 2% and 4% **inulin.** The error bars are representative of the standard deviation between triplicate repeat runs

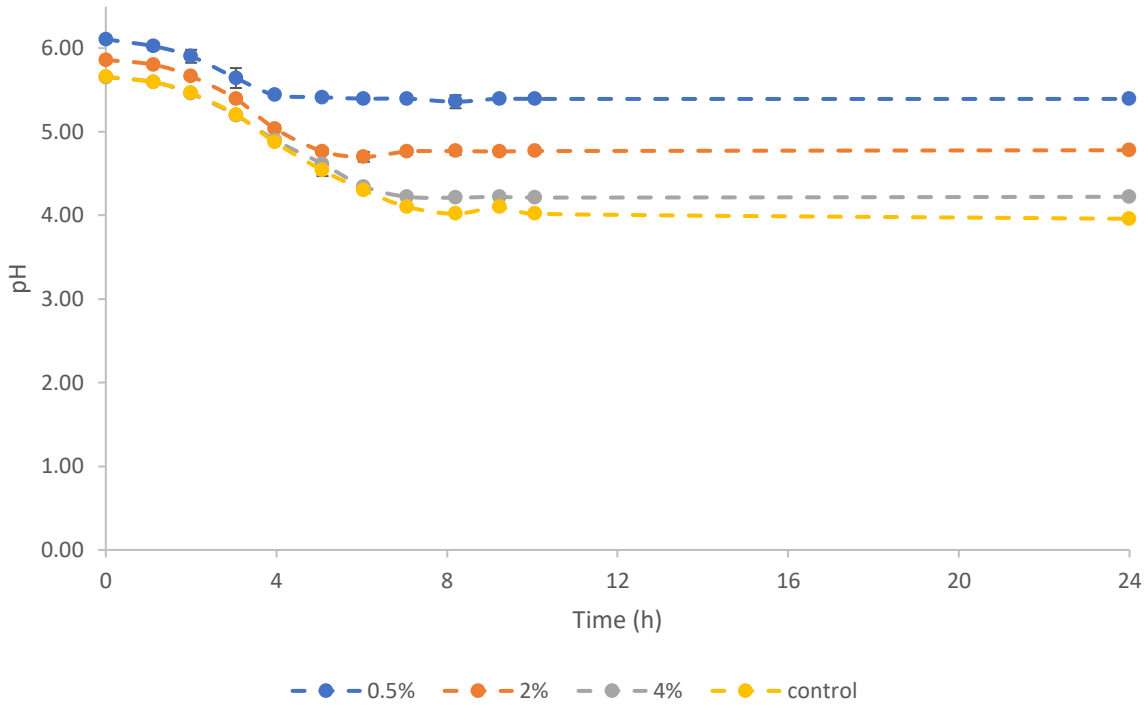

Figure 5-12 pH reduction profiles of *L. plantarum* in the control (MRS media) and glucose-free-MRS media supplemented with 0.5%, 2% and 4% **inulin**. The error bars are representative of the standard deviation between triplicate repeat runs

The reduction in the specific growth rate μ_{max}, between the control media (containing 2% (m/v) glucose) and the runs containing glucose-free-MRS media supplemented with inulin, can be linked to a further hydrolysis step required for the microorganism to break down the oligosaccharide inulin into glucose units before its conversion to pyruvate, a step in the typical LAB metabolism. In doing this, *Lactobacillus* uses more energy for the additional step, resulting in a reduction in the growth rate (Gänzle and Follador, 2012).

However, like the glucose systems, an increase in inulin concentrations in the media resulted in an increase in the OD_{max} by up to about three folds. The explanation for this remains the same as in the case of the glucose systems. The increase in the concentration of inulin means an increase in the availability of the carbon source present in the glucose units for metabolic conversion to cell biomass.

In explaining the reason for an increase in t_{lag}, a change in the substrate nutrient is known to be a cause of delays in the propagation of *Lactobacillus*. The delay is the time spent by the microorganism to adjust to the new substrate media different from the substrate present in the previously grown sub-culture. Another reason for this increased t_{lag} could be the time spent by the microorganism in breaking the complex fructo-oligosaccharide into simpler glucose unit before its conversion to pyruvate.

Table 5-11 Prebiotic test μ_{max}, t_{lag}, and OD_{max} values, and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data on *L. plantarum* cultured in various concentrations of **inulin** (0.5%, 2%, 4% (m/v)) supplemented glucose-free-MRS media and the control

Systems (m/v)		μ_{max} (h^{-1})	Ave	SD	SEM	t_{lag} (h)	Ave	SD	SEM	OD_{max}	Ave	SD	SEM
0.5%	Run 1	0.62	0.57	0.06	0.03	2	2	0	0	1.12	1.02	0.09	0.05
	Run 2	0.57				2				0.97			
	Run 3	0.51				2				0.96			
2%	Run 1	0.59	0.59	0.02	0.01	1	1	0	0	1.85	1.85	0.05	0.03
	Run 2	0.58				1				1.89			
	Run 3	0.61				1				1.80			
4%	Run 1	0.54	0.54	0.00	0.00	1	1	0	0	3.00	2.96	0.04	0.02
	Run 2	0.53				1				2.94			
	Run 3	0.54				1				2.93			
Control (contains 2% (m/v) glucose)	Run 1	0.58	0.61	0.03	0.02	1	1	0	0	3.88	3.87	0.04	0.02
	Run 2	0.63				1				3.90			
	Run 3	0.63				1				3.82			

Table 5-12 Prebiotic test starting and final pH values and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data on *L. plantarum* cultured in various concentrations of **inulin** (0.5%, 2%, 4% (m/v)) supplemented glucose-free-MRS media and the control

Systems (m/v)		Starting pH	Ave	SD	SEM	Final pH	Ave	SD	SEM
0.5%	Run 1	6.15	6.11	0.04	0.02	5.37	5.39	0.02	0.01
	Run 2	6.10				5.41			
	Run 3	6.07				5.40			
2%	Run 1	5.85	5.86	0.02	0.01	4.76	4.78	0.02	0.01
	Run 2	5.85				4.79			
	Run 3	5.88				4.79			
4%	Run 1	5.66	5.65	0.01	0.00	4.20	4.22	0.02	0.01
	Run 2	5.65				4.23			
	Run 3	5.65				4.24			
Control (contains 2% (m/v) glucose)	Run 1	5.63	5.66	0.03	0.02	3.98	3.96	0.02	0.01
	Run 2	5.66				3.96			
	Run 3	5.69				3.94			

5.2.3 Prebiotic potential of **maltodextrin**

The growth curves of *L. plantarum* were developed for each system through the measurement of the increase in cell density with time by reading the absorbance (OD_{660nm}) in the fermentation

culture containing glucose-free-MRS media supplemented with 0.5%, 2.0% and 4% (m/v) maltodextrin. These growth curves are presented in Figure 5-13 and are based on the raw data presented in appendix B.

Table 5-13 presents the μ_{max}, t_{lag} and OD_{max} obtained from the experimental data on the inulin systems. The μ_{max} was significantly lowered to 0.26 ± 0.02 h^{-1} when 0.5% (m/v) of maltodextrin was substituted in the growth media in various concentrations, compared to a μ_{max} of 0.64 ± 0.12 h^{-1} obtained with the control growth media (containing 2% (m/v) of glucose carbon source substrate). However, with increased concentrations of maltodextrin, there was a significant rise in μ_{max}. A maximum value of 0.58 ± 0.01 h^{-1} was obtained with 4% (m/v), the highest concentration of maltodextrin substituted for the glucose substrate. A higher concentration of maltodextrin present in the media led to a higher OD_{max}, with minimum value of 3.59 obtained with 0.5% (m/v) maltodextrin concentration and a maximum value of 3.85 obtained with 4% (m/v) maltodextrin concentration. The higher concentrations of maltodextrin yielding higher biomass indicates that the carbon source in maltodextrin was limiting.

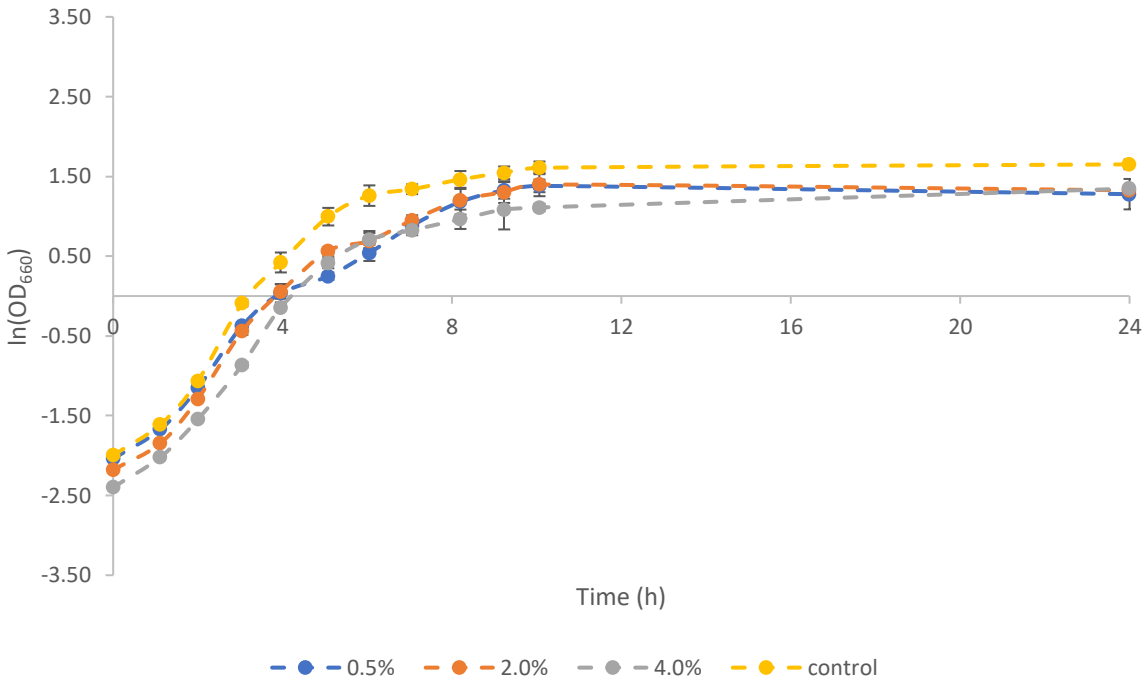

Figure 5-13 Fermentation profiles of *L. plantarum* in the control (MRS media) and glucose-free-MRS media supplemented with 0.5%, 2% and 4% **maltodextrin**. The error bars are representative of the standard deviation between triplicate repeat runs

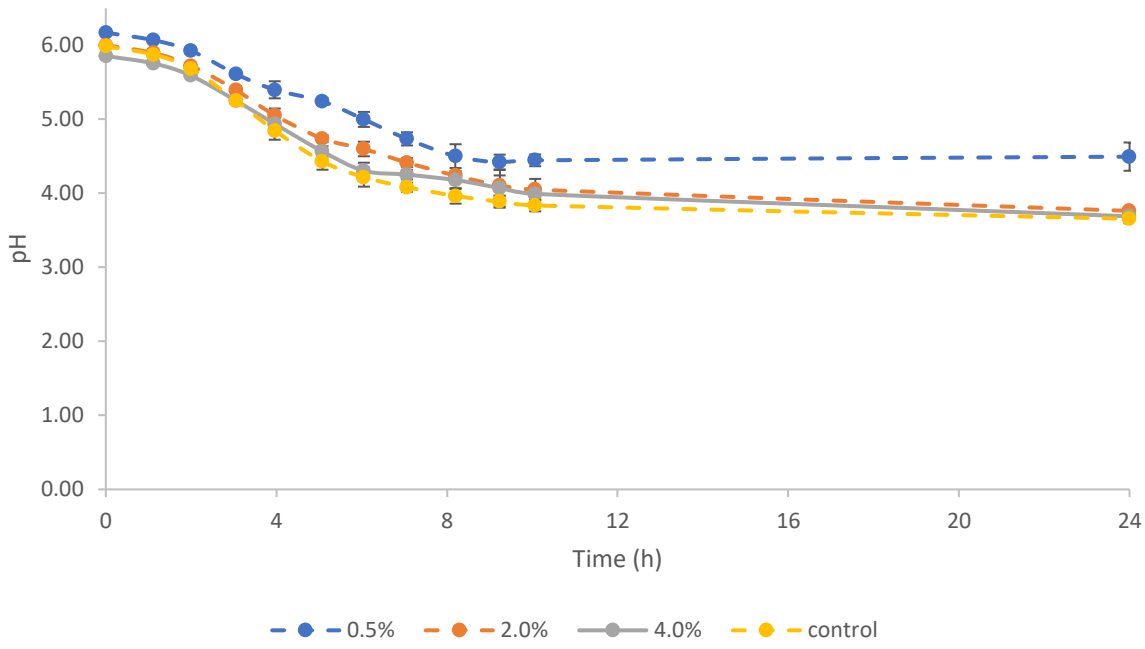

Figure 5-14 pH reduction profiles of *L. plantarum* in the control (MRS media) and glucose-free-MRS media supplemented with 0.5%, 2% and 4% **maltodextrin**. The error bars are representative of the standard deviation between triplicate repeat runs

These results show the ability of *L. plantarum* to proliferate when maltodextrin is present as the sole carbon substrate. The drop in the μ_{max} observed when maltodextrin was substituted for glucose as the sole carbon source suggests that there was a probable shift in the metabolism. Due to the change in the substrate nutrient from glucose to maltodextrin, the carbon source available for the microorganism differed in structure. Maltodextrin is a polymer made up of glucose units, which is more complex compared to single glucose units which are the baseline substrate for the glycolytic pathway and already present in the standard MRS media. As a result, the microorganism would have been expected to have exerted more energy in converting the nutrient substrate to pyruvate because of the additional hydrolysis step involved to the glycolysis metabolic pathway. This can lead to a decline in growth rate compared to the control system containing simple glucose units. Gänzle and Follador (2012) reported that *Lactobacillus* possesses the capacity to hydrolyse starch such as maltodextrin by the release of MalL, MalN and DexB which are $\alpha-$Glucosidases intracellular enzymes present within *Lactobacillus*.

The maximum cell densities, OD_{max}, across all maltodextrin systems were similar but were significantly lower than that of the control. If all the carbon had been converted to biomass, it would have been expected that the maximum cell density would have increased with the additional maltodextrin. A possible explanation for this could be that the maltodextrin nutrient was catabolised for other cell functions such as the conversion of carbon to energy for transportation of substances,

to maintain osmotic balance or the production of cell metabolites rather than channelling this substrate nutrient for cell division.

The results in Figure 5-14 and Table 5-14 show that the fermentation of *L. plantarum* in all concentrations of maltodextrin resulted in a drop in the pH of the culture owing to the production of acidic metabolite. Like the inulin system, *L. plantarum* fermented in 0.5% maltodextrin demonstrated the least acidic restoration ability with the least drop in the pH at the end of the fermentation compared to all other systems, only reaching a pH of 4.50 from an initial value of 6.17. An increase in the concentration of maltodextrin resulted in an increased pH drop, however, the pH value at the end of the fermentations remained the same across all other systems at an approximate value of 3.73.

Table 5-13 Prebiotic test μ_{max}, t_{lag}, and OD_{max} values, and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data on *L. plantarum* cultured in various concentrations of **maltodextrin** (0.5%, 2%, 4% (m/v)) supplemented glucose-free-MRS media and the control

Systems (m/v)		μ_{max} (h^{-1})	Ave (h^{-1})	SD	SEM	t_{lag} (h)	Ave	SD	SEM	OD_{max}	Ave	SD	SEM
0.5%	Run 1	0.65	0.62	0.03	0.02	4	4	0	0	3.79	3.59	0.19	0.11
	Run 2	0.59				4				3.57			
	Run 3	0.62				4				3.41			
2%	Run 1	0.64	0.62	0.02	0.01	1	1	0	0	3.77	3.76	0.02	0.01
	Run 2	0.63				1				3.74			
	Run 3	0.60				1				3.78			
4%	Run 1	0.62	0.60	0.02	0.01	1	1	0	0	3.80	3.85	0.05	0.03
	Run 2	0.59				1				3.89			
	Run 3	0.59				1				3.85			
Control (contains 2% (m/v) glucose)	Run 1	0.68	0.68	0.00	0.00	1	1	0	0	5.15	5.21	0.06	0.03
	Run 2	0.67				1				5.21			
	Run 3	0.68				1				5.27			

Table 5-14 Prebiotic test starting and final pH values and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data on *L. plantarum* cultured in various concentrations of **maltodextrin** (0.5%, 2%, 4% (m/v)) supplemented glucose-free-MRS media and the control

Systems (m/v)		pH	Ave	SD	SEM	pH	Ave	SD	SEM
0.5%	Run 1	6.17	6.17	0.01	0.00	4.50	4.50	0.01	0.00
	Run 2	6.18				4.50			
	Run 3	6.17				4.49			
2%	Run 1	5.99	6.00	0.01	0.00	3.77	3.76	0.02	0.01
	Run 2	6.01				3.74			
	Run 3	6.01				3.78			
4%	Run 1	5.86	5.86	0.01	0.00	3.70	3.69	0.01	0.01
	Run 2	5.86				3.69			
	Run 3	5.86				3.68			
Control (contains 2% (m/v) glucose)	Run 1	5.95	5.99	0.03	0.02	3.67	3.66	0.02	0.01
	Run 2	6.00				3.63			
	Run 3	6.01				3.67			

5.2.4 Prebiotic potential of **sucrose**

The growth curves of *L. plantarum* were developed for each system through the measurement of the increase in cell density with time by reading the absorbance (OD_{660nm}) in the fermentation culture containing glucose-free-MRS media supplemented with 0.5%, 2.0% and 4% (m/v) sucrose. These growth curves are presented in Figure 5-15 and are based on the raw data presented in appendix B.

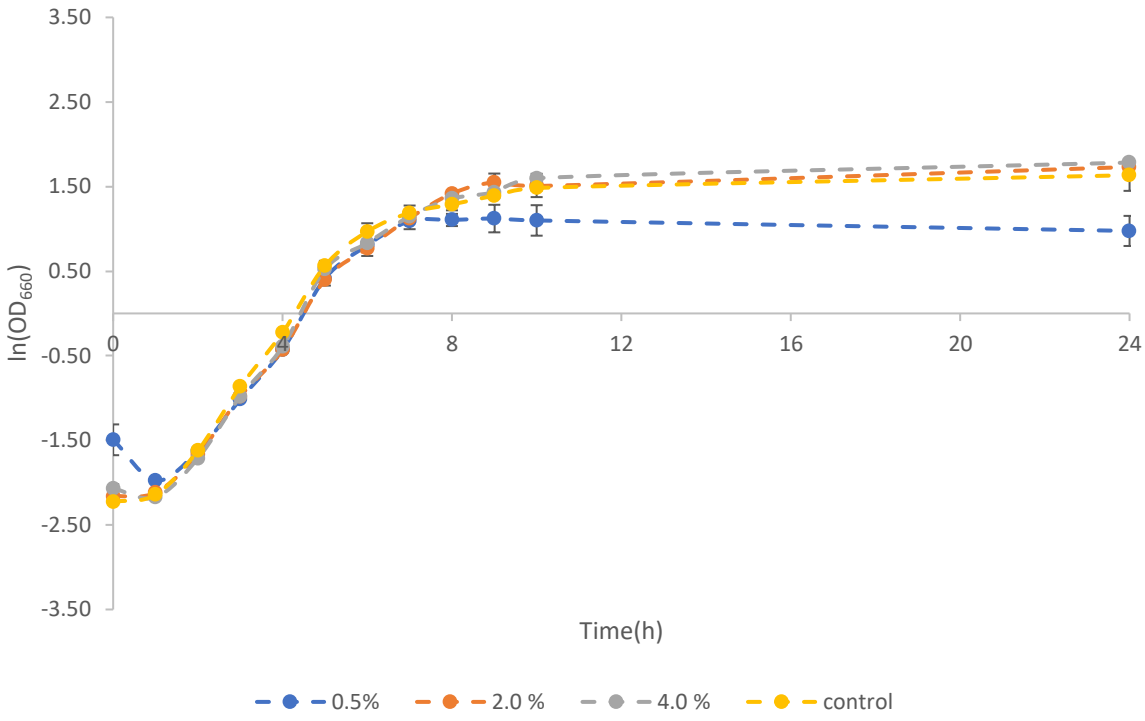

Figure 5-15 Fermentation profiles of *L. plantarum* in the control (MRS media) and glucose-free-MRS media supplemented with 0.5%, 2% and 4% **sucrose**. The error bars are representative of the standard deviation between triplicate repeat runs

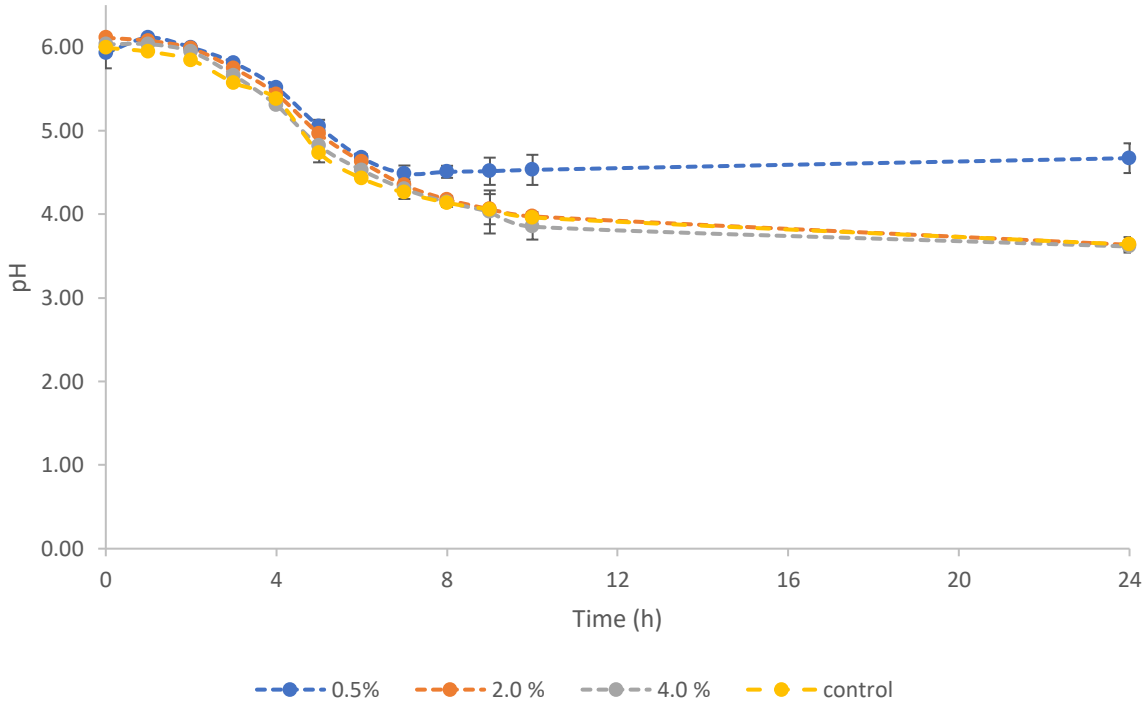

Figure 5-16 pH reduction profiles of *L. plantarum* in the control (MRS media) and glucose-free-MRS media supplemented with 0.5%, 2% and 4% **sucrose**. The error bars are representative of the standard deviation between triplicate repeat runs

Table 5-15 presents the μ_{max}, t_{lag} and OD_{max} obtained from the experimental data on the sucrose systems. The μ_{max} remained the same across all systems. This signifies that *L. plantarum* has high affinity for the digestion of sucrose, like that of glucoses. In contrast to this result, Molina-Ramírez *et al*. (2017) obtained a different metabolic reaction to sucrose fermentability where the growth of *Komagataeibacter medellinensis* was significantly lowered when sucrose was present in the growth culture as the sole carbon substrate compared to glucose. However, Gänzle and Follador (2012) reported that the prebiotic selectivity varies amongst different *Lactobacillus* strains and is based on the varied intrinsic phylogenetic makeup of strains for metabolizing different carbohydrates. This agrees with Petrut *et al*. (2019) where various strains demonstrated different levels of selectivity based on their growth rates in cultures containing these substates.

This faster fermentation by *L. plantarum* observed with sucrose (comparable to glucose) was different from the previous results with other the other carbohydrates (inulin and maltodextrin). This could be linked to the dimer nature of sucrose which is made up of shorter glucose units compared to the longer polymer units of maltodextrin and inulin. This agrees with Hernandez-Hernandez *et al*. (2012) that shorter length carbohydrates ferment faster than carbohydrates with longer lengths.

Table 5-15 Prebiotic test μ_{max}, t_{lag}, and OD_{max} values, and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data on *L. plantarum* cultured in various concentrations of **sucrose** (0.5%, 2%, 4% (m/v)) supplemented glucose-free-MRS media and the control

Systems (m/v)		μ_{max} (h^{-1})	Ave	SD	SEM	t_{lag} (h)	Ave	SD	SEM	OD_{max}	Ave	SD	SEM
0.5%	Run 1	0.62	0.63	0.01	0.01	1	1	0	0	3.29	3.14	0,13	0,08
	Run 2	0.63				1				3.09			
	Run 3	0.64				1				3.04			
2%	Run 1	0.63	0.63	0.00	0.00	1	1	0	0	5.70	5.65	0,09	0,05
	Run 2	0.62				1				5.70			
	Run 3	0.63				1				5.54			
4%	Run 1	0.66	0.66	0.01	0.01	1	1	0	0	6.01	5.95	0,05	0,03
	Run 2	0.67				1				5.93			
	Run 3	0.65				1				5.91			
Control (contains 2% (m/v) glucose)	Run 1	0.67	0.66	0.01	0.00	1	1	0	0	5.02	5.13	0.18	0.11
	Run 2	0.66				1				5.34			
	Run 3	0.66				1				5.02			

Table 5-16 Prebiotic test starting and final pH values and the corresponding average (ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data on *L. plantarum* cultured in various concentrations of **sucrose** (0.5%, 2%, 4% (m/v)) supplemented glucose-free-MRS media and the control

Systems (m/v)		pH	Ave	SD	SEM	pH	Ave	SD	SEM
0.5%	Run 1	5.64	5.93	0.25	0.14	4.62	4.67	0.04	0.03
	Run 2	6.04				4.69			
	Run 3	6.10				4.70			
2%	Run 1	6.11	6.11	0.00	0.00	3.64	3.63	0.02	0.01
	Run 2	6.11				3.65			
	Run 3	6.11				3.61			
4%	Run 1	5.98	6.03	0.05	0.03	3.61	3.61	0.02	0.01
	Run 2	6.08				3.60			
	Run 3	6.03				3.63			
Control (contains 2% (m/v) glucose)	Run 1	5.98	5.99	0.03	0.02	3.61	3.64	0.03	0.02
	Run 2	5.97				3.67			
	Run 3	6.02				3.63			

5.2.5 Summary

These *in vitro* tests revealed that the synthesis of the various carbohydrates by *L. plantarum* resulted in the proliferation and a drop in the pH of the monoculture. This indicates that glucose, inulin, maltodextrin, and sucrose possess prebiotic abilities to promote the delivery of desired outcomes by pharmabiotic containing *L. plantarum* when administered. Furthermore, it has been confirmed that the concentration of the cryoprotectants as substrates influences the resulting cell densities (with the exception of maltodextrin) and the resulting drop in the pH of the immediate growth environment of the microorganism.

However, it must be noted that behaviour and interaction between the microorganism and the substrates during *in vitro* tests might translate differently when *in vivo* tests are performed. Prebiotics able to stimulate the propagation in media may not promote growth in the same way on the human body where other carbon sources are present and other microorganisms may compete for the prebiotic.

6 RESULTS AND DISCUSSION II: IMPACT OF CRYOPROTECTANTS ON FREEZE DRIED *LACTOBACILLUS PLANTARUM*

Having confirmed the probable impact of the cryoprotectants on *L. plantarum* growth, this study proceeded to investigate the protective effects of the skimmed milk, inulin, maltodextrin, and sucrose on the candidate strain, *L. plantarum*, during freeze drying and over storage.

The viability and the vitality of cells upon re-growth was examined. The vitality of cells was demonstrated by the ability of cells to grow in fresh MRS media after freeze drying and storage. After a thorough search for literature, no such study was found that demonstrate the behaviour of dried probiotic cells upon re-growth.

Skimmed milk was now introduced as another cryoprotectant, while cells freeze dried in water without excipients was the negative control. The skimmed milk was not previously considered in the prebiotic experiments as it could not be autoclaved. The cells were cultured in standard MRS media, harvested, washed, and re-suspended in different drying media containing 10% (m/v) of these various cryoprotectants.

In initial testing, glucose failed to form a glassy matrix during freeze drying and instead formed a rubbery matrix (a qualitatively assessed result and thus result not shown). Its use was therefore discontinued.

The following subsections discuss in detail the results obtained from freeze drying *L. plantarum* cells embedded in skimmed milk, inulin, maltodextrin, sucrose and without cryoprotectants.

6.1 Moisture content of freeze dried *L. plantarum*

The data presented in Table 6-1 represent the resulting moisture content of *L. plantarum* cells after freeze drying at -85^{o}C, -32^{o}C primary drying temperature, and 0.0004 bar primary drying pressure. Results are of various drying media containing the cryoprotectants skimmed milk, inulin, maltodextrin, and sucrose, and for drying media without cryoprotectants (control).

As seen in Table 6-1, the average final moisture content achieved under these conditions was in a range of 1.39% to 7.15%. This wide range of moisture content could have been a function of the different relative molecular weights of the cryoprotectants. Lower molecular weight sucrose resulted in the highest moisture content of 7.15%. The resulting moisture content in the samples

then reduced as the molecular weight of the carbohydrates increased. Skimmed milk being the most complex excipient is made up of carbohydrate protein and fatty acids (Cavalcanti *et al.*, 2019). It resulted in the lowest moisture content of 1.39%. A similar observation was made by Michalska-Ciechanowska *et al*. (2020) when freeze dried cranberry powder containing inulin resulted in a higher moisture content than freeze dried powder containing maltodextrin. These researchers reported that higher content carrier of excipients in the cranberry powder resulted in the lower moisture content.

Table 6-1 Moisture content of freeze dried probiotics samples

Moisture content (wt%)					
Drying media	Control	Skimmed Milk	Inulin	Maltodextrin	Sucrose
Run 1	2.62	1.02	4.00	0.87	6.06
Run 2	3.77	1.76	5.68	2.29	8.25
Run 3	2.36	1.38	4.73	6.12	-
Average	2.92	1.39	4.80	3.09	7.15
SD	0.43	0.21	0.49	1.57	0.89

6.2 Morphology and structural form of freeze dried *L. plantarum*

The introduction of various cryoprotectants into the drying media influenced both the physicochemical structure of the probiotic product and the micro-nature of resulting cell membranes. Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) methods were applied respectively to analyse the nature of these influences.

6.2.1 Powder structure of product

The freeze dried *L. plantarum* cells in various cryoprotectants varied in its physiochemical structure. The cells in water without cryoprotectant resulted in probiotic cells that were clogged loosely in a powder form. In contrast to this, *L. plantarum* cells freeze dried in skimmed milk resulted in a cake-like matrix that could be broken up to form a powder when crushed. Freeze dried maltodextrin and sucrose with embedded *L. plantarum*, as shown in Figure 6-1, were also in a cake-like matrix. However, the freeze dried maltodextrin-*L. plantarum* matrix was more loosely packed compared to skimmed milk and sucrose. Freeze dried cells embed in inulin resulted in a mix of partially sticky and cake-like matrices.

Figure 6-1 Photographic images of freeze dried *L. plantarum* cells without cryoprotectant, embedded in maltodextrin, and sucrose (from left to right)

6.2.2 Scanning electron microscopic images

The SEM micrographs presented in Figure 6-2 show freeze dried *L. plantarum* cells in the control media, i.e., water without cryoprotectants. The cells appear to be rod-like in shape (Hernandez *et al.*, 2012). They also appear to be exposed and packed, while some are seen to be free flowing.

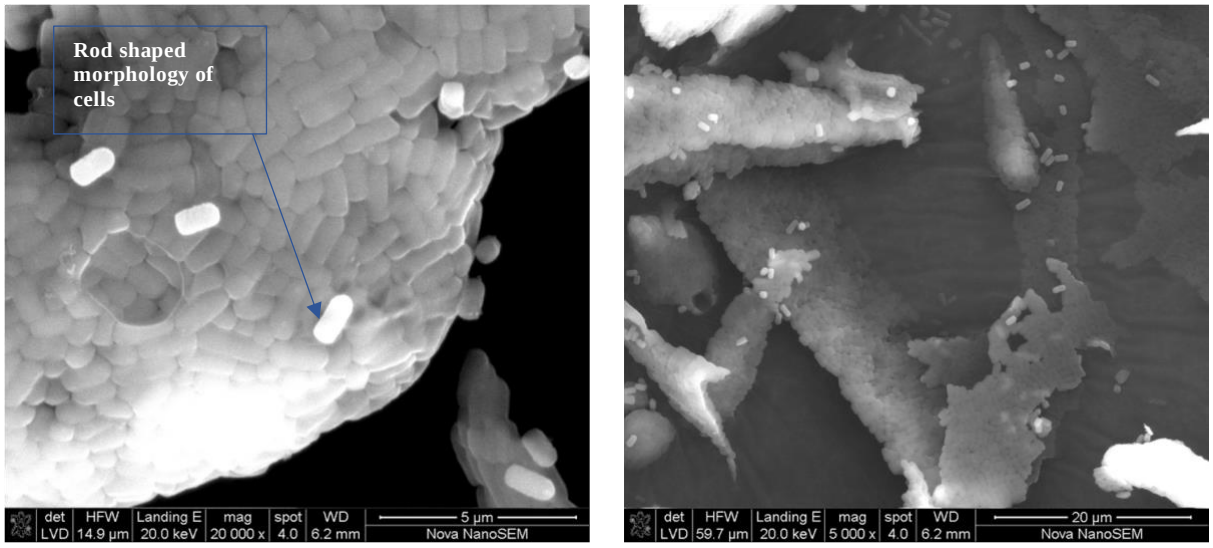

Figure 6-2 Scanning Electron Microscopic images of freeze dried probiotic cells **without cryoprotectants (control)**

The morphology of *L. plantarum* freeze dried in skimmed milk was visualized by the SEM images presented in Figure 6-3. In contrast to the exposed cells in the negative control, freeze dried *L.*

plantarum in skimmed milk were embedded in a dense protective matrix. However, some exposed cells were visible at the corners and cracks of the embedding material.

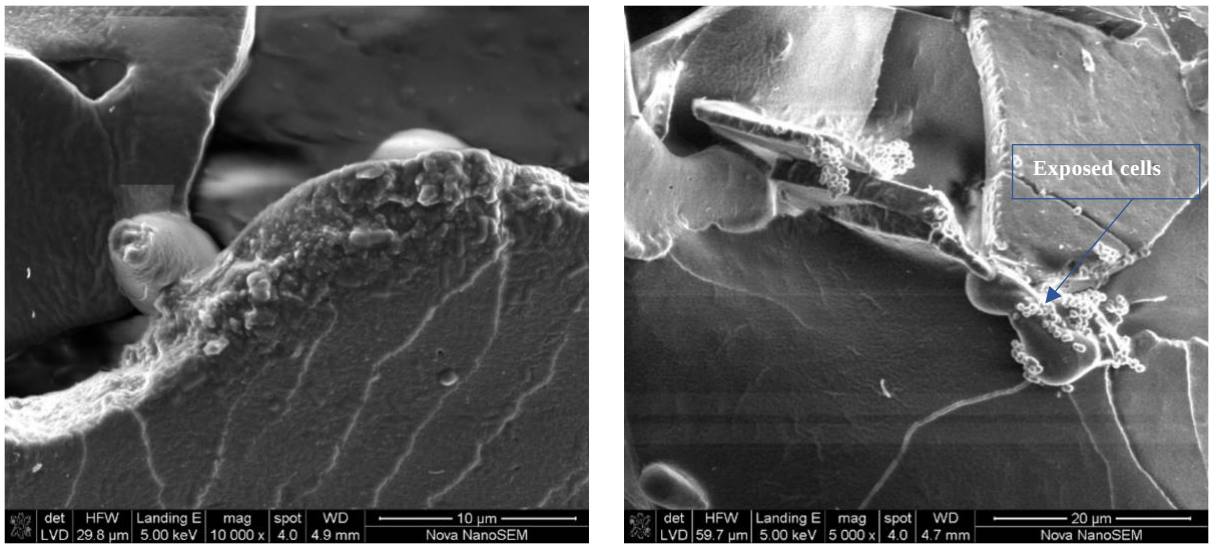

Figure 6-3 Scanning Electron Microscopic images of freeze dried probiotic cells embedded in **skimmed milk**

SEM visualization of the freeze dried probiotic cells in inulin is presented in Figure 6-4. The images show that the cells are embedded in a protective layer of inulin. In contrast to the skimmed milk system, the morphology of the cell membrane could be seen slightly through the outer matrix.

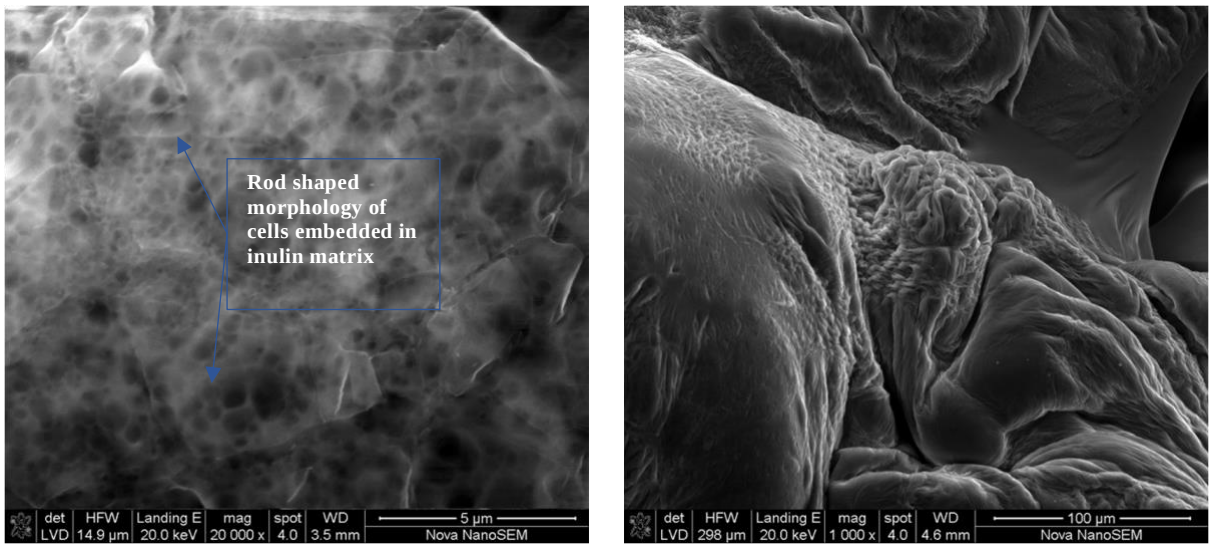

Figure 6-4 Scanning Electron Microscopic images of freeze dried probiotic cells embedded in **inulin**

The SEM images presented in Figure 6-5 for the maltodextrin system show *L. plantarum* cells embedded in a protective matrix, similar to that of inulin. The rod-shaped morphology of the cells could be seen through the protective matrix.

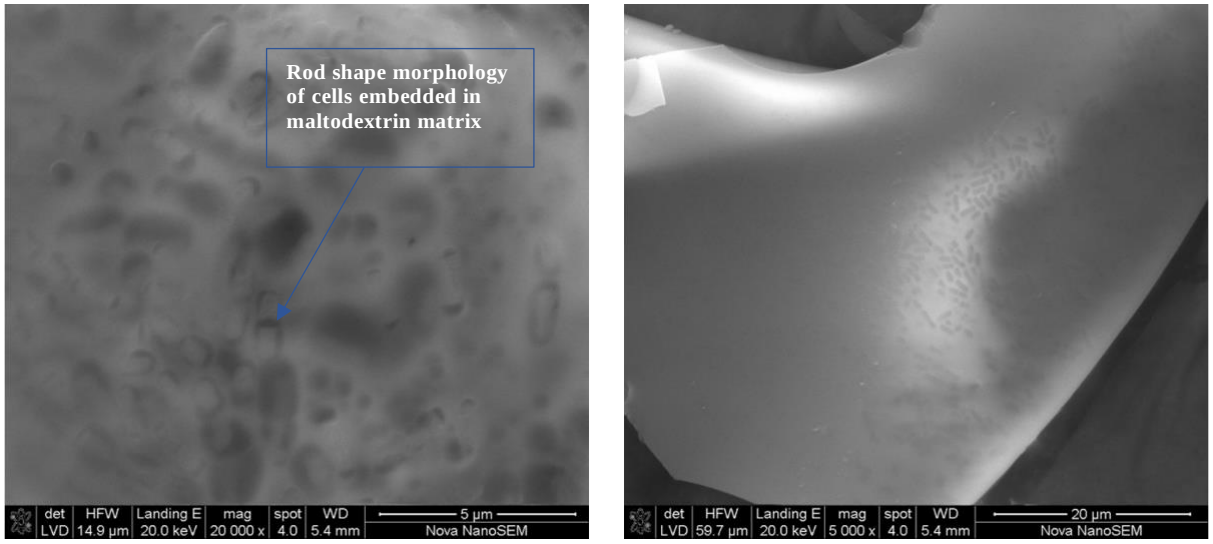

Figure 6-5 Scanning Electron Microscopic images of freeze dried probiotic cells embedded in **maltodextrin**

Figure 6-6 shows *L. plantarum* cells embedded in sucrose. Similar to maltodextrin, the cells appear to bulge through the protective covering matrix but in this case, they appear more visible and to be more clustered together in the matrix.

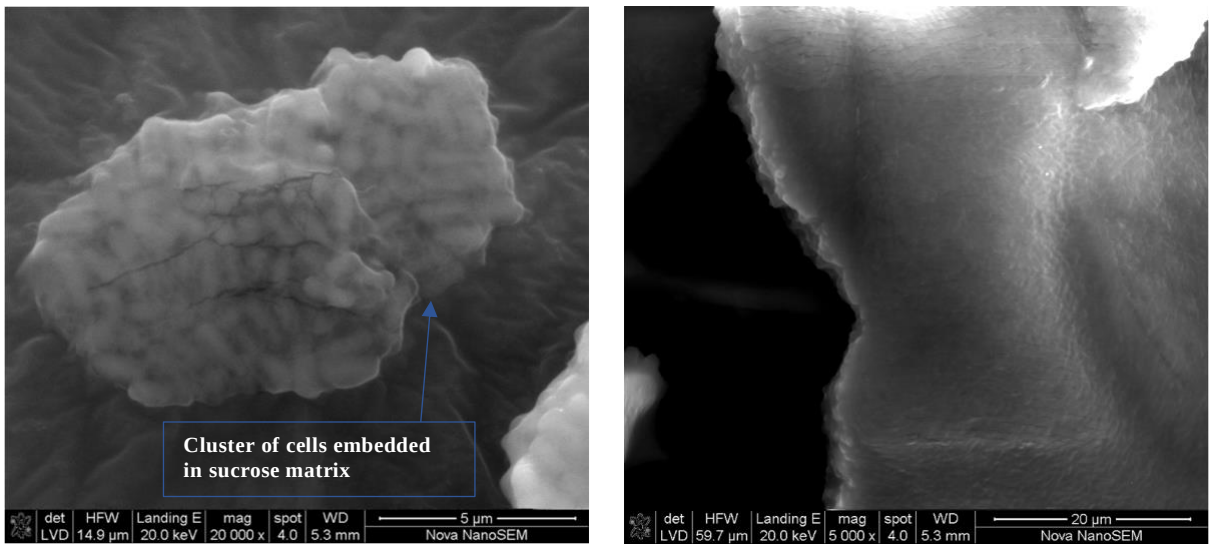

Figure 6-6 Scanning Electron Microscopic images of freeze dried probiotic cells embedded in **sucrose**

The SEM images showed the presence of a cell matrix with a protective layer when freeze drying with different cryoprotectants. However, it remains inconclusive from the images as to how the matrices formed would influence the viability of the cells after freeze drying. The images suggest that cells remain intact and protected but it would seem a closer inspection on the cell structure may give better understanding on the cell's physical state.

6.2.3 Transmission electron microscopic images

To examine the influence of freeze drying on *L. plantarum* cell membrane and the role of cryoprotectants in retaining the native structure of the membrane, the freeze dried *L. plantarum* was analysed by TEM images. Two samples which demonstrated highest survival were selected for further TEM investigations to understand the nature of the cell membranes after freeze drying in comparison to the control without cryoprotectants. These samples were *L. plantarum* embedded in skimmed milk and inulin.

The TEM revealed close-up and cross-sectional images of the cell membranes, compared to SEM images which revealed the physical surface structure of the freeze dried product.

The TEM images in Figure 6-7 of freeze dried *L. plantarum* in the control without cryoprotection show distortion of the native rod-like structure of *L. plantarum* owing to the detrimental impact of the freeze drying process on cell membranes (Aschenbrenner, Foerst and Kulozik, 2015). In comparing the images obtained by SEM and TEM on freeze dried cells without cryoprotectants, the rod-like structure of *L. plantarum* was not visible in the latter. However, a possibility lies where sample preparation during the TEM procedure might have caused damage to the cell membranes.

With the introduction of cryoprotectants in the drying media, TEM images presented in Figure 6-8 revealed that the *L. plantarum* cells still maintained the structure of their membranes after freeze drying in the presence of inulin. This agrees with results by Moayyedi *et al.* (2018), that probiotic cells retain their native state in membrane structure by the addition of a protective material. However, the cells embedded in skimmed milk (Figure 6-9) were difficult to identify. An explanation for this could be that the obscured cells were covered by the cryoprotectant matrix, as shown in the SEM images in Figure 6-3.

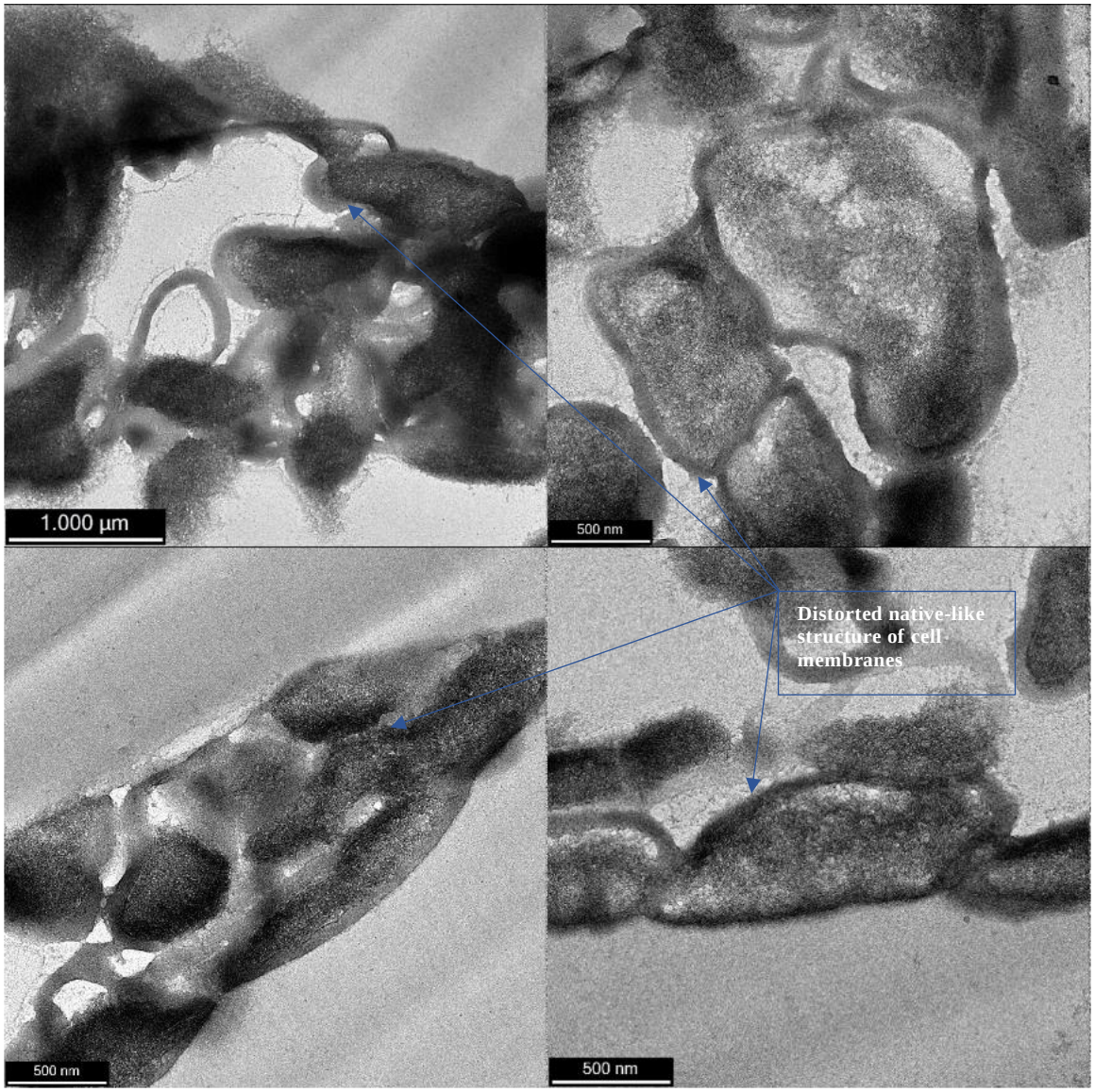

Figure 6-7 Transmission Electron Microscopic images of freeze dried probiotic cells **without cryoprotectants (control)**

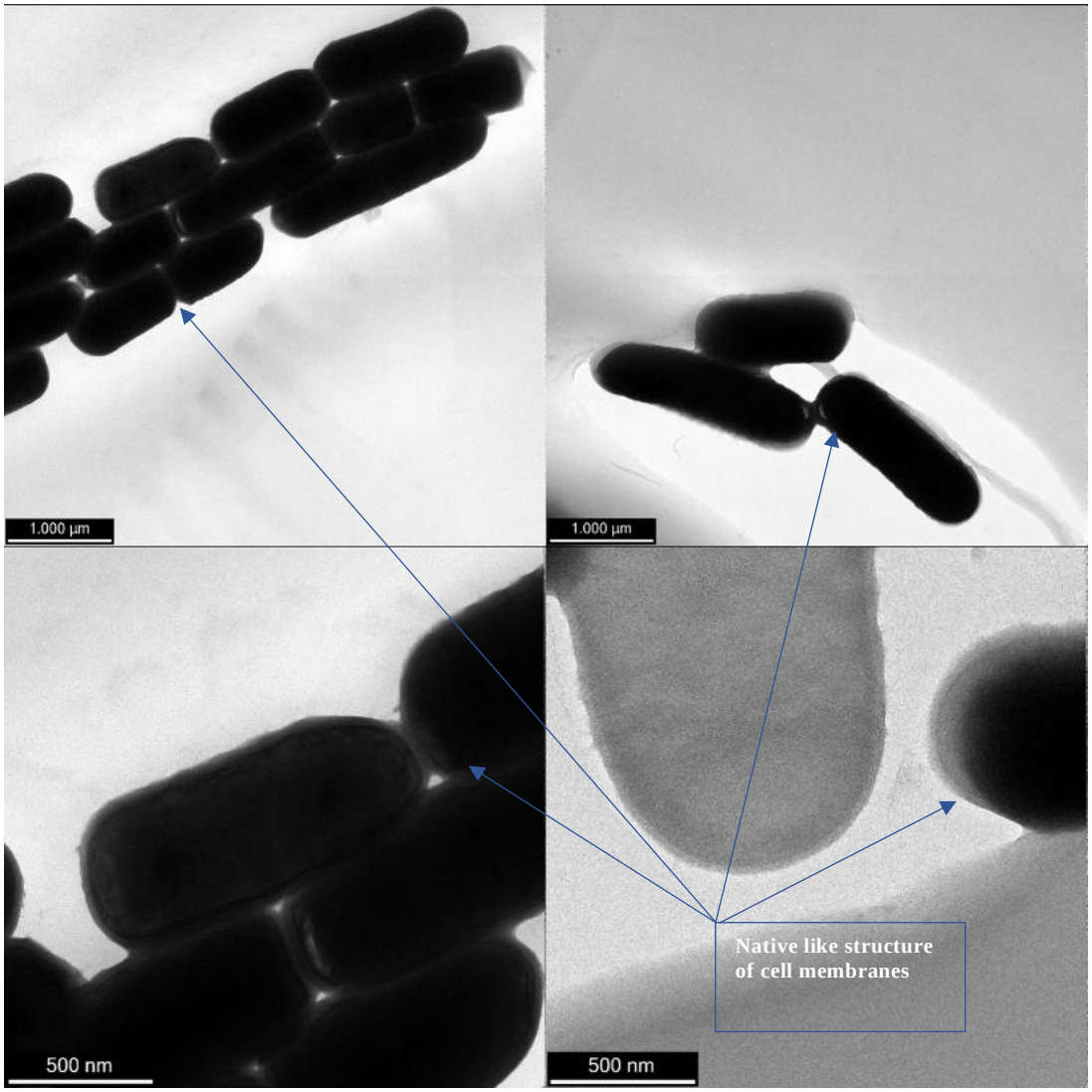

Figure 6-8 Transmission Electron Microscopic images of freeze dried probiotic cells embedded in **inulin**

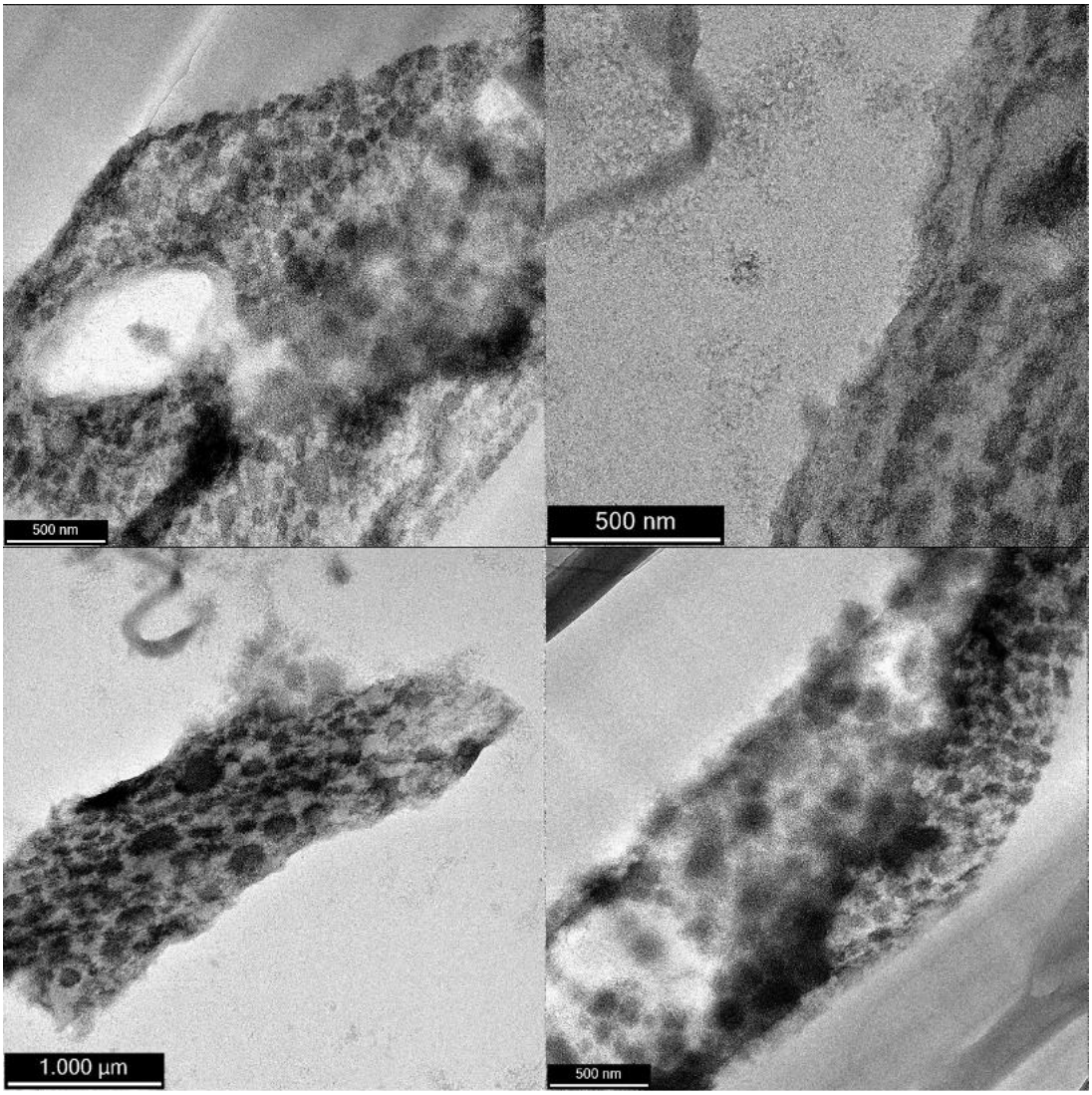

Figure 6-9 Transmission Electron Microscopic images of freeze dried probiotic cells embedded in **skimmed milk**

6.3 Impact of cryoprotectants on *L. plantarum* during freeze drying dehydration

To understand the impact of the inclusion of the cryoprotectants with *L. plantarum*, the viability and the vitality of freeze dried cells were examined after freeze drying by assessing the CFU per ml and growth kinetic parameters obtained for the rehydrated cells compared to what was obtained before the drying process. These results are presented and discussed below.

6.3.1 Survival of *L. plantarum* during freeze drying

The viability of *L. plantarum* when the cells were freeze dried in various drying media each containing 10% (m/v) inulin, skimmed milk, maltodextrin, and sucrose respectively and no cryoprotectant media are presented in Figure 6-10, with the raw data presented in appendix C.

The results show that there was a marked improvement in the survival of cells compared to that of the control (without excipients). A survival rate of up to 91% was achieved in the sample of cells freeze dried with skimmed milk, compared to the cells freeze dried in water which demonstrated a survival rate of 22%. Amongst the other cryoprotective candidates, inulin followed skimmed milk by demonstrating the second-highest survival rate of 85% during freeze drying of *L. plantarum*, followed by sucrose and maltodextrin with survival rates of 33% and 31% respectively.

This means that the presence of sugars in the freeze drying media provided protection to cell membranes during the freeze drying process. This protective ability can be linked to various mechanisms, one of which is the ability of the cryoprotectants presented in this study to form a protective glassy matrix during the freezing process, in which the cells are embedded. These protective matrices can be seen in the SEM micrographs presented in Figure 6-3, Figure 6-4, Figure 6-5, and Figure 6-6 for skimmed milk, inulin, maltodextrin and sucrose.

Another mechanism that could contribute to the improved survival with the inclusion of cryoprotectants in the drying media, is the ability of sugars molecules to permeate through the cell membranes and replace the removed water molecules that were initially present between the bi-phospholipid layers within the cells. The water molecules act as spacers between the phospholipid heads that prevent them from rubbing against each other and subsequently causing an acyl chain reaction. This chain reaction leads to Van der Waals forces that shift the fluid state within cells to a more viscous gel-like phase. Upon rehydration, the transition back to the liquid crystal-like phase is non-homogenous due to the inhomogeneous nature of the phospholipid layers. This results in packing defects within cells and the cells lose their membrane integrity which consequently leads to death (Aschenbrenner, Foerst and Kulozik, 2015).

Therefore, its replacement during dehydration by the sugar molecules helps to retain the native robust structure of cell membranes after the freeze drying process. This native cell membrane form is seen in TEM images of inulin present in Figure 6-8, however, the loss in the native cell membrane is observable in Figure 6-7 with cells freeze dried in water without cryoprotectants. Another mechanism that could possibly elucidate the improved survival with the inclusion of cryoprotectants in the drying media is the vitrification hypothesis.

This result is similar to those reported by Reddy *et al*. (2009), where *L. plantarum* demonstrated high survival rates of 100% when freeze dried in skimmed milk. However, the survival rates obtained when *L. plantarum* was freeze dried in maltodextrin and sucrose ($\geq$ 90%) and in water without cryoprotectants ($\geq$ 40%), were significantly higher than the rates obtained in this work. Two possible reasons could have contributed to the improved survival rates obtained by Reddy *et al*. (2009). One reason is that due to pre-exposure to cold shock prior to freeze drying, the cells

developed cold shock resistance. Derzelle *et al.* (2003) explains that this developed resistance by *L. plantarum* to cold shock is linked to the release of three cold shock proteins CspC, CspP and CspL during exposure to cold shock in the early log phase. Reddy *et al.* (2009) confirmed this by identifying induced cold shock proteins during the exposure to *L. plantarum* cells in the early log phase of growth. Another probable explanation for a higher rate of survival obtained by *Reddy et al.* (2009) is the higher concentration of cryoprotectants in the drying media. It is possible that the higher concentration of 20% (m/v) compared to 10% used in this study led to higher survival rates.

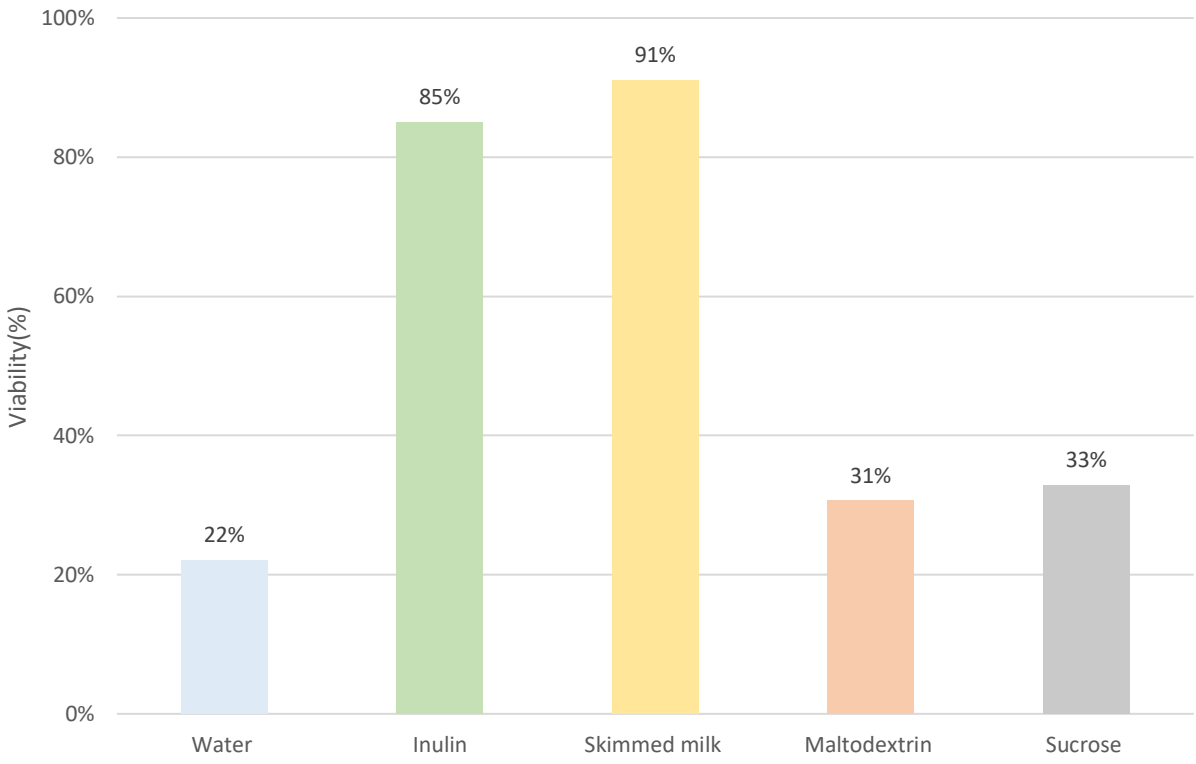

Figure 6-10 Percentage survival *of L. plantarum* after freeze drying in water (control), inulin, skimmed milk, maltodextrin, and sucrose

6.3.2 Propagation and pH reduction potential of *L. plantarum* after freeze drying

To investigate the impact of the various cryoprotectants on the vitality of *L. plantarum* during the drying process, freeze dried samples of *L. plantarum* in skimmed milk, inulin, maltodextrin, and sucrose were rehydrated to their original volume and inoculated into standard MRS media for fermentation. The cell density and the pH of the fermentation culture in the systems were measured at an hourly time interval.

The results presented in Figure 6-11 and Figure 6-13 show the fermentation profiles of *L. plantarum* in growth media before and after freeze drying, with the latter achieved by inoculation of the media with the rehydrated freeze dried cells. These results are drawn from data presented in appendix D.

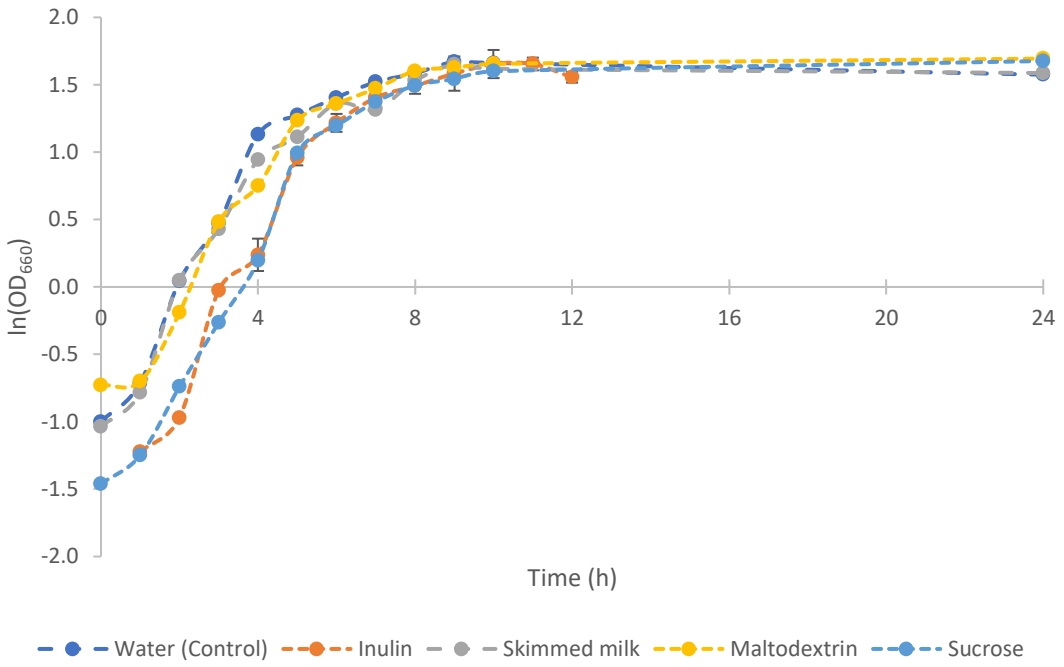

Figure 6-11 Fermentation profiles during re-growth in MRS media of freshly harvested *L. plantarum* cells suspended in different drying media: water (control), inulin, skimmed milk, maltodextrin, and sucrose. The error bars are representative of the standard deviation between triplicate repeat runs

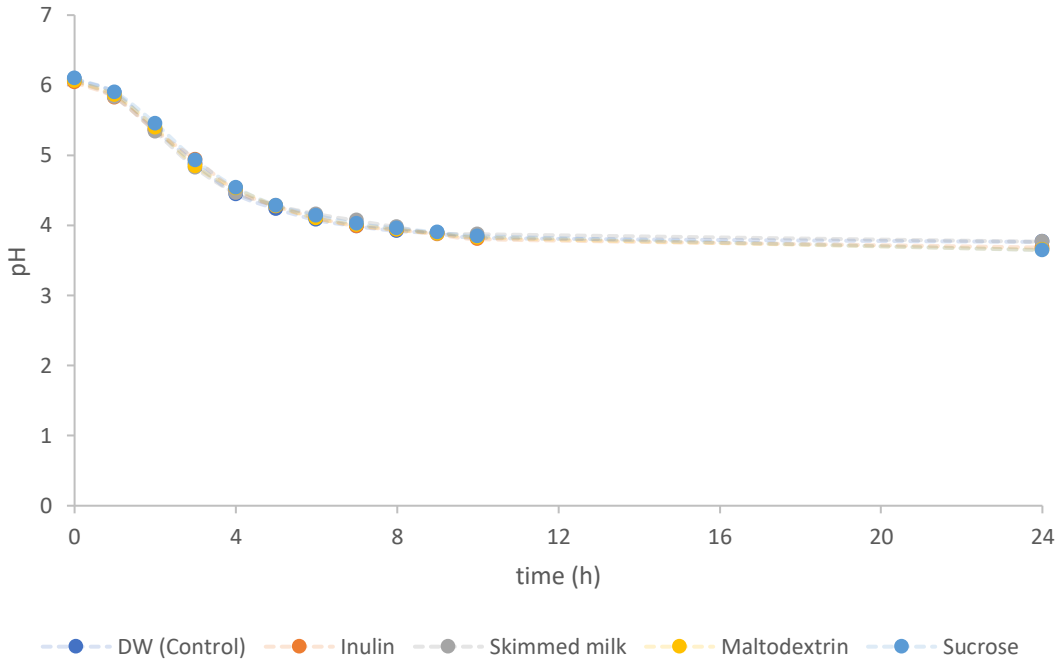

Figure 6-12 pH profiles due to *L. plantarum* lactic acid production during re-growth in MRS media of freshly harvested *L. plantarum* cells suspended in different drying media water (control), inulin, skimmed milk, maltodextrin, and sucrose. The error bars are representative of the standard deviation between triplicate repeat runs

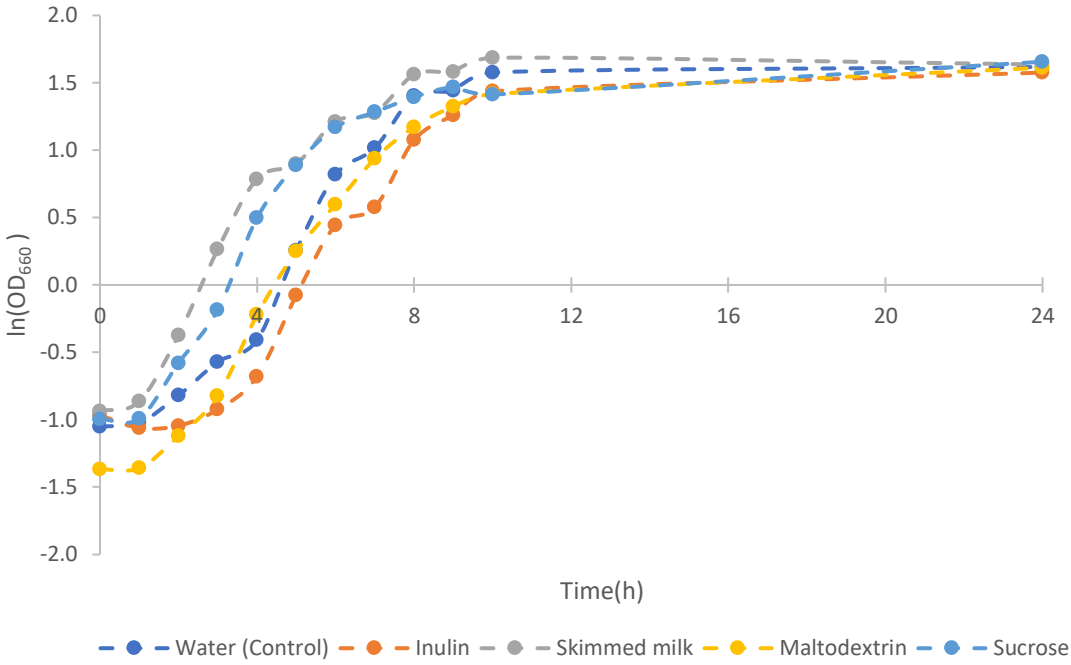

Figure 6-13 Fermentation profiles of *L. plantarum* cells freeze dried in water (control), inulin, skimmed milk, maltodextrin, and sucrose upon re-growth in MRS media. The error bars are representative of the standard deviation between triplicate repeat runs

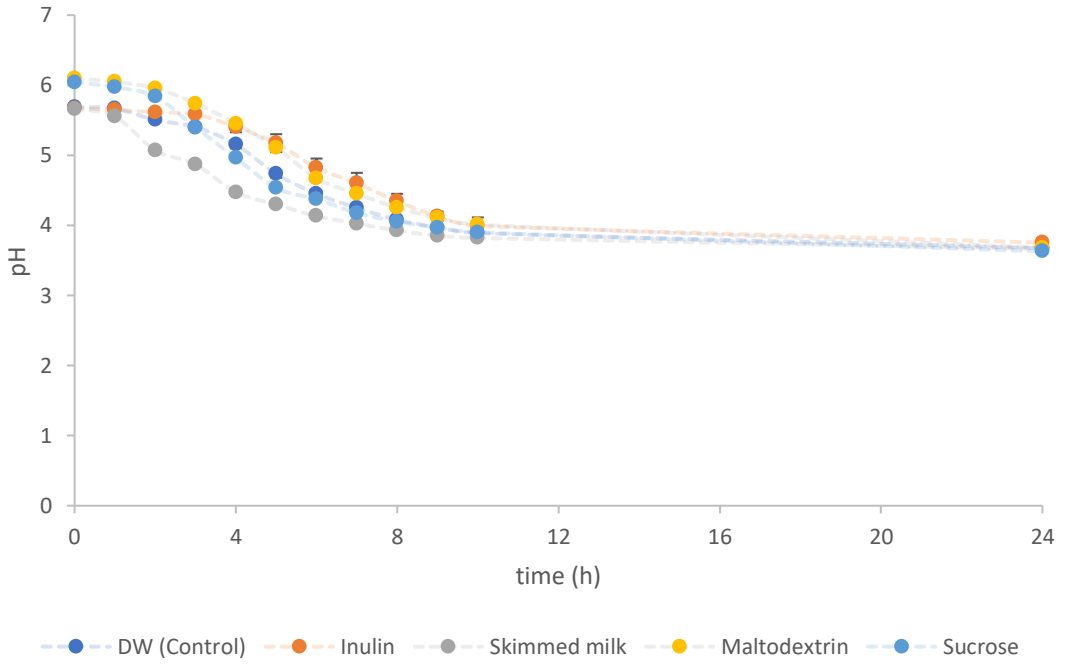

Figure 6-14 pH profiles due to *L. plantarum* lactic acid production of *L. plantarum* cells freeze dried in water (control), inulin, skimmed milk, maltodextrin, and sucrose upon re-growth in MRS media. The error bars are representative of the standard deviation between triplicate repeat runs

The growth profiles of *L. plantarum* during fermentation in all systems were similar to each other. However, the cells freeze dried in the different cryoprotectants exhibited different growth curves during this fermentation relative to the cells that were not freeze dried.

A similar pattern can be observed in the case of pH reduction profiles as seen in Figure 6-12 and Figure 6-14. The single line curve across all systems inoculated before freeze drying signifies that there was no significant change in the pH reduction rate and final pH at the end of fermentation. However, differences in the pH reduction rates from the cultures grown post freeze drying with different cryoprotectants can be seen in Figure 6-14.Table 6-2 presents the $\mu_{max,}$ t_{lag} and OD_{max} obtained from the experimental data presented in appendix D. Although some small variations were observed in the μ_{max} calculated from the data, across all systems, there was no significant change between the μ_{max} in the propagation of cells before freeze drying and after freeze drying. However, the lowest μ_{max} value of 0.45 ± 0.06 h^{-1} was found after freeze drying in cells freeze dried in maltodextrin. Skimmed milk appeared better at retaining the normal growth behaviour by exhibiting the exact same μ_{max} values before and after freeze drying compared to cells freeze dried in other cryoprotects and the control drying media. The OD_{max} values did not significantly change across all systems before and after freeze drying, however, the lowest value of 4.8 was obtained in cells freeze dried in maltodextrin. There was no significant drop in the t_{lag} of 1 h across all cryoprotectants except for cells freeze dried in inulin and the control drying media which showed t_{lag} of 4 h after freeze drying.

This indicates that the freeze drying process had a negative impact on the propagation and lactic acid production of *L. plantarum*. The presence of cryoprotectants provides reduction in the attenuating effect on the propagation of cells. Cells freeze dried in water showed increase in $t_{lag.}$ The delay in propagation can be linked to the low survival rates obtained in freeze dried cells in water. Low viable cell densities lead to increase in dilution and reduce the proximity of viable cells. The reduce proximity of cells limits cells to interact with each other and share proteins required for propagation (Maier, 2000). As a result of this the cells take longer time to metabolize available nutrients for propagation and production of lactic acid for pH reduction.

It should be noted that for the freeze drying experiments, the fermentations began at slightly different pH compared to the fermentation of the samples before freeze drying. This could be a result of possible biomolecular interactions between the cells and the various cryoprotectants during the rehydration.

Table 6-2 μ_{max}, t_{lag}, and OD_{max} values, and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data on the growth of fresh *L. plantarum* cells in various cryoprotectants before and after freeze drying

Systems (m/v)		μ_{max} (h^{-1})	Ave	SD	SEM	t_{lag} (h)	Ave	SD	SEM	OD_{max}	Ave	SD	SEM
Before freeze drying													
Water	Run 1	0.68	0.60	0.09	0.05	1	1	0	0	5.45	5.44	0.12	0.07
	Run 2	0.50				1				5.32			
	Run 3	0.60				1				5.56			
Skimmed milk	Run 1	0.51	0.56	0.04	0.03	1	1	0	0	5.13	5.45	0.42	0.24
	Run 2	0.55				1				5.29			
	Run 3	0.60				1				5.92			
Inulin	Run 1	0.61	0.60	0.02	0.01	1	1	0	0	5.25	5.27	0.12	0.07
	Run 2	0.59				1				5.16			
	Run 3	0.61				1				5.40			
Maltodextrin	Run 1	0.47	0.50	0.03	0.02	1	1	0	0	5.45	5.44	0.12	0.07
	Run 2	0.50				1				5.56			
	Run 3	0.53				1				5.32			
Sucrose	Run 1	0.63	0.57	0.06	0.04	1	1	0	0	5.20	5.35	0.16	0.09
	Run 2	0.56				1				5.33			
	Run 3	0.50				1				5.51			
After freeze drying													
Water (control)	Run 1	0.56	0.50	0.05	0.03	4	1	0	0	5.12	5.04	0.23	0.13
	Run 2	0.50				4				4.99			
	Run 3	0.46				4				5.01			
Skimmed milk	Run 1	0.55	0.56	0.02	0.01	1	1	0	0	5.25	5.40	0.13	0.08
	Run 2	0.58				1				5.51			
	Run 3	0.55				1				5.43			
Inulin	Run 1	0.40	0.48	0.07	0.04	4	4	0	0	4.89	4.84	0.07	0.04
	Run 2	0.49				4				4.76			
	Run 3	0.54				4				4.88			
Maltodextrin	Run 1	0.52	0.45	0.06	0.03	1	1	0	0	5.06	5.01	0.11	0.06
	Run 2	0.45				1				5.09			
	Run 3	0.40				1				4.89			
Sucrose	Run 1	0.45	0.47	0.04	0.02	1	1	0	0	5.25	5.24	0.06	0.03
	Run 2	0.44				1				5.18			
	Run 3	0.51				1				5.29			

6.4 Probiotic stability of *L. plantarum* over storage

The remaining freeze dried samples were sealed in airtight vials and transferred for storage under two different conditions: at room temperature and 4°C (standard refrigeration temperature). At equal time intervals of 4 weeks, for a total of 3 months, a vial representing each system was taken, rehydrated to the original volume, and analysed to determine the impact of the various cryoprotectants on the probiotic cells over storage. The survival rates were determined by calculating the viability of cells by the plate method. The viability was quantified relative to the cells present before freeze drying. The impacts on cell vitality, i.e. the ability to proliferate and pH reduction potential, were determined by the re-growth of rehydrated cells in MRS media. The cell density by measurement of the OD at 660 nm and pH were read at hourly time intervals.

6.4.1 Survival over storage

Survival without cryoprotectants-**water (control)**

The results of the viability of freeze dried *L. plantarum* cells without a cryoprotectant over a 12 week storage period at 4°C and room temperature are presented in Figure 6-15 and These results are based on the raw data presented in appendix C.

When freeze dried *L. plantarum* cake powder without excipients was rehydrated after being stored under dry airtight conditions, there was a further decline in its viability beyond the viability found at the end of the dehydration process. A reduction in the number of cells from 2.2×10^8 ($\frac{CFU}{ml}$) immediately after freeze drying to 4.5×10^7 ($\frac{CFU}{ml}$) (representing a viability of 4%) and 5.8×10^5 ($\frac{CFU}{ml}$) (representing a viability of 0.05%) after 4 weeks storage at 4°C and room temperature respectively. The number of cells dropped further at the end of 8 weeks storage at 4°C and room temperature respectively to 3.1×10^7 ($\frac{CFU}{ml}$) (representing a viability of 3%) and 3.1×10^4 ($\frac{CFU}{ml}$) (representing a viability of $\leq 1\%$) respectively. The number of cells that remained viable at the end of the 12 weeks storage at 4°C and room temperature was 5.4×10^6 ($\frac{CFU}{ml}$) and 2.5×10^3 ($\frac{CFU}{ml}$), in both cases this amounted to less than 1% viability remaining.

The absence of protective material around stored cells accounts for the high rate of the observed decrease in cell viability. The exposure of cells to harsh environmental factors causes stress to cells and eventually leads to cell death. Furthermore, the biochemical activity of cells is enhanced when cells are stored at higher temperature compared to a lower temperature storage, and these reactions release metabolites which are detrimental to cells and eventually causes cell death.

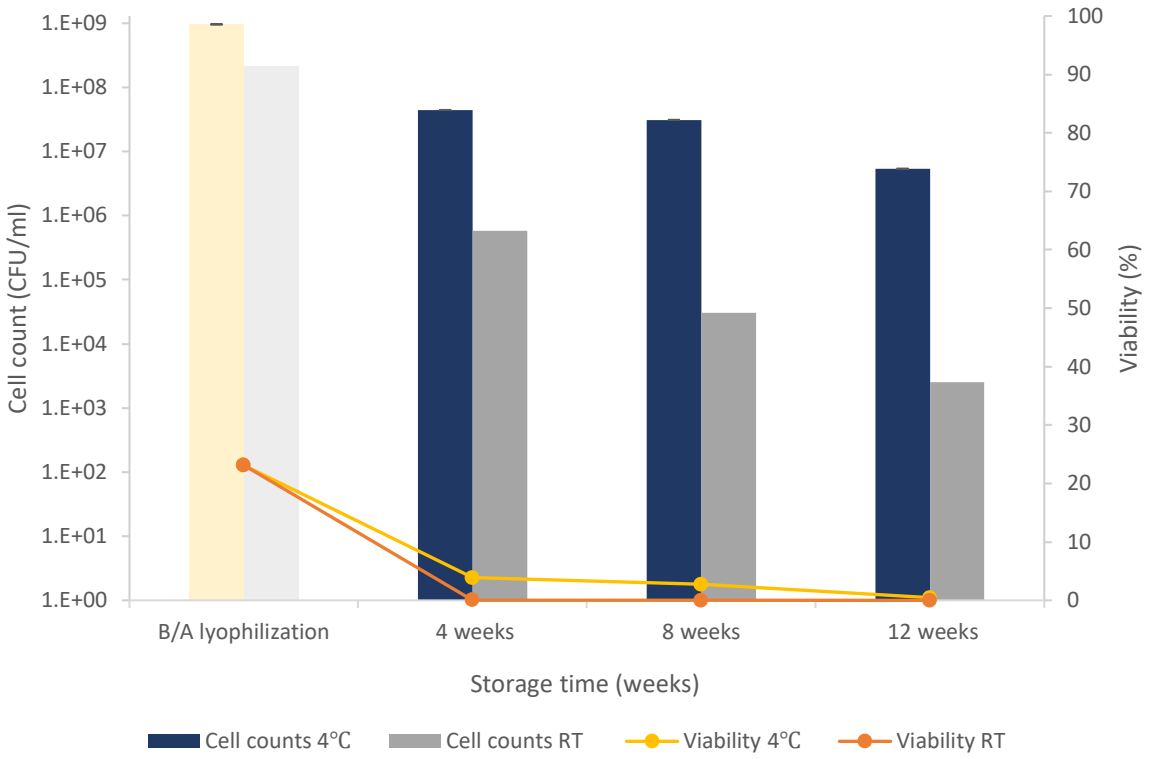

Figure 6-15 Survival of *L. plantarum* freeze dried in **water (control)** before and after (B/A) freeze drying and over 12 weeks of storage at 4°C and at room temperature (RT)

Survival in **skimmed milk** powder

The results of the viability of freeze dried *L. plantarum* cells in skimmed milk over a 12 week storage period at 4°C and room temperature are presented in Figure 6-16. These results are based on the raw data presented in appendix C.

The results show improved survival rates compared to that of the control at both 4°C and room temperature. A reduction in the number of cells was observed from 6.9×10^8 $\left(\frac{CFU}{ml}\right)$ before freeze drying to 6.2×10^8 $(\frac{CFU}{ml})$ (representing a viability of 89%) and 4.4×10^8 $(\frac{CFU}{ml})$ (representing a viability of 63%) after 4 weeks storage at 4°C and room temperature respectively. At the end of 8 weeks storage at 4°C and room temperature 6.4×10^8 $(\frac{CFU}{ml})$ cells (representing a viability of 93%) and 4.0×10^8 $(\frac{CFU}{ml})$ cells (representing a viability of 67%) were present respectively. The number of cells that remained viable at the end of the 12 weeks storage at 4°C and room temperature was 6.3×10^8 $(\frac{CFU}{ml})$ (representing a viability of 91%) and 4.2×10^8 $(\frac{CFU}{ml})$ (representing a viability of 61%) respectively. Therefore, a high degree of product stability was observed under refrigerated

conditions, with little degradation of the cell viability. The product also appeared to be relatively stable at room temperature following the initial decrease in viability during the first month.

These high survival rates observed for dehydration with skimmed milk can be attributed to the stabilizing effect on cell membrane contents, with the presence of protein in skimmed milk known to provide additional protective coating to cells (Carvalho *et al.*, 2004). The exposed cells that appear in the SEM images presented in Figure 6-3 showing bulges at surfaces of the product could account for the loss of viable cells at the end of the dehydration process because there was less or no protective covering around them.

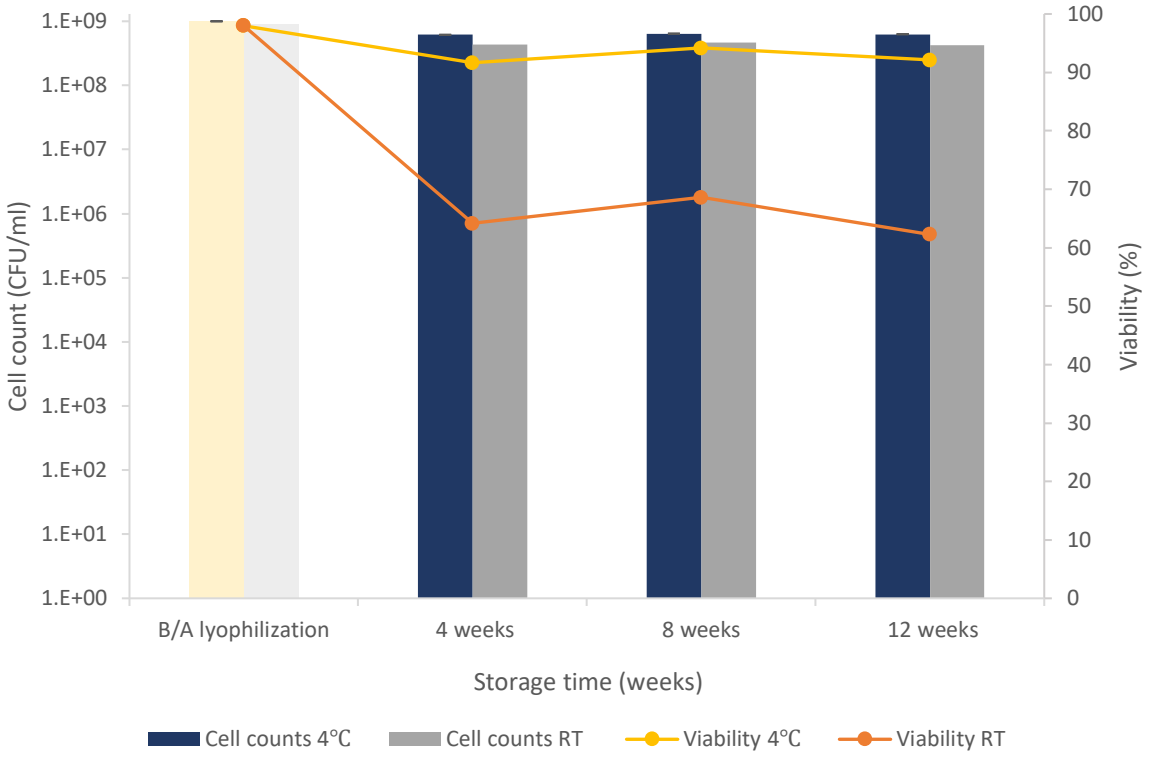

Figure 6-16 Survival of *L. plantarum* freeze dried in **skimmed milk** before and after (B/A) freeze drying and over 12 weeks of storage at 4°C and at room temperature (RT)

Survival in **inulin**

The results of the viability of freeze dried *L. plantarum* cells in inulin over a 12 week storage period at 4°C and room temperature are presented in Figure 6-17. These results are based on the raw data presented in appendix C.

The results show a significant decline in the survival rates over the first 4 weeks. A reduction in the number of cells was observed from 1.1×10^9 $(\frac{CFU}{ml})$ before freeze drying to 1.6×10^7 $(\frac{CFU}{ml})$

(representing a viability of 1%) and 3.8 $\times 10^5$ ($\frac{CFU}{ml}$) (representing a viability of less than 1%) after 4 weeks storage at 4°C and room temperature respectively. The number of cells that appeared to be present at the end of 8 weeks storage at 4°C was 4.5 $\times 10^5$ ($\frac{CFU}{ml}$). No cells appeared viable for cells stored at room temperature at the end of this storage period. The number of cells that remained viable at the end of the 12 weeks storage at 4°C and room temperature was 1.2 $\times 10^6$ ($\frac{CFU}{ml}$) and 6.3 $\times 10^3$ ($\frac{CFU}{ml}$). By the end of 4 weeks storage until 12 weeks, at both storage temperature cases amounted to less than 1% viability remaining.

The absence of viable cells when analysed at the end of 8 weeks, could have been a result of a higher cell dilution factor used during the analysis of viable cells when compared to a lower dilution factor at the end of 12 weeks. It is possible that some viable cells were present in the sample but failed to be accounted for due to a dilution factor not low enough to detect viable cells.

Regardless, even though the inulin maintained 87% cell viability during the freeze drying process, it performed poorly as a protectant over storage.

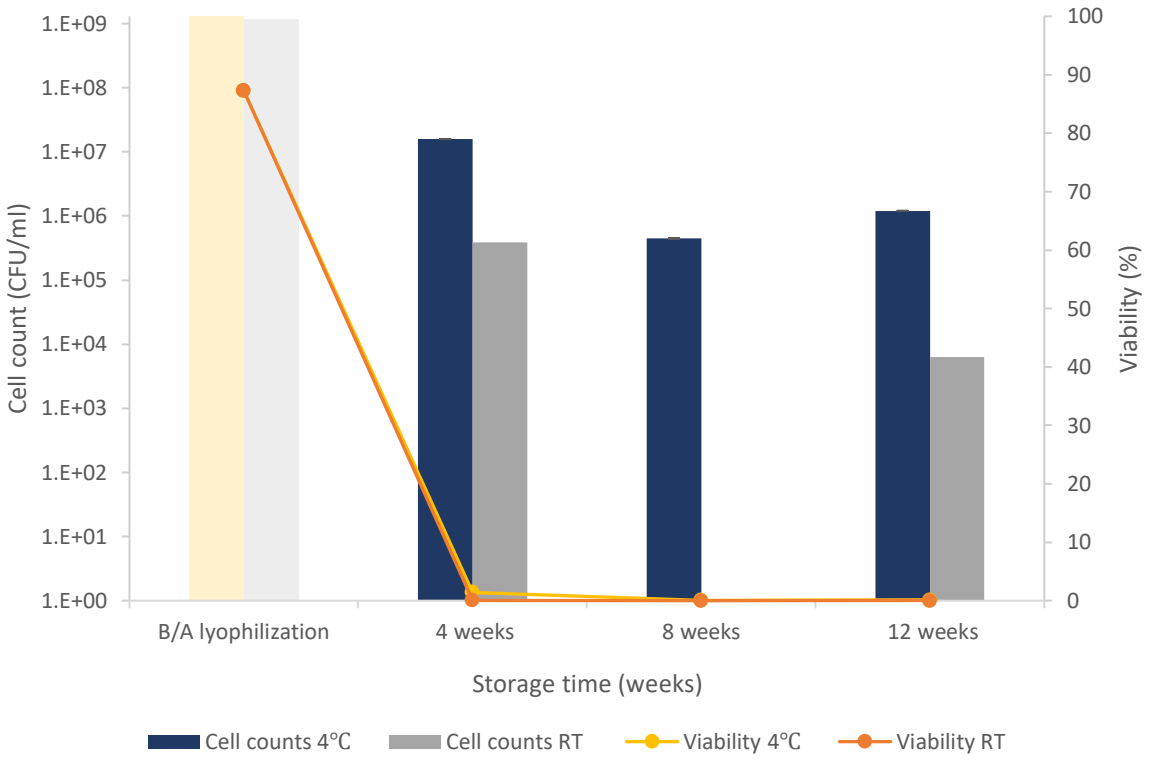

Figure 6-17 Survival of *L. plantarum* freeze dried in **inulin** before and after (B/A) freeze drying and over 12 weeks of storage at 4°C and at room temperature (RT)

Survival in **maltodextrin**

The results of the viability of freeze dried *L. plantarum* cells in maltodextrin over a 12 week storage period at 4°C and room temperature are presented in Figure 6-18. These results are based on the raw data presented in appendix C.

The results show that *L. plantarum* stored under ambient conditions in the presence of maltodextrin demonstrated a decline in survival from 3.1×10^8 ($\frac{CFU}{ml}$) immediately after freeze drying to 4.2×10^7 ($\frac{CFU}{ml}$) (representing a viability of 4%) at the end of 4 weeks at room temperature. At the end of 8 weeks, 6.6×10^5 ($\frac{CFU}{ml}$) cells (representing a viability of less than 1%) were present at room temperature. A decline from this to 1.3×10^5 ($\frac{CFU}{ml}$) was observed by the end of the 12 weeks. Comparing these numbers to the numbers obtained when the cells were stored at 4°C, a slower decline trend was observed from 3.1×10^8 $\left(\frac{CFU}{ml}\right)$ to 1.3×10^8 $\left(\frac{CFU}{ml}\right)$ (representing a final viability of 13%) by the end of the 12 week shelf life. Overall, the protective performance of maltodextrin over storage was therefore significantly better than that of inulin but still not as good as skimmed milk.

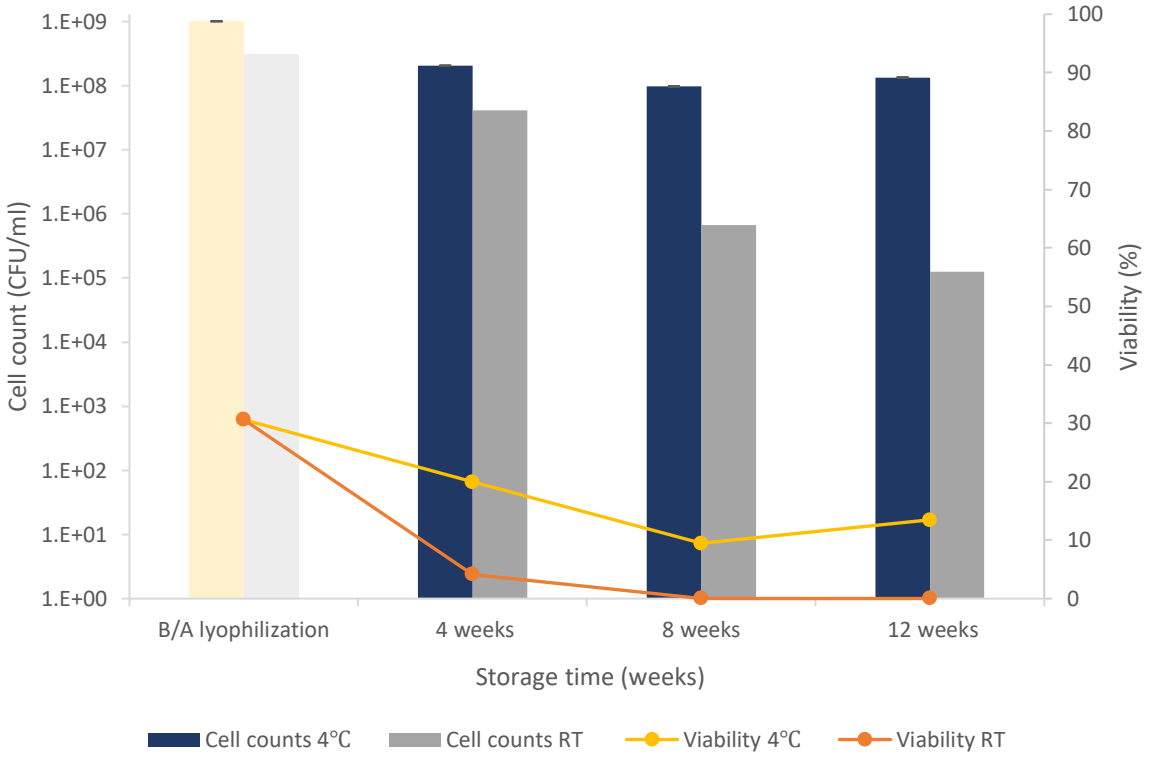

Figure 6-18 Survival of *L. plantarum* freeze dried in **maltodextrin** before and after (B/A) freeze drying and over 12 weeks of storage at 4°C and at room temperature (RT)

Survival in **sucrose**

The results on the viability of freeze dried *L. plantarum* cells in sucrose over a 12 week storage period at 4°C and room temperature are presented in Figure 6-19. These results are based on the raw data presented in appendix C.

A reduction in the number of cells from 3.4×10^8 $\left(\frac{CFU}{ml}\right)$ immediately after freeze drying to 3.2×10^8 $\left(\frac{CFU}{ml}\right)$ (representing a viability of 31%) and 1.8×10^7 $\left(\frac{CFU}{ml}\right)$ (representing a viability of 2%) after 4 weeks storage at 4°C and room temperature respectively. The number of cells dropped further at the end of 8 weeks storage at 4°C and room temperature respectively to 2.6×10^8 $\left(\frac{CFU}{ml}\right)$ (representing a viability of 25%) and 1.2×10^5 $\left(\frac{CFU}{ml}\right)$ (representing a viability of less than 1%) respectively. The number of cells that remained viable at the end of the 12 weeks storage at 4°C and room temperature was 3.4×10^8 $(\frac{CFU}{ml})$ (representing a viability of 33%) and 5.1×10^5 $(\frac{CFU}{ml})$ with less than 1% viability remaining.

The results with sucrose at room temperature showed a similar trend to the other cryoprotectants, with a significant decrease in CFU counts of cells by week 12. *L. plantarum* demonstrated better stability during storage at 4°C compared to storage at room temperature, a similar behaviour observed for the other cryoprotectants. Allowing for slight variations in the CFU, it would appear that there was relatively negligible loss of viability when the cells were stored at 4°C, with viabilities of between 25% and 35% recorded across the storage period.

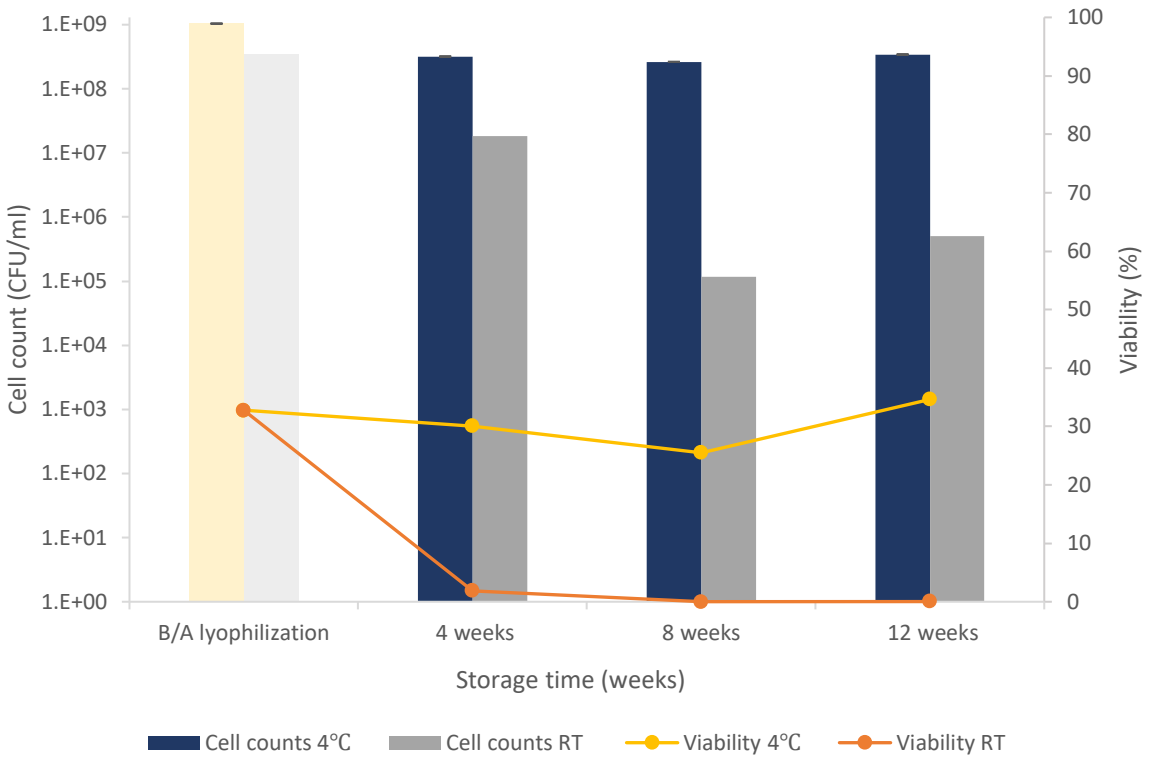

Figure 6-19 Survival of *L. plantarum* freeze dried in **sucrose** before and after (B/A) freeze drying and over 12 weeks of storage at 4°C and at room temperature (RT)

6.4.2 Impact on cell propagation over storage

To investigate the impact of the various cryoprotectants on the vitality of freeze dried and stored *L. plantarum* over 12 weeks at 4ºC and room temperature, the freeze dried and stored samples of *L. plantarum* in skimmed milk, inulin, maltodextrin, and sucrose were rehydrated to their original volume and inoculated into standard MRS media for fermentation. The following results were obtained when the cell density and the pH of the fermentation systems were measured at hourly time intervals. These are compared to the growth and pH reduction profiles of fresh cells before freeze drying.

The μ_{max} values for some of the systems were not calculated due to the absence of data points in the log phase because of the prolonged lag time.

Propagation of cells freeze dried in **water (control)** upon re-growth

The results presented in Figure 6-20 and Figure 6-21 show the fermentation and pH profiles of *L. plantarum* in the control when re-grown at different time points over the 12 weeks of storage at 4ºC and in Figure 6-22 and Figure 6-23 at room temperature, respectively. Table 6-3 presents the

associated $\mu_{max,}$ t_{lag} and OD_{max} upon re-growth of the *L. plantarum*. These values were drawn and calculated from the experimental data presented in appendix D.

The cells freeze dried in water demonstrated low ability to retain the vitality upon re-growth following storage at both temperature conditions. There is a significant deviation in the fermentation curve of the cells during storage from that of fresh cells. This deviation increased with increased storage time. The cells did not retain their growth pattern at the end of the storage time when stored at both 4°C and room temperature. However, all samples eventually appeared to obtain approximately the same OD.

This is similar for the pH reduction profiles of freeze dried *L. plantarum* in the control, during re-growth in growth media over 12 weeks of storage at 4°C and room temperature respectively. The reduction in pH upon re-growth of *L. plantarum* freeze dried in the control over storage, demonstrated slower pH reduction in pH profiles compared to that of the fresh cells.

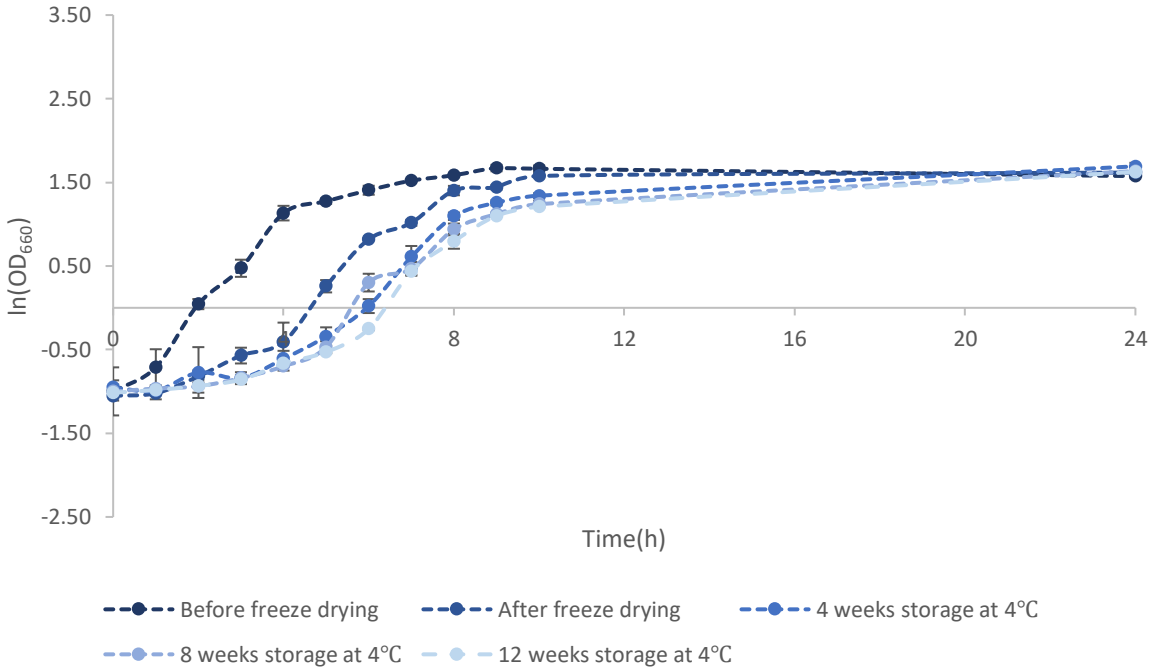

Figure 6-20 Fermentation profiles during re-growth in MRS media of *L. plantarum* cells in **water (control)** before freeze drying, after freeze drying and over storage at 4°C. The error bars are representative of the standard deviation between triplicate repeat runs.

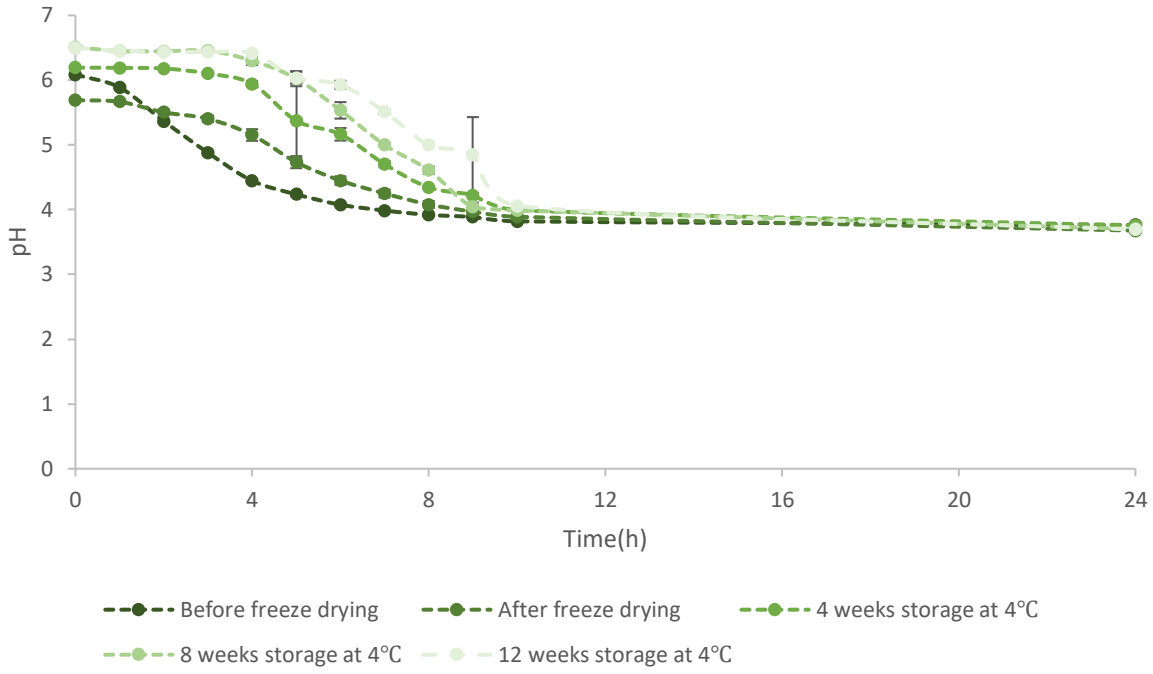

Figure 6-21 pH profiles due to *L. plantarum* lactic acid production during re-growth in MRS media of *L. plantarum* cells in **water (control)** before freeze drying, after freeze drying and over storage at 4°C. The error bars are representative of the standard deviation between triplicate repeat runs

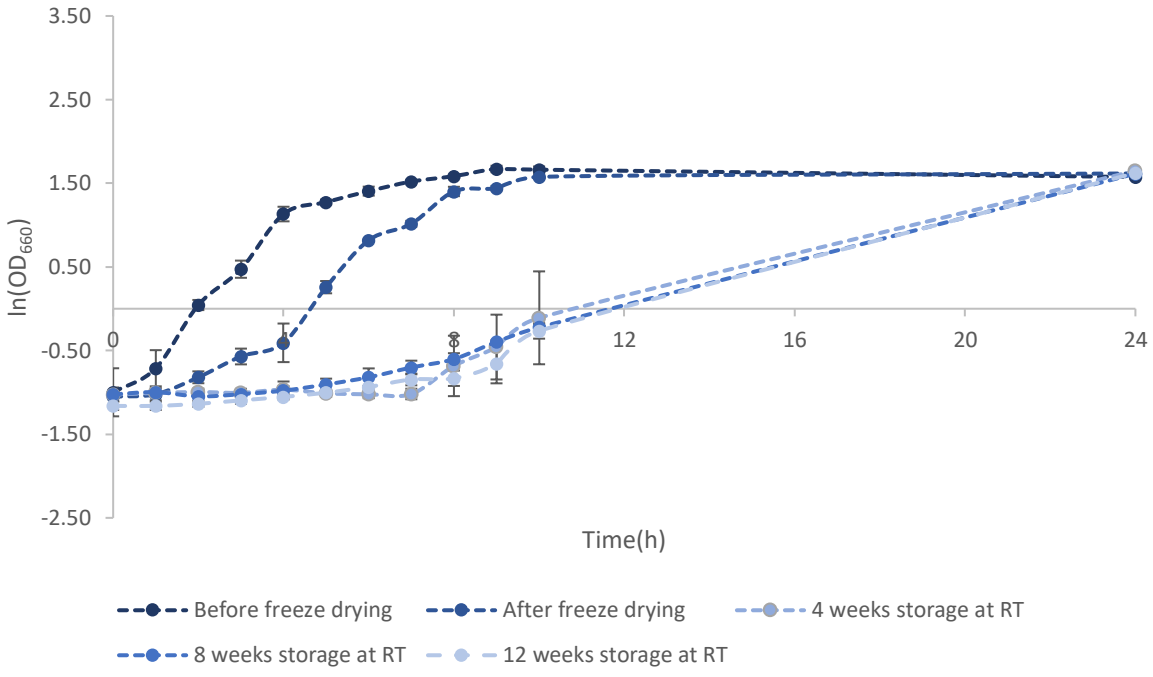

Figure 6-22 Fermentation profiles during re-growth in MRS media of *L. plantarum* cells in **water (control)** before freeze drying, after freeze drying and over storage at room temperature. The error bars are representative of the standard deviation between triplicate repeat runs.

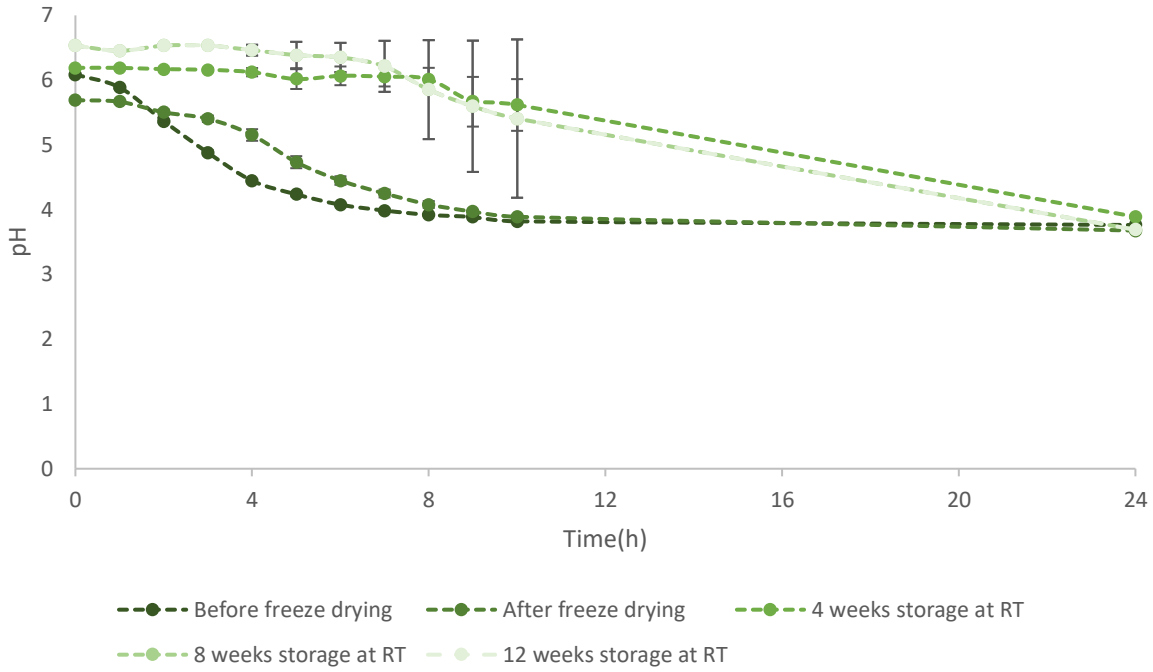

Figure 6-23 pH profiles due to *L. plantarum* lactic acid production during re-growth in MRS media of *L. plantarum* cells in **water (control)** before freeze drying, after freeze drying and over storage at room temperature. The error bars are representative of the standard deviation between triplicate repeat runs.

A drop is seen in the μ_{max} to 0.41 h^{-1} after 8 weeks of storage at 4°C from 0.60 h^{-1} before freeze drying. However, some small variations were observed in the μ_{max} calculated from the data, over the storage period. There was a delay in cell propagation upon re-growth with increased storage time. Furthermore, this delay increases when the storage temperature increased. Cells stored at 4°C showed a t_{lag} of 5 h, 5 h and 6 h after 4, 8 and 12 weeks of storage respectively. At room temperature, a higher increase in the t_{lag} of >8 h was observed at 4, 8 and 12 during re-growth compared to cells stored 4°C.

The drop in pH was only observed after 4 h of the fermentation time for cells stored at 4°C after 4, 8 and 12 weeks. However, when the storage temperature increased, this prolonged the time to 7h for the pH to drop. This corresponds to the delay observed in the propagation of cells at these times and temperatures and means that the propagation of cells corresponded to the production of lactic acid which in turn led to a drop in pH.

Table 6-3 μ_{max}, t_{lag}, and OD_{max} values, and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data during re-growth in MRS media of *L. plantarum* cells in **water (control)** before freeze drying, after freeze drying and over storage at 4°C and room temperature

Systems (m/v)		μ_{max} (h^{-1})	Ave (h^{-1})	SD	SEM	t_{lag} (h)	Ave	SD	SEM	OD_{max}	Ave	SD	SEM
Before freeze drying	Run 1	0.68	0.60	0.05	0.05	1	1.00	0.00	0.00	5.45	5.44	0.12	0.07
	Run 2	0.50				1				5.32			
	Run 3	0.60				1				5.56			
After freeze drying	Run 1	0.56	0.50	0.03	0.03	4	4	0	0	5.12	5.04	0.23	0.13
	Run 2	0.50				4				4.99			
	Run 3	0.46				4				5.01			
Storage at 4°C													
After 4 weeks storage	Run 1	0.48	0.48	0.00	0.00	5	5	0	0	5.40	5.13	0.02	0.01
	Run 2	0.48				5				5.67			
	Run 3	0.48				5				5.21			
After 8 weeks storage	Run 1	0.41	0.41	0.03	0.03	5	5	0	0	5.15	5.04	0.07	0.04
	Run 2	0.36				5				5.14			
	Run 3	0.46				5				5.11			
After 12 weeks storage	Run 1	0.44	0.51	0.05	0.05	6	6	0	0	5.05	5.43	0.23	0.13
	Run 2	0.60				6				5.11			
	Run 3	0.48				6				5.07			
Storage at room temperature													
After 4 weeks storage	Run 1	-	-	-	-	8	8	0	0	5.23	5.21	0.03	0.01
	Run 2	-				8				5.20			
	Run 3	-				8				5.19			
After 8 weeks storage	Run 1	-	-	-	-	8	8	0	0	5.02	5.03	0.02	0.01
	Run 2	-				8				5.00			
	Run 3	-				8				5.05			
After 12 weeks storage	Run 1	-	-	-	-	8	8	0	0	5.04	5.07	0.03	0.02
	Run 2	-				8				5.10			
	Run 3	-				8				5.06			

Propagation of cells freeze dried in **skimmed milk** upon re-growth

The results presented in Figure 6-24 and Figure 6-25 show respectively, the fermentation and pH profiles of L. plantarum in skimmed milk when re-grown at different time points over the 12 weeks of storage at 4°C. Figure 6-26 and Figure 6-27 show the same for cells stored at room temperature.

Table 6-4 presents the associated μ_{max}, t_{lag} and OD_{max} upon re-growth of the *L. plantarum*. These values were drawn and calculated from the experimental data presented in appendix D.

In contrast to the cells from the control system, freeze dried cells in skimmed milk demonstrated higher ability to retain the vitality upon re-growth following storage at both temperature conditions. There is no significant difference in the propagation curves as the growth curves are very similar and synchronized in growth patterns to each other. From the curves shown, there was no significant attenuation of propagation with increased storage time. The cells retained their growth pattern at the end of the storage time when stored at both 4°C and room temperature.

This is similar for the pH reduction profiles of freeze dried *L. plantarum* in skimmed milk, during re-growth in growth media over 12 weeks of storage at 4°C and room temperature respectively. No delays or reduced gradients were observed, as was done for the control. This means that skimmed milk was efficient in preserving the normal pH reduction ability of cells upon re-growth in the fermentation media.

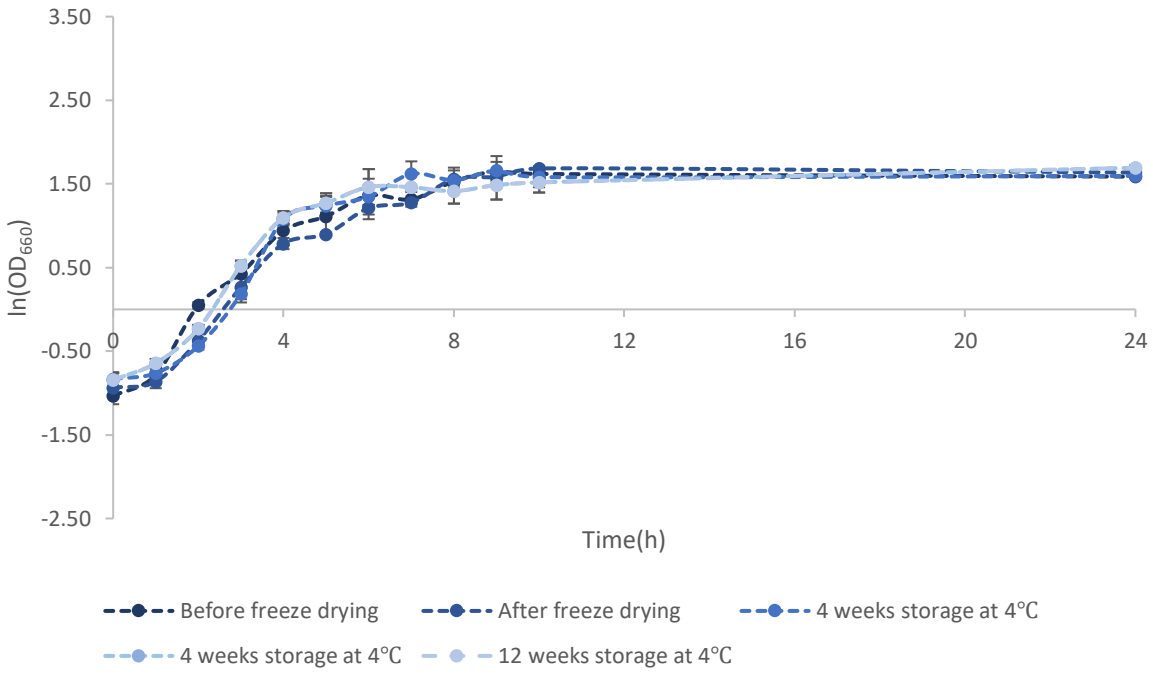

Figure 6-24 Fermentation profiles during re-growth in MRS media of *L. plantarum* cells in **skimmed milk** before freeze drying, after freeze drying and over storage at 4°C. The error bars are representative of the standard deviation between triplicate repeat runs.

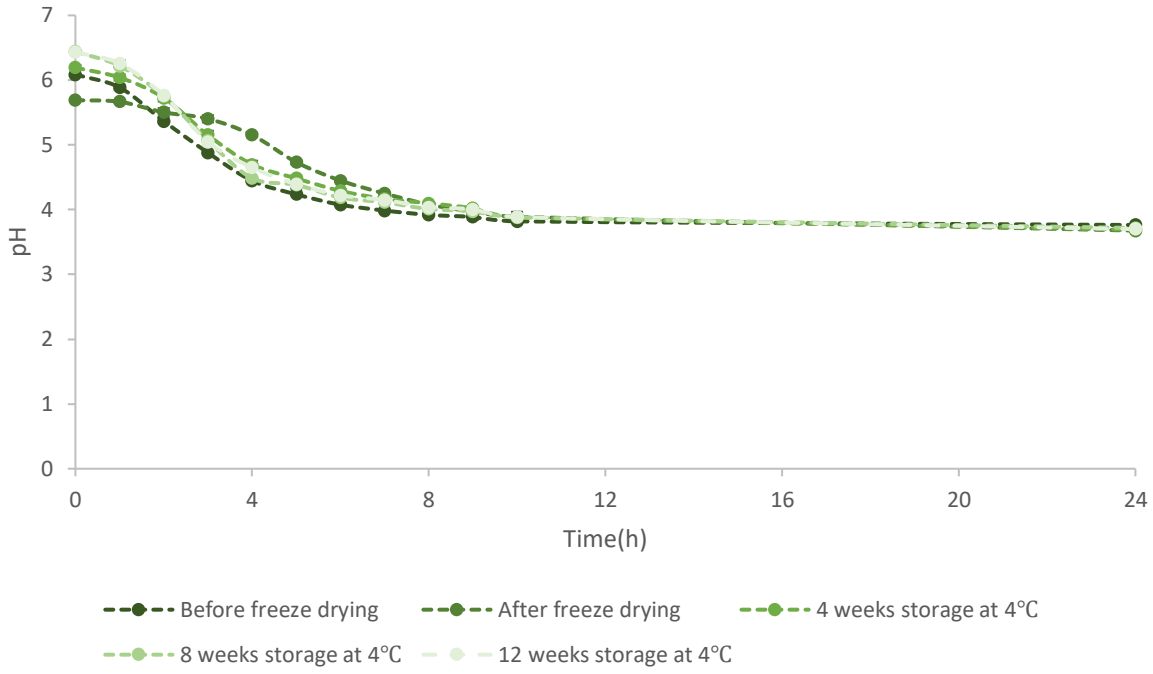

Figure 6-25 pH profiles due to *L. plantarum* lactic acid production during re-growth in MRS media of *L. plantarum* cells in **skimmed milk** before freeze drying, after freeze drying and over storage at 4°C. The error bars are representative of the standard deviation between triplicate repeat runs.

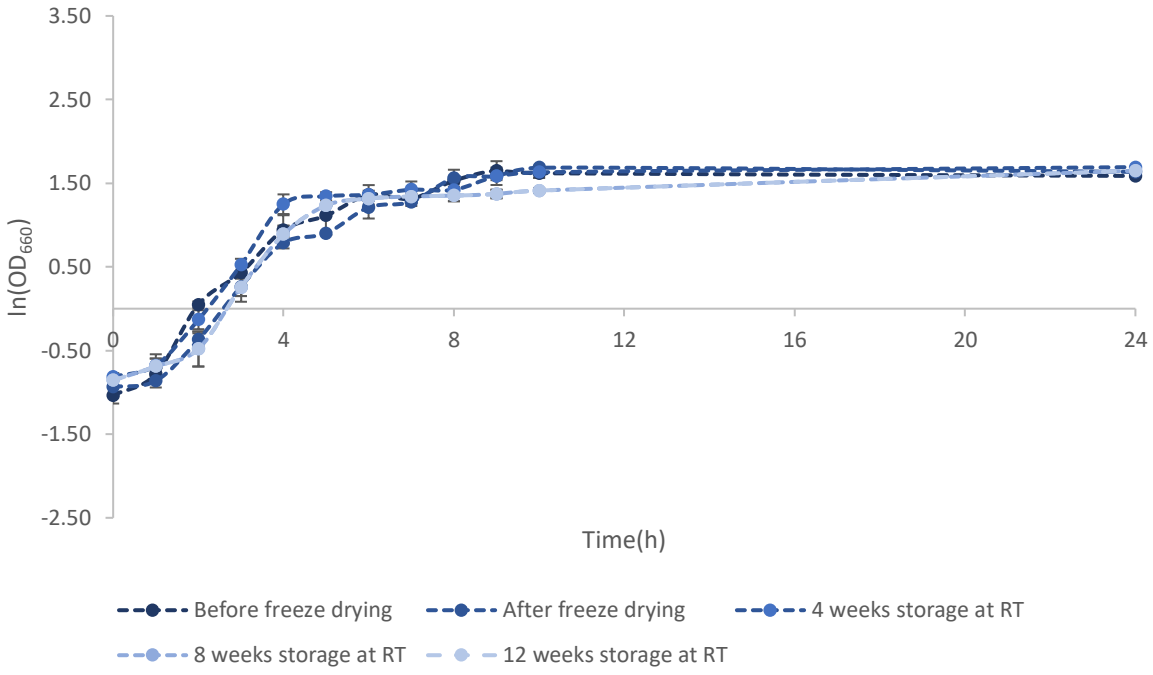

Figure 6-26 Fermentation profiles during re-growth in MRS media of *L. plantarum* cells in **skimmed milk** before freeze drying, after freeze drying and over storage at room temperature. The error bars are representative of the standard deviation between triplicate repeat runs.

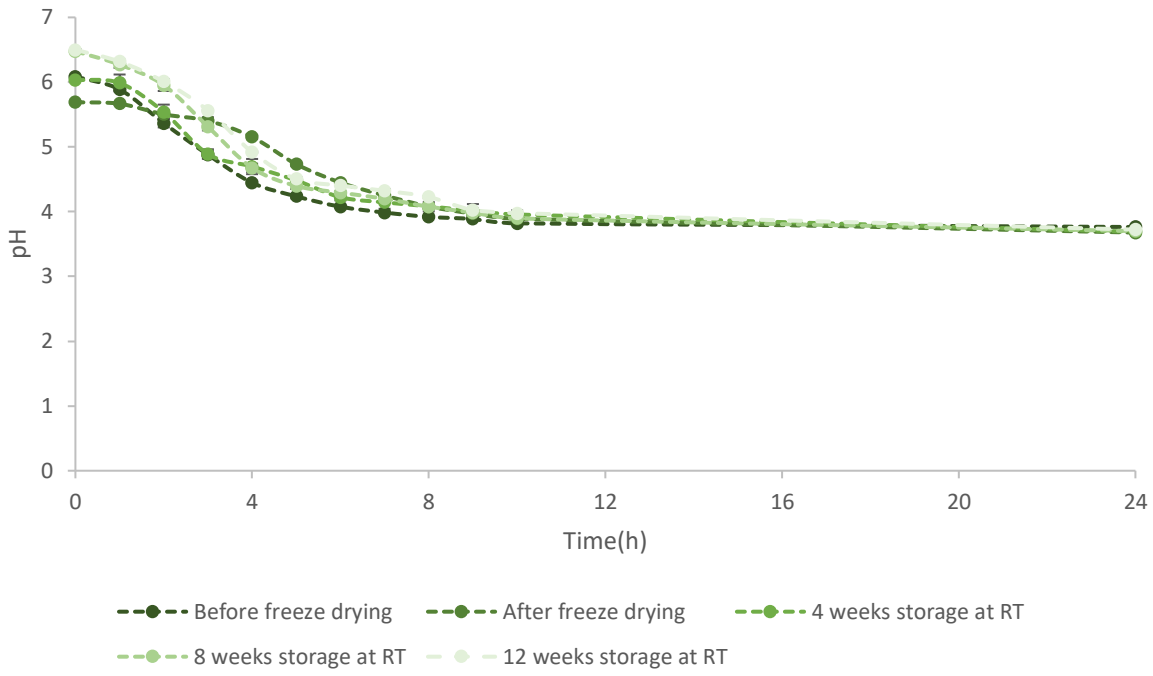

Figure 6-27 pH profiles due to *L. plantarum* lactic acid production during re-growth in MRS media of *L. plantarum* cells in **skimmed milk** before freeze drying, after freeze drying and over storage at room temperature. The error bars are representative of the standard deviation between triplicate repeat runs.

The μ_{max} of cells freeze dried in skimmed milk remained the same over storage at both storage temperatures, with a value of 0.46 ± 0.06 h^{-1}. This is except for cells stored after 12 weeks at 4ºC and 4 weeks at room temperature with values of 0.60 ± 0.00 h^{-1} and 0.56 ± 0.02 h^{-1} respectively. There was no delay in the propagation of cells upon re-growth throughout the storage period at both temperatures with t_{lag} of 1 h. The OD_{max} obtained remained roughly similar, with highest value of 5.68 ± 0.43 and lowest value of 5.20 ± 0.01 obtained after 4 weeks at 4ºC and 8 weeks at room temperature respectively.

There was no significant change or reduction in this efficiency of skimmed milk with increased storage time or temperature. Similar to the propagation of cells, a drop in pH was observed after 1 h during the fermentation time for cells stored at both temperatures over 12 weeks. The pH of the culture at the end of each fermentation remained at the same magnitude across all cell cultures with no significant difference. From these results skimmed milk demonstrated high protective efficiencies in maintaining the stability of probiotic cells.

Table 6-4 μ_{max}, t_{lag}, and OD_{max} values, and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data during re-growth in MRS media of *L. plantarum* cells in **skimmed milk** before freeze drying, after freeze drying and over storage at 4°C and room temperature.

Systems (m/v)		μ_{max} (h^{-1})	Ave (h^{-1})	SD	SEM	t_{lag} (h)	Ave	SD	SEM	OD_{max}	Ave	SD	SEM
Before freeze drying	Run 1	0.51	0.56	0.04	0.03	1	1	0	0	5.13	5.45	0.42	0.24
	Run 2	0.55				1				5.29			
	Run 3	0.60				1				5.92			
After freeze drying	Run 1	0.55	0.56	0.02	0.01	1	1	0	0	5.25	5.40	0.13	0.08
	Run 2	0.58				1				5.51			
	Run 3	0.55				1				5.43			
Storage at 4°C													
After 4 weeks storage	Run 1	0.43	0.46	0.05	0.03	1	1	0	0	5.48	5.68	0.43	0.25
	Run 2	0.44				1				5.38			
	Run 3	0.51				1				6.17			
After 8 weeks storage	Run 1	0.52	0.48	0.06	0.04	1	1	0	0	5.45	5.44	0.01	0.01
	Run 2	0.41				1				5.43			
	Run 3	0.51				1				5.44			
After 12 weeks storage	Run 1	0.60	0.60	0.00	0.00	1	1	0	0	5.45	5.44	0.01	0.01
	Run 2	0.60				1				5.43			
	Run 3	0.60				1				5.44			
Storage at room temperature													
After 4 weeks storage	Run 1	0.56	0.56	0.02	0.01	1	1	0	0	5.41	5.43	0.02	0.01
	Run 2	0.58				1				5.45			
	Run 3	0.54				1				5.44			
After 8 weeks storage	Run 1	0.50	0.46	0.05	0.03	1	1	0	0	5.21	5.20	0.01	0.00
	Run 2	0.46				1				5.20			
	Run 3	0.41				1				5.20			
After 12 weeks storage	Run 1	0.50	0.48	0.02	0.01	1	1	0	0	5.21	5.20	0.00	0.00
	Run 2	0.46				1				5.20			
	Run 3	0.46				1				5.20			

Propagation of cells freeze dried in **inulin** upon re-growth

The results presented in Figure 6-28 and Figure 6-29 show respectively, the fermentation and pH profiles of *L. plantarum* in inulin when re-grown at different time points over the 12 weeks of storage at 4°C and in Figure 6-30 and Figure 6-31 at room temperature, respectively. Table 6-5 presents the associated μ_{max}, t_{lag} and OD_{max} upon re-growth of the *L. plantarum*. These values were drawn and calculated from the experimental data presented in appendix D.

Inulin demonstrated low protective efficiencies in retaining the vitality of cells upon re-growth following storage at both temperature conditions. There is a significant deviation in the

fermentation curve of the cells during storage from that of fresh cells. This deviation increased with increased storage time. The cells did not retain their growth pattern at the end of the storage time when stored at both 4ºC and room temperature. However, all samples eventually appeared to obtain approximately the same OD.

This is similar for the pH reduction profiles of freeze dried *L. plantarum* in inulin, during re-growth in growth media over 12 weeks of storage at 4ºC and room temperature respectively. The reduction in pH upon re-growth of *L. plantarum* freeze dried in inulin, demonstrated slower pH reduction in pH profiles compared to that of the fresh cells.

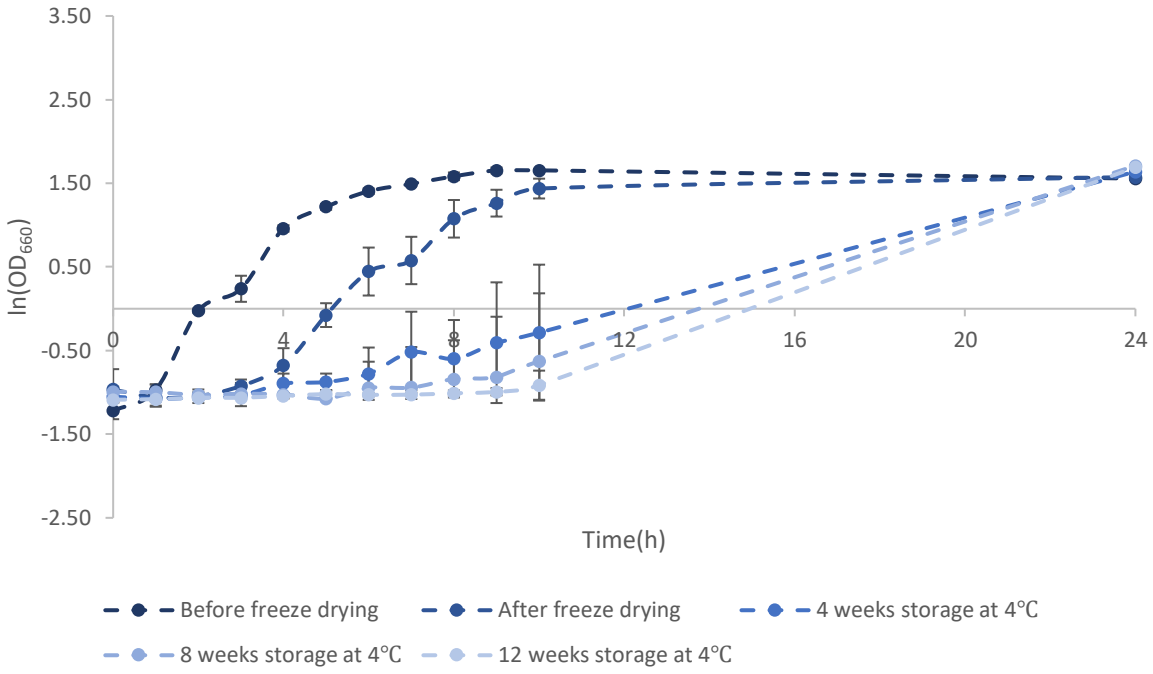

Figure 6-28 Fermentation profiles during re-growth in MRS media of *L. plantarum* cells in **inulin** before freeze drying, after freeze drying and over storage at 4ºC. The error bars are representative of the standard deviation between triplicate repeat runs.

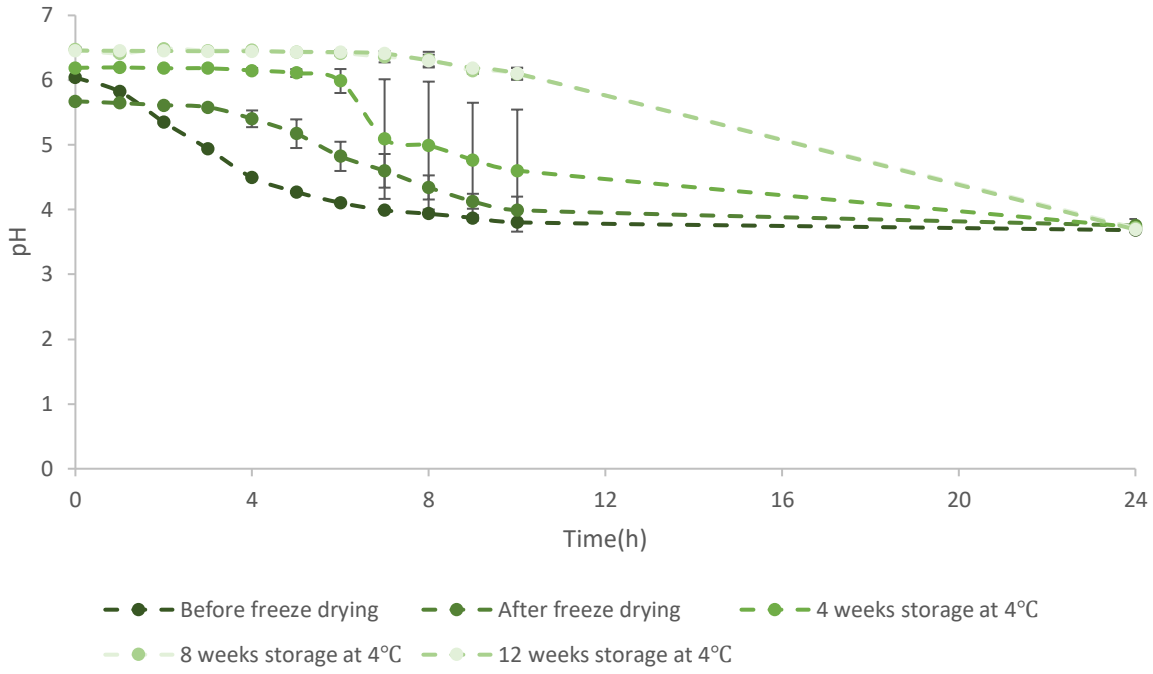

Figure 6-29 pH reduction profiles during re-growth in MRS media of *L. plantarum* cells in **inulin** before freeze drying, after freeze drying and over storage at 4°C. The error bars are representative of the standard deviation between triplicate repeat runs.

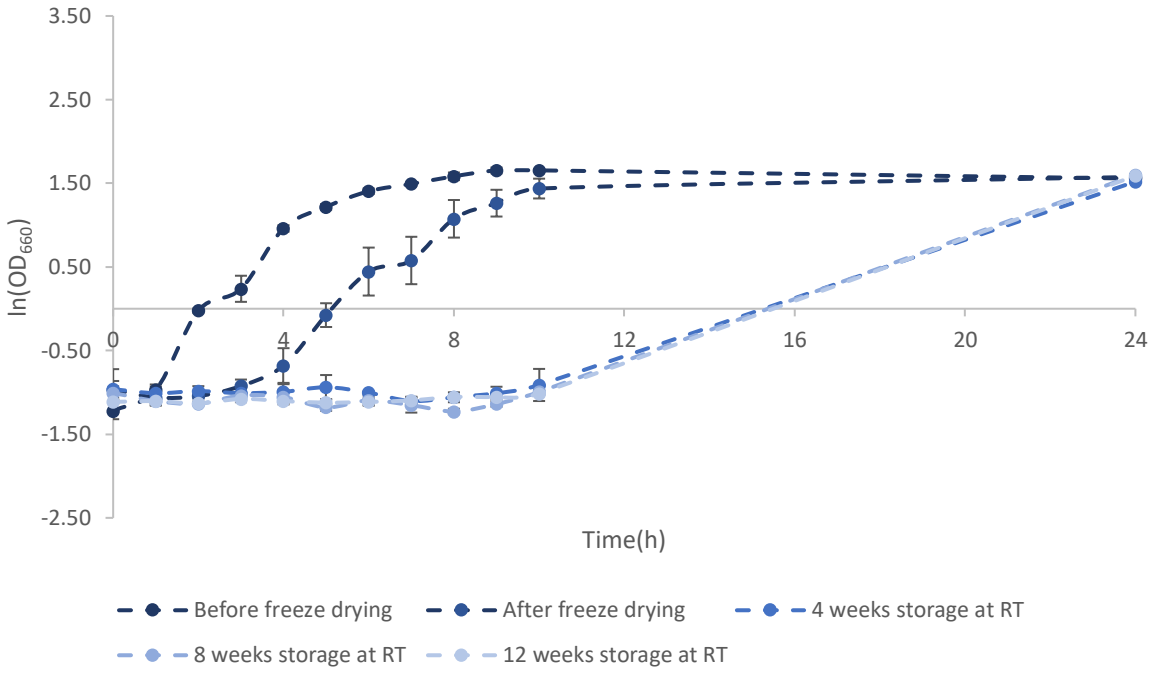

Figure 6-30 Fermentation profiles during re-growth in MRS media of *L. plantarum* cells in **inulin** before freeze drying, after freeze drying and over storage at room temperature. The error bars are representative of the standard deviation between triplicate repeat runs.

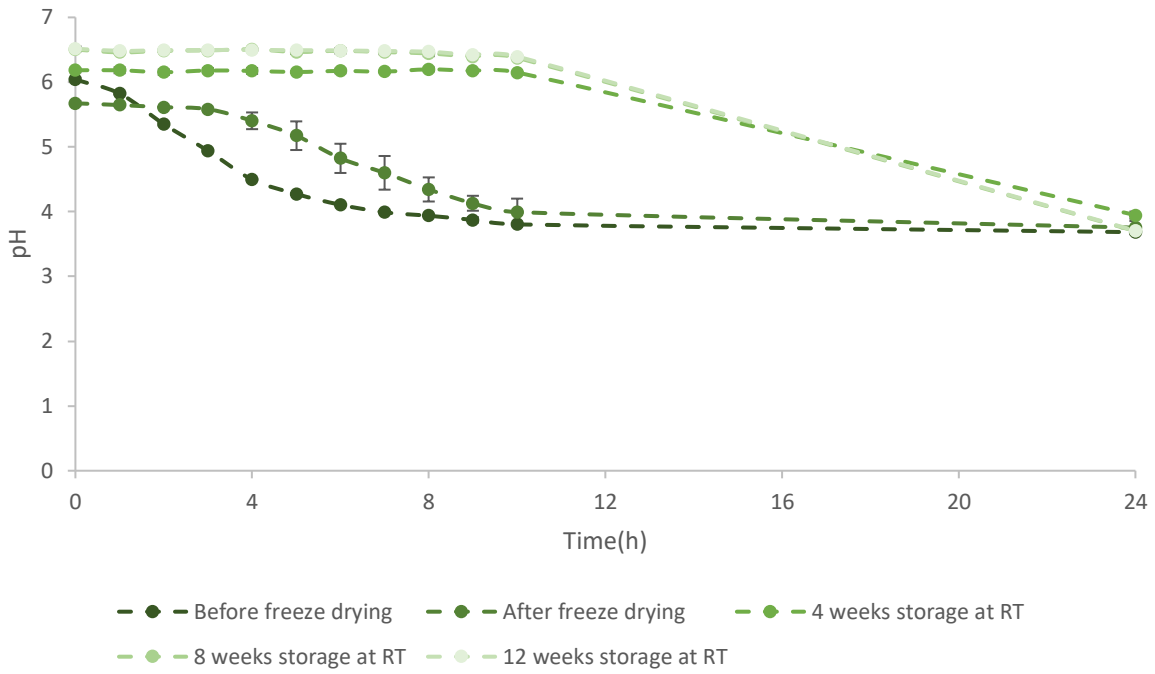

Figure 6-31 pH reduction profiles during re-growth in MRS media of *L. plantarum* cells in **inulin** before freeze drying, after freeze drying and over storage at room temperature. The error bars are representative of the standard deviation between triplicate repeat runs.

The μ_{max} values for inulin systems were not calculated due to the absence of data points in the log phase because of the prolonged lag time. There was an increase in delay of the propagation of cells upon re-growth increased as storage time increased. Furthermore, the delay increases when the storage temperature increased. Cells stored at 4° C showed a t_{lag} of 8 h, 9 h and 10 h after, 8 and 12 weeks of storage respectively. At room temperature, a higher increase in the t_{lag} of >9 h was observed at 4, 8 and 12 during re-growth compared to cells stored 4°C.

The drop in pH is seen to be gradual, signifying a delay in the production of acidic metabolite required to confer probiotic health benefits upon rehydration. An increase in storage temperature further increased this deviation. The drop in pH was only observed after 6 h of the fermentation time for cells stored at 4°C after 4 weeks. This delay in pH drop increased further beyond 5 h after 8 weeks of storage. The cells showed a delay in the pH up to 10 hours when they were stored at room temperature. This corresponds to the delay observed in the propagation of cells at these times and temperatures. This means that the propagation of cells accompanied the production of lactic acid which led to a drop in pH at the respective times.

At room temperature, there was no significant drop in the pH over 10 hours of fermentation. However, at the end of the fermentation process, the final pH had decreased. There was no significant difference in the final pH across all cultures at the end of the fermentation period.

Table 6-5 μ_{max}, t_{lag}, and OD_{max} values, and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data during re-growth in MRS media of *L. plantarum* cells in **inulin** before freeze drying, after freeze drying and over storage at 4°C and room temperature.

Systems (m/v)		μ_{max} (h^{-1})	Ave (h^{-1})	SD	SEM	t_{lag} (h)	Ave	SD	SEM	OD_{max}	Ave	SD	SEM
Before freeze drying	Run 1	0.61	0.60	0.02	0.01	1	1	0	0	5.25	5.27	0.12	0.07
	Run 2	0.59				1				5.16			
	Run 3	0.61				1				5.40			
After freeze drying	Run 1	0.40	0.48	0.07	0.04	1	1	0	0	4.89	4.84	0.07	0.04
	Run 2	0.49				1				4.76			
	Run 3	0.54				1				4.88			
Storage at 4°C													
After 4 weeks storage	Run 1	-	-	-	-	8	8	0	0	5.13	5.15	0.02	0.01
	Run 2	-	-	-	-	8				5.15			
	Run 3	-				8				5.17			
After 8 weeks storage	Run 1	-	-	-	-	9	9	0	0	5.53	5.53	0.02	0.01
	Run 2	-	-	-	-	9				5.51			
	Run 3	-				9				5.54			
After 12 weeks storage	Run 1	-	-	-	-	10	10	0	0	5.40	5.42	0.08	0.04
	Run 2	-	-	-	-	10				5.36			
	Run 3	-				10				5.51			
Storage at room temperature													
After 4 weeks storage	Run 1	-	-	-	-	10	10	0	0	4.68	4.59	0.09	0.05
	Run 2	-	-	-	-	10				4.57			
	Run 3	-				10				4.51			
After 8 weeks storage	Run 1	-	-	-	-	10	10	0	0	4.90	4.96	0.05	0.03
	Run 2	-	-	-	-	10				4.97			
	Run 3	-				10				5.00			
After 12 weeks storage	Run 1	-	-	-	-	10	10	0	0	5.40	5.42	0.08	0.04
	Run 2	-	-	-	-	10				5.36			
	Run 3	-				10				5.51			

Propagation of cells freeze dried in **maltodextrin** upon re-growth

The results presented in Figure 6-32 and Figure 6-33 show respectively, the fermentation and pH profiles of *L. plantarum* in inulin when re-grown at different time points over the 12 weeks of storage at 4°C and in Figure 6-34 and Figure 6-35 at room temperature, respectively. Table 6-6

presents the associated $\mu_{max,}$ t_{lag} and OD_{max} upon re-growth of the *L. plantarum*. These values were drawn and calculated from the experimental data presented in appendix D.

In contrast to the performance of inulin in enhancing propagation upon re-growth, maltodextrin demonstrated better performance in maintaining the stability of cells at both room temperature and 4ºC. The deviation of the growth curve of cells regrown after storage did not deviate as much as that of the control and inulin. However, an increase in storage temperature led to increased growth attenuation of the freeze dried cells. This deviation appears to increase as storage time increased. The delay in propagation of cells upon re-growth increased with storage time and with temperature. However, the extent of delay was not greater than inulin. Despite the propagation delays, all samples eventually appeared to obtain approximately the same OD.

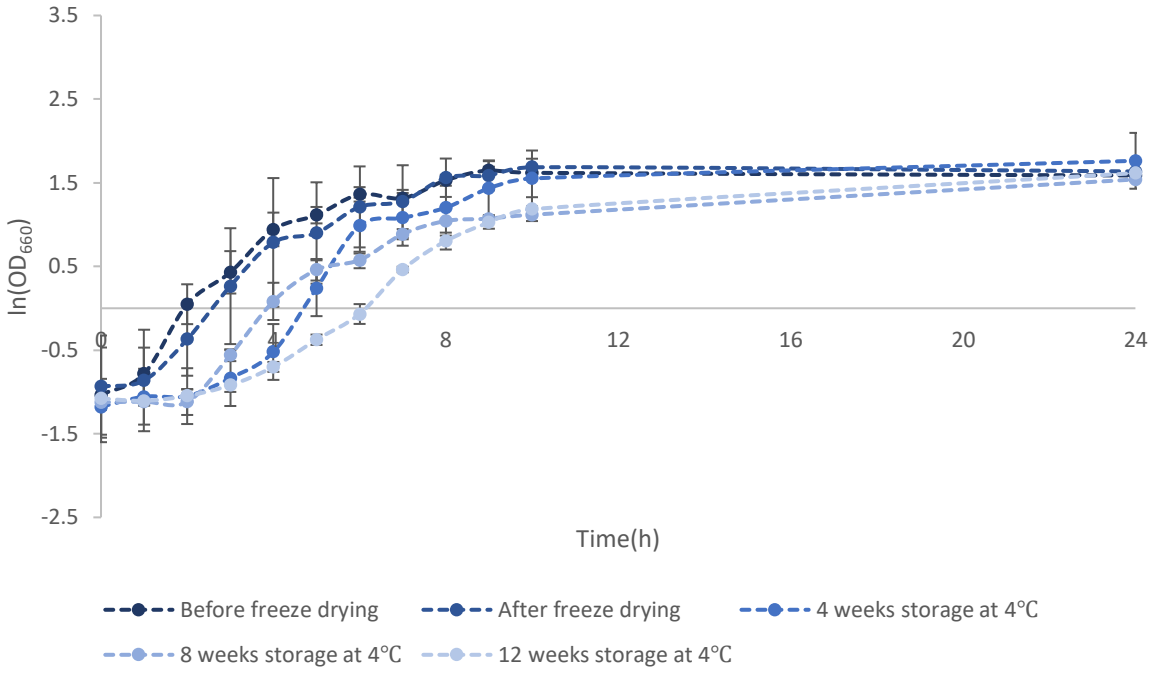

Figure 6-32 Fermentation profiles during re-growth in MRS media of *L. plantarum* cells in **maltodextrin** before freeze drying, after freeze drying and over storage at 4ºC. The error bars are representative of the standard deviation between triplicate repeat runs.

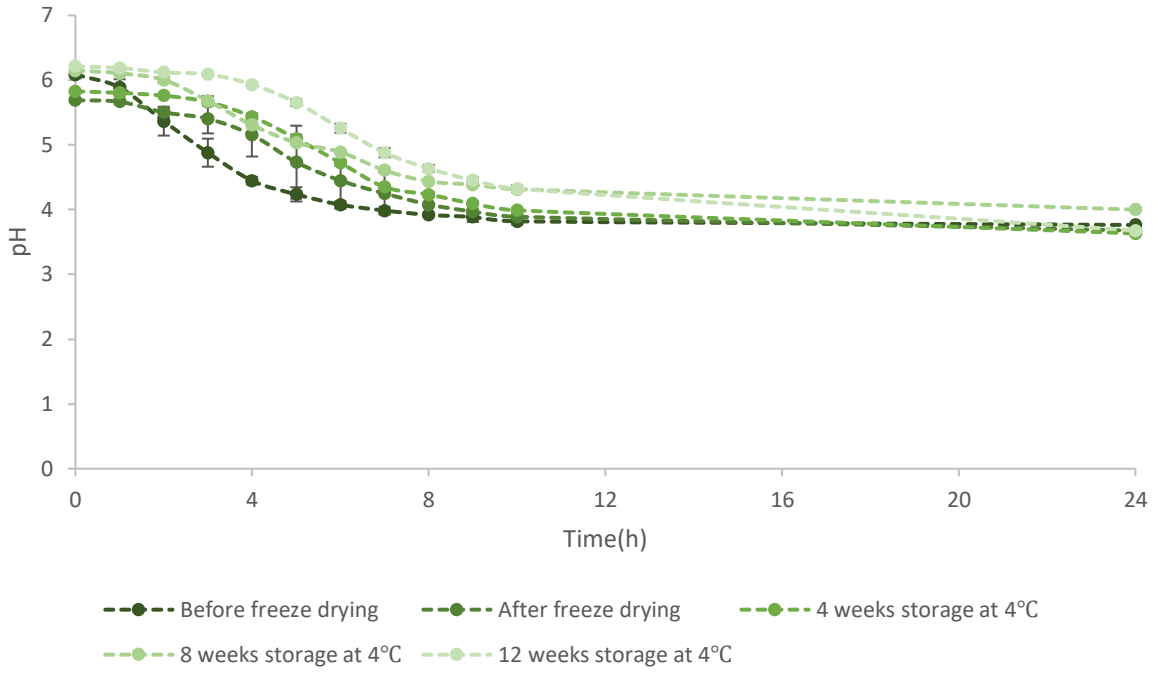

Figure 6-33 pH profiles due to *L. plantarum* lactic acid production during re-growth in MRS media of *L. plantarum* cells in **maltodextrin** before freeze drying, after freeze drying and over storage at 4°C. The error bars are representative of the standard deviation between triplicate repeat runs.

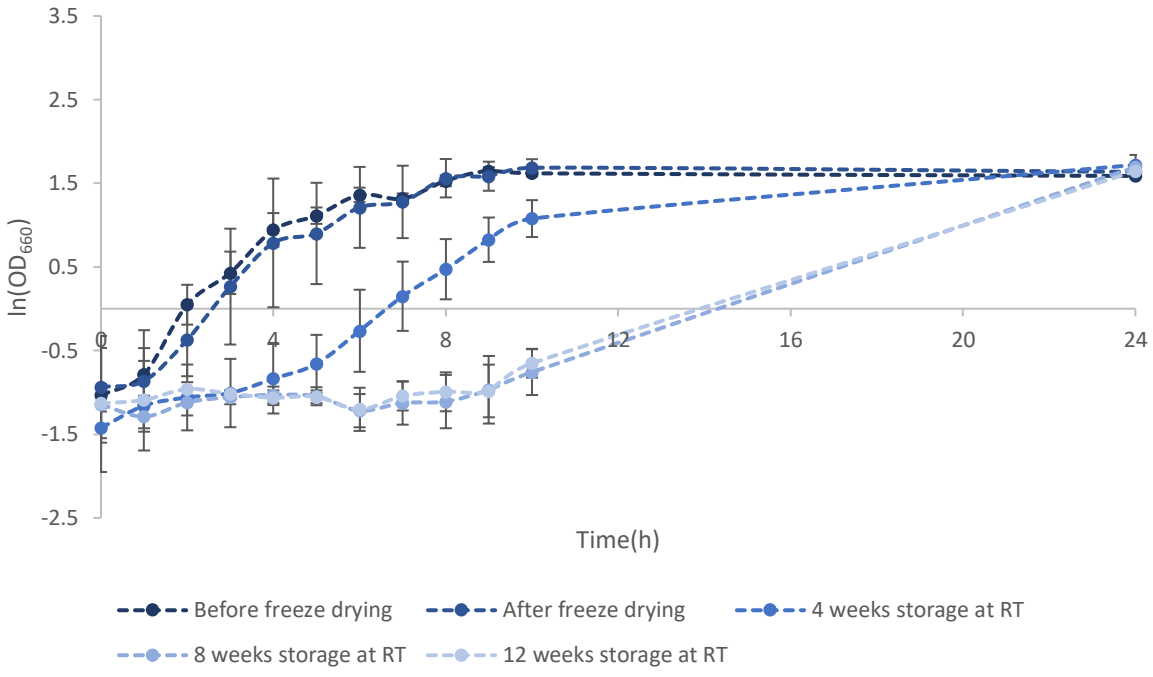

Figure 6-34 Fermentation profiles during re-growth in MRS media of *L. plantarum* cells in **maltodextrin** before freeze drying, after freeze drying and over storage at room temperature. The error bars are representative of the standard deviation between triplicate repeat runs.

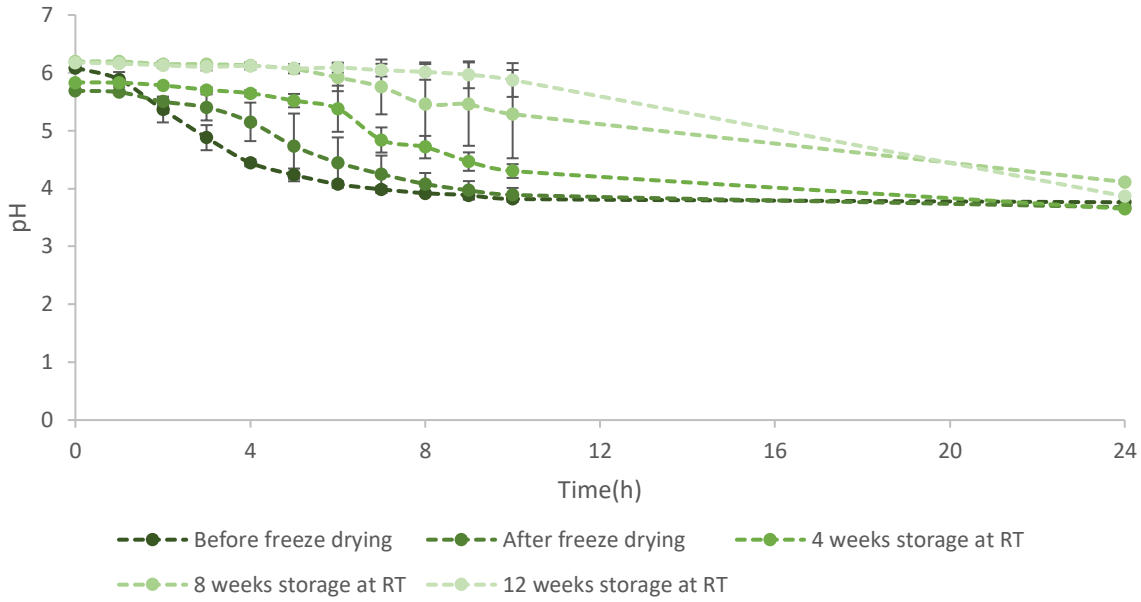

Figure 6-35 pH profiles due to *L. plantarum* lactic acid production during re-growth in MRS media of *L. plantarum* cells in **maltodextrin** before freeze drying, after freeze drying and over storage at room temperature. The error bars are representative of the standard deviation between triplicate repeat runs

The μ_{max} values over storage for maltodextrin systems were not calculated due to the absence of data points in the log phase because of the prolonged lag time when the cells were stored. This increase in delay of the propagation of cells upon re-growth did not increase with storage time when cells were stored at 4°C with a t_{lag} of 3 h, however, as storage temperature increased when cells were stored at room temperature, a higher increase in the t_{lag} of 5 h was observed after 4 weeks of storage and this increased to >9 h after 8 weeks of storage.

In contrast to the behaviour of cells freeze dried in inulin, the influence of maltodextrin in preserving the pH reduction potential of freeze dried probiotic cells at 4°C was more efficient. There was a slight deviation from the pH reduction profile of fresh cells compared to that of cells after freeze drying and over storage, with the pH dropping more slowly. This deviation increased with an increase in storage time. The re-growth of cells after storage at a higher temperature condition, showed a greater deviation for cells stored at 4°C. The drop in pH was only observed after 3 h of the fermentation time for cells stored at 4°C after 4, 8 and weeks and showed further delay in pH drop of 6 h for cells stored at room temperature after 8 weeks. There was no drop in pH up until 10 hours of fermentation time after 12 weeks of storage at room temperature. This corresponds to the delay observed in the propagation of cells at these times and temperatures. This means that the propagation of cells accompanied the production of lactic acid which led to a drop in pH.

Table 6-6 $\mu_{max,}$ $t_{lag,}$ and OD_{max} values, and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data during re-growth in MRS media of *L. plantarum* cells in **maltodextrin** before freeze drying, after freeze drying and over storage at 4°C and room temperature

Systems (m/v)	μ_{max} (h^{-1})	Ave (h^{-1})	SD	SEM	t_{lag} (h)	Ave	SD	SEM	OD_{max}	Ave	SD	SEM
Before freeze drying	Run 1	0.47	0.50	0.03	0.02	1	1	0	0	5.45	5.44	0.12	0.07
	Run 2	0.50				1				5.56			
	Run 3	0.53				1				5.32			
After freeze drying	Run 1	0.52	0.45	0.06	0.03	1	1	0	0	5.06	5.01	0.11	0.06
	Run 2	0.45				1				5.09			
	Run 3	0.40				1				4.89			
Storage at 4°C													
After 4 weeks storage	Run 1	-	-	-	-	3	3	0	0	5.93	5.83	0.17	0.10
	Run 2	-	-	-	-	3				5.63			
	Run 3	-	-	-	-	3				5.93			
After 8 weeks storage	Run 1	-	-	-	-	3	3	0	0	4.66	4.67	0.10	0.06
	Run 2	-	-	-	-	3				4.57			
	Run 3	-	-	-	-	3				4.77			
After 12 weeks storage	Run 1	-	-	-	-	3	3	0	0	5.10	5.03	0.06	0.04
	Run 2	-	-	-	-	3				4.99			
	Run 3	-	-	-	-	3				5.00			
Storage at room temperature													
After 4 weeks storage	Run 1	-	-	-	-	5	5	0	0	5.76	5.61	0.65	0.38
	Run 2	-	-	-	-	5				6.17			
	Run 3	-	-	-	-	5				4.89			
After 8 weeks storage	Run 1	-	-	-	-	9	9	0	0	5.35	5.44	0.14	0.08
	Run 2	-	-	-	-	9				5.37			
	Run 3	-	-	-	-	9				5.61			
After 12 weeks storage	Run 1	-	-	-	-	9	9	0	0	5.12	5.18	0.13	0.07
	Run 2	-	-	-	-	9				5.10			
	Run 3	-	-	-	-	9				5.33			

Propagation of cells freeze dried in **sucrose** upon re-growth

The results presented in Figure 6-36 and Figure 6-37 show respectively, the fermentation and pH profiles of *L. plantarum* in inulin when re-grown at different time points over the 12 weeks of storage at 4°C and in Figure 6-38 and Figure 6-39 at room temperature, respectively. Table 6-7 presents the associated $\mu_{max,}$ t_{lag} and OD_{max} upon re-growth of the *L. plantarum*. These values were drawn and calculated from the experimental data presented in appendix D.

The influence of sucrose on the propagation of freeze dried *L. plantarum* upon re-growth after storage was like that of maltodextrin. Over storage at 4ºC, sucrose enhanced the propagation of cells better than that of the control, as seen in the respective fermentation profiles. However, when cells were stored at room temperature, the ability of sucrose to improve the propagation decreased compared to when they were stored at 4ºC. There was a significant increase in deviation of fermentation profiles between the growth of fresh cells and cells stored at both temperature conditions. This deviation increases with increased storage time and temperature. Again, despite initial differences in the plots, all samples eventually appeared to obtain approximately the same OD.

Sucrose demonstrated a similar influence on the pH reducing potential of the probiotic cells to that of maltodextrin. A slight deviation in the profiles of cells regrown after freeze drying and storage at 4ºC can be seen in the respective profiles. However, an increase in the storage temperature led to a greater deviation from the pH reduction profiles of fresh cells, with the pH decreasing more slowly. By the end of the experiment period, the cells reduced the pH to the same value with no significant difference across all cultures.

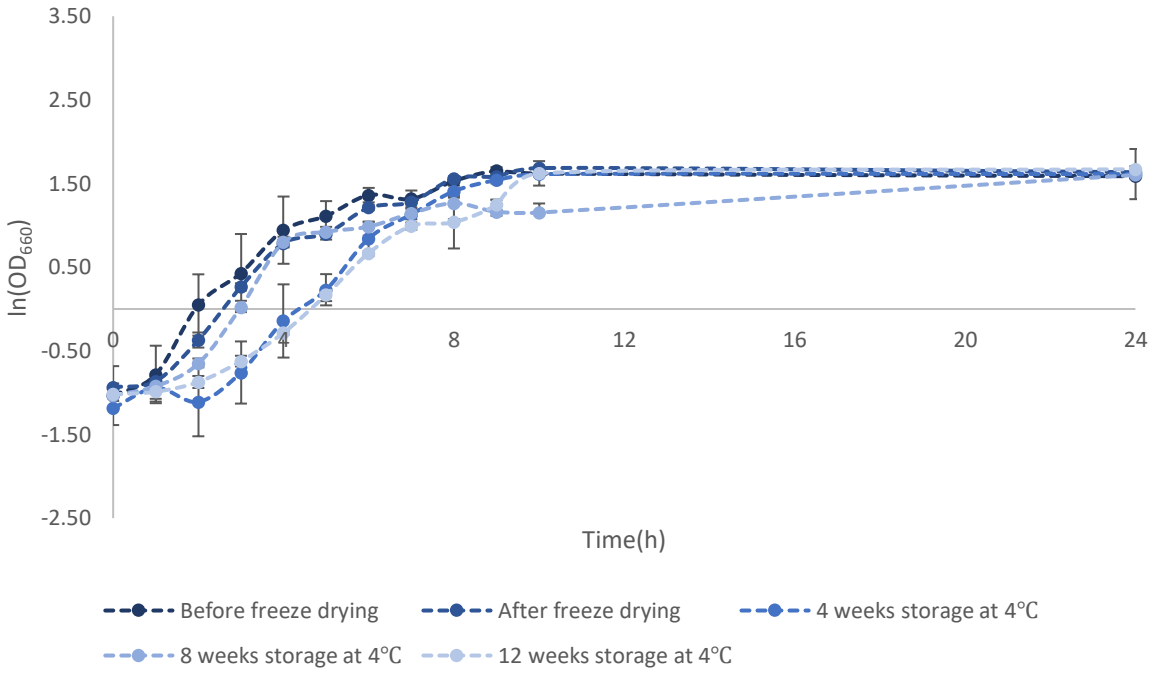

Figure 6-36 Fermentation profiles during re-growth in MRS media of *L. plantarum* cells in **sucrose** before freeze drying, after freeze drying and over storage at 4ºC. The error bars are representative of the standard deviation between triplicate repeat runs.

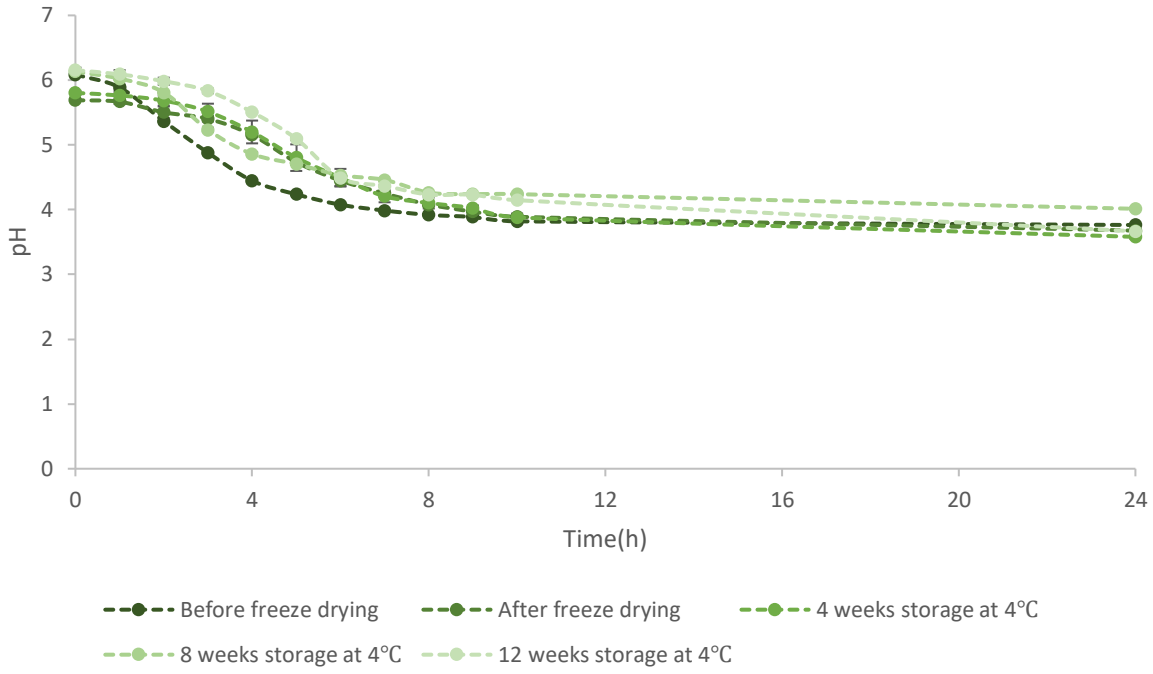

Figure 6-37 pH profiles due to *L. plantarum* lactic acid production during re-growth in MRS media of *L. plantarum* cells in **sucrose** before freeze drying, after freeze drying and over storage at 4°C. The error bars are representative of the standard deviation between triplicate repeat runs.

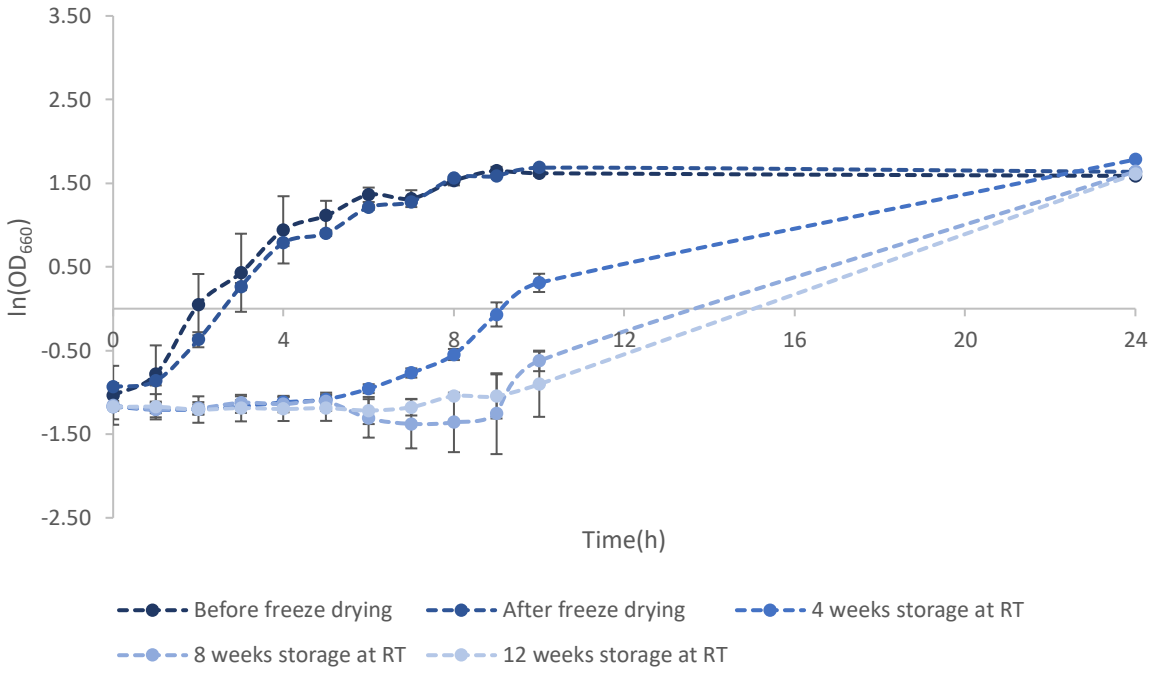

Figure 6-38 Fermentation profiles during re-growth in MRS media of *L. plantarum* cells in **sucrose** before freeze drying, after freeze drying and over storage at room temperature. The error bars are representative of the standard deviation between triplicate repeat runs.

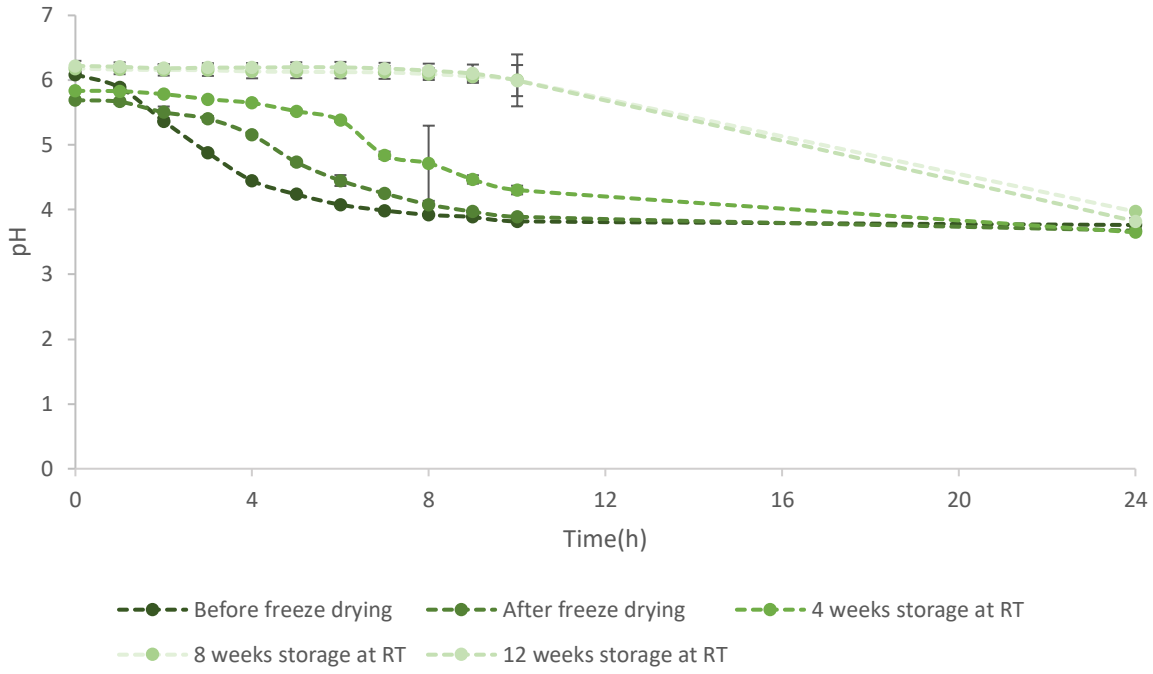

Figure 6-39 pH profiles due to *L. plantarum* lactic acid production during re-growth in MRS media of *L. plantarum* cells in **sucrose** before freeze drying, after freeze drying and over storage at room temperature. The error bars are representative of the standard deviation between triplicate repeat runs

The μ_{max} values over storage for sucrose systems were not calculated due to the absence of data points in the log phase because of the prolonged lag time when the cells were stored. This increase in delay of the propagation of cells upon re-growth did not increase with storage time when cells were stored at 4°C with a t_{lag} of 3 h, however, as storage temperature increased to room temperature, a higher increase in the t_{lag} of 8h was observed after 4 weeks of storage and this increased to 9 h after 8 weeks of storage and >8 h at the end of 12 weeks.

The influence of sucrose in preserving the pH reduction potential of freeze dried probiotic cells was similar to the behaviour of cells freeze dried in maltodextrin. The drop in pH was only observed after 3 h of the fermentation time for cells stored at 4°C after 4, 8 weeks and showed further delay in pH drop of 6 h for cells stored at room temperature after 8 weeks. There was no drop in pH up until 7 hours of fermentation time after 8 weeks of storage at room temperature. This corresponds to the delay observed in the propagation of cells at these times and temperatures. This means that the propagation of cells accompanied the production of lactic acid which led to a drop in pH.

Table 6-7 μ_{max}, t_{lag}, and OD_{max} values, and the corresponding average (Ave), standard deviation (SD) and standard error (SEM) values calculated from experimental data during re-growth in MRS media of *L. plantarum* cells in **sucrose** before freeze drying, after freeze drying and over storage at 4°C and room temperature

Systems (m/v)		μ_{max} (h^{-1})	Ave (h^{-1})	SD	SEM	t_{lag} (h)	Ave	SD	SEM	OD_{max}	Ave	SD	SEM
Before freeze drying	Run 1	0.63	0.57	0.06	0.04	1	1.00	0.00	0.00	5.20	5.35	0.16	0.09
	Run 2	0.56				1				5.33			
	Run 3	0.50				1				5.51			
After freeze drying	Run 1	0.45	0.47	0.04	0.02	1	1.00	0.00	0.00	5.25	5.24	0.06	0.03
	Run 2	0.44				1				5.18			
	Run 3	0.51				1				5.29			
Storage at 4°C													
After 4 weeks storage	Run 1	-	-	-	-	3	3.00	0.00	0.00	6.05	5.66	0.54	0.31
	Run 2	-	-	-	-	3				5.88			
	Run 3	-				3				5.04			
After 8 weeks storage	Run 1	-	-	-	-	3	3.00	0.00	0.00	4.94	4.97	0.02	0.01
	Run 2	-	-	-	-	3				4.98			
	Run 3	-				3				4.98			
After 12 weeks storage	Run 1	-	-	-	-	3	3.00	0.00	0.00	5.89	5.43	0.40	0.23
	Run 2	-	-	-	-	3				5.21			
	Run 3	-				3				5.20			
Storage at room temperature													
After 4 weeks storage	Run 1	-	-	-	-	8	8.00	0.00	0.00	6.00	5.95	0.05	0.03
	Run 2	-	-	-	-	8				5.94			
	Run 3	-				8				5.91			
After 8 weeks storage	Run 1	-	-	-	-	9	9.00	0.00	0.00	5.14	5.16	0.15	0.08
	Run 2	-	-	-	-	9				5.31			
	Run 3	-				9				5.02			
After 12 weeks storage	Run 1	-	-	-	-	9	9.00	0.00	0.00	4.99	5.01	0.02	0.01
	Run 2	-	-	-	-	9				5.02			
	Run 3	-				9				5.01			

6.5 Relating the protective efficiencies to the propagation upon re-growth

From the results obtained for the survival of probiotic cells after freeze drying with the various cryoprotectants and the propagation behaviour upon rehydration in media, it was observed that a relationship existed between the cell viability and vitality.

The lag phase in the microbial growth profile is affected by the inoculum size or low cell density. Exoenzymes and cell nutrients which are released or leaked from cell membranes when they are inoculated into fresh media, are shared, and utilized by cells in proximity. However, with high dilution caused by low cell density, these enzymes and nutrients are not easily accessed by the cells and hence results in more time spent to reach the exponential phase (Maier, 2000).

Although it was observed that across all systems and cryoprotectants, the cell density at the onset of re-growth in the monoculture remained the same, all systems demonstrated a varying delay in propagation. In explaining this, Maier (2000) noted that the enumeration of cells by turbidity does not identify the non-viable or dead cells when comparing this method to the CFU count method. This suggests that the varied delay in propagation may have been as result of differences in the number of viable cells present upon re-growth.

The relationship between the number of viable cells present at the time of inoculation, given as log (CFU/ml), and the lag time upon re-growth for freeze dried *L. plantarum* in each cryoprotectant is presented in Figure 6-40, Figure 6-41, Figure 6-42, Figure 6-43 and Figure 6-44. As the number of viable cells decreased, the delay in propagation upon re-growth increased. This signifies that the delay in the propagation of cells upon re-growth is linked to the number of viable cells present. Furthermore, as discussed in the previous section, this delay in propagation of cells correspond to the delay in the pH drop during fermentation.

Skimmed milk had the best efficiency across all other cryoprotectants in improving the survival of cells after freeze drying and over subsequent storage. Additionally, it demonstrated the highest performance in providing high vitality upon re-growth by reducing the delay in propagation greatly compared to cells without cryoprotection or in the other cryoprotectants. This can be linked to the high number of viable cells that were present upon re-growth. Furthermore, a propagation delay increased with an increase in storage temperature.

The presence of inulin provided good protection of the *L. plantarum* cells during dehydration but not over storage. This is due to the loss of its glassy matrix over storage, and thereby a loss in its ability to protect cells. The loss in the glassy state could be a result of absorbed moisture due to the high hygroscopic nature of inulin compared to the other cryoprotectants (Leyva-Porras *et al.*, 2014).

With inulin as a cryoprotectant resulting in low numbers of viable cells upon hydration, the propagation delay in cells protected by inulin was highest compared to other cryoprotectants and in the control without cryoprotectants.

These results elucidate why maintaining high numbers of viable cells present at the time the pharmabiotic is being administered is important. A low number of viable cells results in propagation delay and inhibits proliferation.

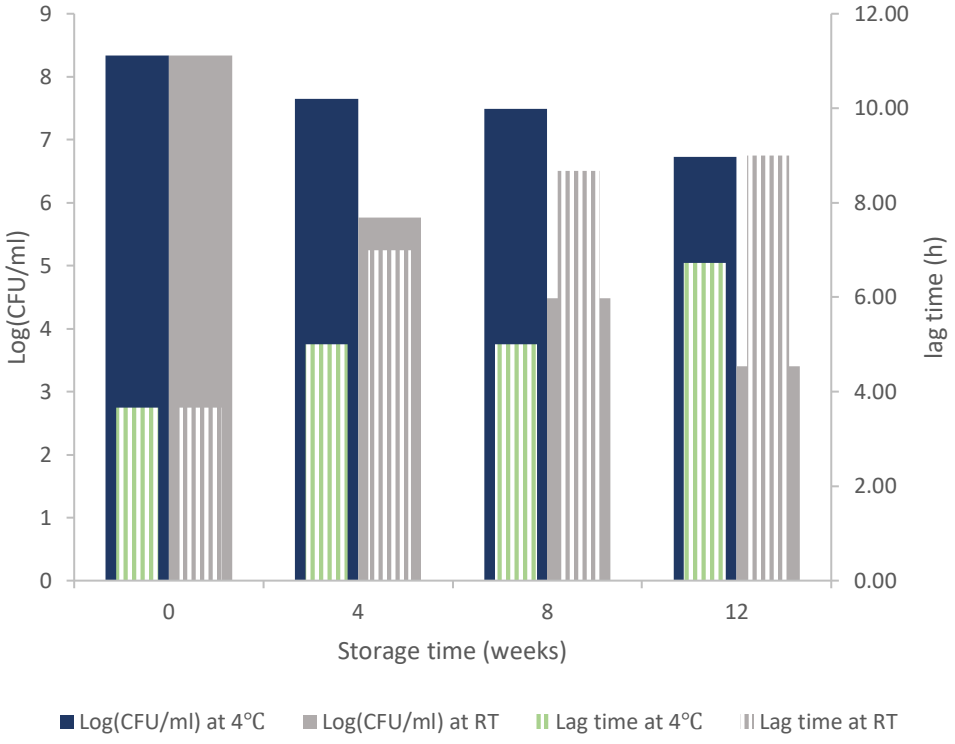

Figure 6-40 Relationship between the number of viable cells present at the onset of re-growth in MRS media and the lag time after storage at 4°C and room temperature of cells freeze dried in **water (control)**

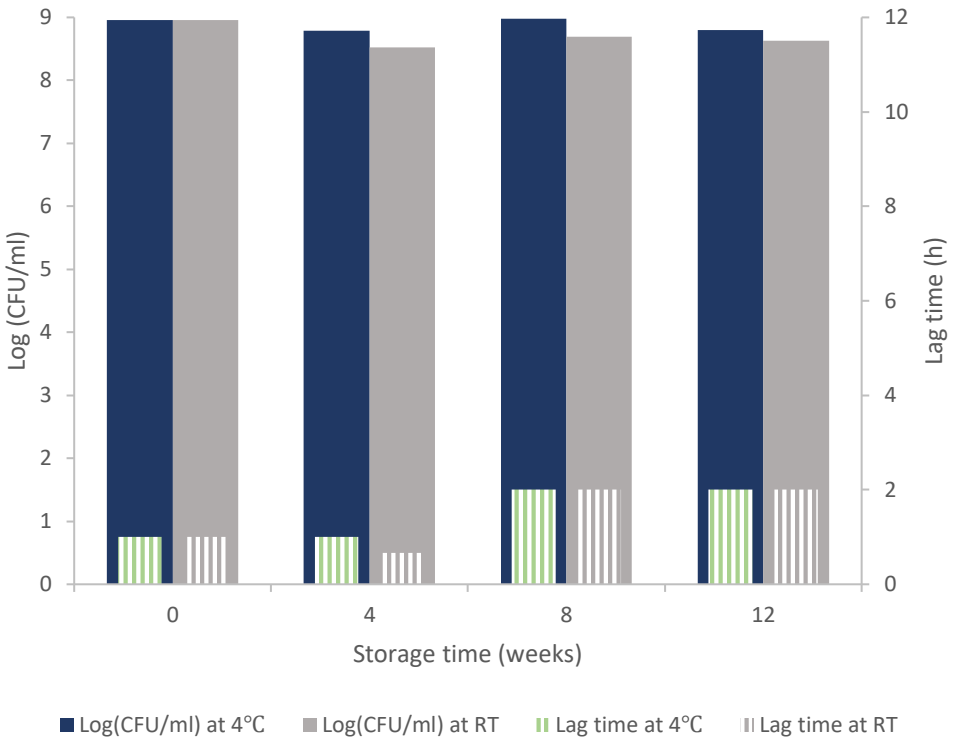

Figure 6-41 Relationship between the number of viable cells present at the onset of re-growth in MRS media and the lag time after storage at 4°C and room temperature of cells freeze dried in **skimmed milk**

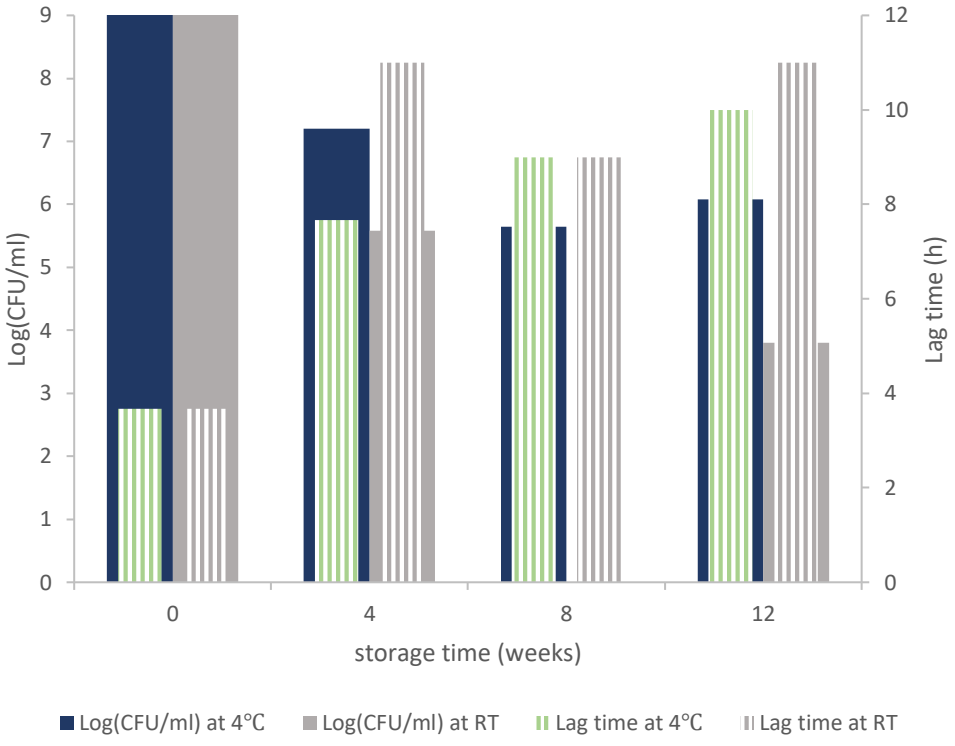

Figure 6-42 Relationship between the number of viable cells present at the onset of re-growth in MRS media and the lag time after storage at 4°C and room temperature of cells freeze dried in **inulin**

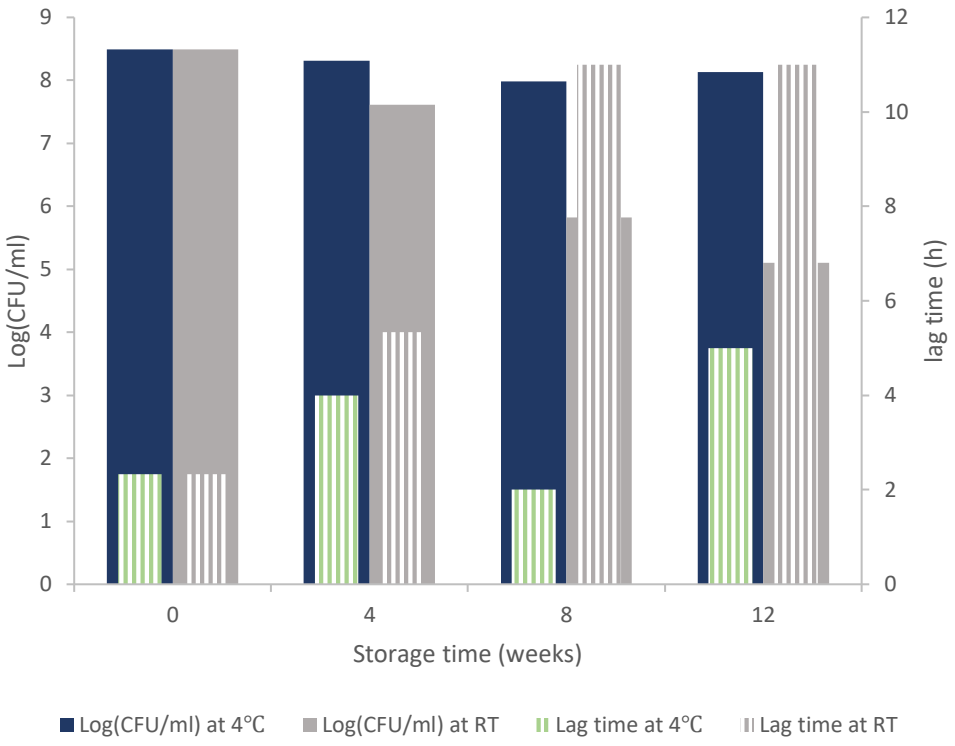

Figure 6-43 Relationship between the number of viable cells present at the onset of re-growth in MRS media and the lag time after storage at 4°C and room temperature of cells freeze dried in **maltodextrin**

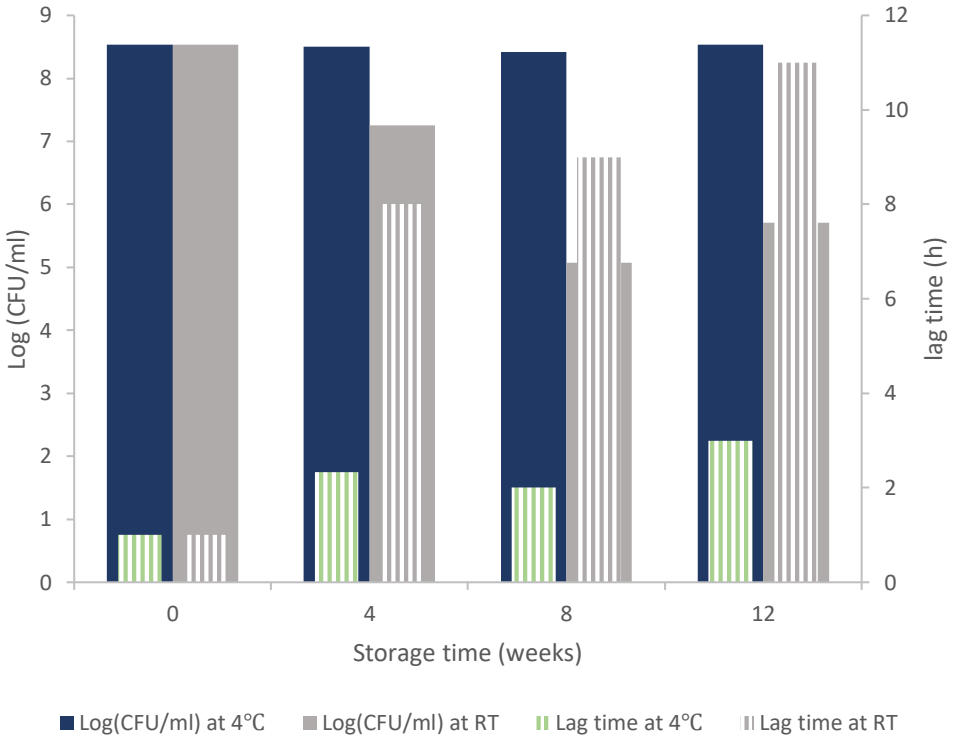

Figure 6-44 Relationship between the number of viable cells present at the onset of re-growth in MRS media and the lag time after storage at 4°C and room temperature of cells freeze dried in **sucrose**

6.6 Summary assessment of cryoprotectant candidates

The rationale behind the selection of each cryoprotectant used for this work to identify potential synbiotics-saccharide candidate(s) towards the development of probiotics for potential vaginal and skin applications are now summarised. Each candidate is discussed in turn, addressing aspects of why it was chosen for investigation in this study and noting important findings. A qualitative comparison of the cryoprotectants' properties and relative performance is presented in Table 6-8.

Skimmed milk

It appears that skimmed milk is an excellent choice of cryoprotectant based on its performance against other cryoprotectants to improve the survival of cells during the drying process and over storage. High survival rate of 91% was achieved after freeze drying using skimmed milk as a cryoprotectant. At the end of the 12 weeks storage period, the survival rate remained high at 91% (for cells stored at 4^{o}C) and 61% (for cells stored at room temperature). This agrees with other studies (Otero, Espeche and Nader-Macías, 2007; Reddy *et al.*, 2009). Skimmed milk provided high efficiency in retaining the vitality of on *L. plantarum* during freeze drying where $\mu_{max,}$ and t_{lag} remained unchanged with values of 0.5 h^{-1} and 1 h respectively.

Glucose

It is advised that the first option to consider in selecting a dehydration excipient should be the carbon source present in the fermentation media (Carvalho *et al.*, 2004). The reason for this is the possible elimination of a significant lag period upon rehydration for cells. The time required for cells to adjust to a new substrate is excluded in this case. Since glucose is the main carbon source in MRS, it was the first choice to consider. However, due to technological challenges around the inability of glucose to form glassy matrix during freeze drying, the protective efficiencies of glucose were not determined. However, *L. plantarum* demonstrated high substrate selectivity for glucose.

Inulin

For industrial and pharmaceutical purposes, inulin is a preferential choice as a prebiotic. This is because it can be readily available and most importantly is an indigestible fibre that remains intact through its journey in the gastrointestinal tract for stimulation by LAB within the gut flora (Leyva-Porras *et al.*, 2014). In this study inulin demonstrated good potential for its use as a prebiotic and showing no inhibiting effect on the growth of *L. plantarum*. As a cryoprotectant, it demonstrated high protective efficiency during freeze drying of 85%. However, over storage, it demonstrated

poor protective efficiencies. At the end of the 12 weeks storage, less than 1% of cells remained viable at both storage temperatures. This low protective capacity of inulin over storage is proposed to be due to its high hygroscopic nature and low dry state T_g (Franck, 2002; Saavedra-Leos *et al.*, 2014; Moayyedi *et al.*, 2018). These limit the use of inulin as a cryoprotectant.

Maltodextrin

Maltodextrin is a commonly used excipient and filler/bulk agent used to produce probiotics commercially. This is because of its physiochemical properties (i.e., low $T_{g'}$), ease of particle powder formation, its affordability and availability (Saavedra-Leos *et al.*, 2015). For these reasons, maltodextrin was considered a good choice. In this study, maltodextrin demonstrated good prebiotic potential and moderately protected cells during freeze drying with a survival rate of 31%. However, low survival rates of 13% and less than 1% were achieved at the end of the 12-week storage period with prolonged delay in propagation for up to 9 hours. Maltodextrin did not demonstrate to be a good contender in improving the stability of *L. plantarum* over storage. However, further strategies such as microencapsulation of cells can be explored to improve the stability of cells over storage.

Sucrose

Sucrose was added at the end of the experiments to substitute glucose, due to experimental issues experienced. Previous studies show that that sucrose has good protective efficiency on *Lactobacillus* (Reddy *et al.*, 2009). Furthermore, it is a readily available and affordable carbohydrate, therefore it was considered a potential candidate for this study. In this study, sucrose demonstrated prebiotic potential with no inhibiting effect on *L. plantarum*. Furthermore, it demonstrated moderate protective efficiency with viabilities of 33 % which remained the same with a t_{lag} of 3h at the end of 12 weeks storage at 4^{o}C. However, sucrose demonstrated low protective efficiency on the vitality of *L. plantarum* at room temperature resulting to a delay in propagation of up to 9 h when stored at room temperature.

Overall, and putting all these factors into consideration, in recommending a suitable candidate that could play double role as a prebiotic and a cryoprotectant, sucrose will be a good choice following skimmed milk. However, further strategies such as cold shock exposure prior to freeze drying, to improve survival during freeze drying and microencapsulation to improve the survival of storage can be explored to strengthen the protective efficiency of sucrose and its choice as a suitable probiotic cryoprotectant.

Table 6-8 Criteria for section of a cryoprotectant candidate including usage factors and relative performance

Factors		Skimmed milk	Inulin	Maltodextrin	Sucrose	Glucose
Influence on fermentability	Prebiotic potential	n/a	yes	yes	yes	yes
	Non-Inhibitory effect	n/a	no	no	no	no
Protective impact on stability during freeze drying	Viability	high	high	moderate	moderate	n/a
	Propagation	high	high	high	high	n/a
	pH reduction potential	yes	yes	yes	yes	n/a
Protective impact on stability over storage	Viability	high	low	low	moderate	n/a
	Propagation	high	low	low	moderate	n/a
	pH reduction potential	yes	yes	yes	yes	n/a
Protective impact on membrane structure		n/a	yes	n/a	n/a	n/a
Availability		high	high	high	high	high
Cost (R/kg)		[2]110	[3]440	[4]39	[5]143	[6]45

[2] Chem lab supplies

[3] Chem lab supplies

[4] Chem lab supplies

[5] Chem lab supplies

[6] Qinhuangdao Lihua starch .co China

7 CONCLUSIONS AND RECOMMENDATIONS

The aim of this study was to investigate the impact of various saccharides on the growth and functionality of *L. plantarum* (as a model strain) and their potential to enhance the stability of its concentrates after dehydration and over shelf life. The goal was to identify a cryoprotectant candidate(s) that could play a double role to protect cells and act as a prebiotic to stimulate proliferation upon topical probiotic administration.

The selected microorganism proved to be compatible with glucose, inulin, maltodextrin, and sucrose investigated in this study. None of the saccharides investigated demonstrated significant inhibiting effects by supplementing 0.5%, 2%, and 4% (m/v) of the saccharides in MRS media. All the saccharides demonstrated stimulation of *Lactobacillus* growth and the simultaneous reduction of pH by the production of lactic acid by supplementing 0.5%, 2%, and 4% (m/v) of the saccharides in glucose-free-MRS media. Thus, all the investigated options showed prebiotic potential. However, this conclusion is only limited to *in vitro* testing, and the prebiotic suitability of these saccharides for *Lactobacillus* on human tissues and microbiome is still to be confirmed.

The incorporation of 10% (m/v) skimmed milk, inulin, maltodextrin, and sucrose demonstrated a significant role in protecting *Lactobacillus* during freeze drying. The type and extent of protection was dependent on the cryoprotectant used.

SEM and TEM images revealed that the native structure of cell membranes was retained after freeze drying with the cryoprotectants compared to cells without cryoprotective protection which showed deformities in the cell membrane structure. This suggested that the cryoprotectants would provide both protection during freeze drying and stability over storage to probiotic cells.

Skimmed milk demonstrated the highest protection after freeze drying, with a survival rate of 91% and viable cell counts of 9.1×10^8 ($\frac{\text{CFU}}{\text{ml}}$) from an initial cell count prior to drying of 1.0×10^9 ($\frac{\text{CFU}}{\text{ml}}$). Inulin demonstrated high protective efficiency, with 85% viability maintained during freeze drying which resulted in final cell counts of 1.1×10^9 ($\frac{\text{CFU}}{\text{ml}}$) from an initial cell count of 1.3×10^9($\frac{\text{CFU}}{\text{ml}}$). However, inulin provided the least protection over the 12 week storage period compared to cells dried in the presence of maltodextrin, sucrose, and skimmed milk, with cell counts of only 1.2×10^6 ($\frac{\text{CFU}}{\text{ml}}$) at 4°C and 6.3×10^3 ($\frac{\text{CFU}}{\text{ml}}$) at room temperature recorded at the end of the period. Following skimmed milk, sucrose performed second best in maintaining the stability

of cells at 4ºC storage at the end of the 12 weeks with a survival rate of 33% which resulted in final cell counts of 3.4×10^8 ($\frac{CFU}{ml}$) from an initial cell count of 1.0×10^9 ($\frac{CFU}{ml}$).

The results of the shelf-life experiments confirmed that the *Lactobacillus* demonstrated improved stability over 3 months with the inclusion of cryoprotectants. This stability was better under refrigeration conditions compared to storage at room temperature. The degree of stability also varied amongst the various cryoprotectants. Skimmed milk provided the highest stability over storage across all cryoprotectants investigated.

Furthermore, the inclusion of cryoprotectants was found to play a significant role in the propagation of cells upon *in vitro* re-growth of the freeze dried cells. Importantly, the cryoprotectants enhanced the propagation of cells upon re-growth by reducing the time delay in proliferation. This finding insinuates that the presence of cryoprotectants in the pharmabiotic formulation has the potential to enhance the efficacy of the biotherapeutic treatment upon administration.

This study showed that the drying process does not significantly affect the ability of *L. plantarum* to restore acidity at the end of fermentation. However, the rate of the drop in pH at the early stages of fermentation was often reduced after freeze drying.

The following recommendations are proposed for further investigation:

Methods such as pre-stressing cells before freeze drying to improve the survival of *Lactobacillus* during freeze drying in the presence of maltodextrin and sucrose should be explored.

The use of inulin can be further investigated for its ability to protect probiotic cells over storage period by providing extra protection through micro-encapsulation. This would prevent the moisture absorption by the hygroscopic inulin and is therefore expected to improve the freeze dried product stability over storage.

Although findings from this study show that skimmed milk demonstrated significantly higher protective efficiencies throughout processing and over storage in terms compared to the other cryoprotectants which makes it a highly rated candidate, excipient without animal origin is generally preferred for development of pharmabiotics when considering persons with allergies to dairy or vegetarians. However, this may not necessarily be a limitation, in recommending a solution, the development of pharmabiotics separately, for patients with specific needs using an alternative cryoprotectant of non-animal origin such as sucrose can be proposed. However, a downfall to this may be the increase in cost by better performing cryoprotectants of non-animal origin, e.g., trehalose or the increase in cost in optimizing protective performance of the other

substituted cryoprotectants. Furthermore, the assessment of skimmed milk as a prebiotic should be considered as this was not established in this study due to unsuccessful quantification of the growth of *Lactobacillus* by OD measurement method because of coagulation in skimmed milk during fermentation.

The prebiotic potential of cryoprotectants can be further investigated via *in vivo* testing to confirm the prebiotic potential of these substrates and how they perform in a mixed microbial environment on human tissues.

The effect of the presence of cryoprotectant in reducing the propagation delay on epithelial tissues can be further investigated.

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APPENDICES

The following select results are presented in this thesis appendix, with the full results files available at: https://doi.org/10.25375/uct.15089883 or https://figshare.com/s/78f3508bf53032517424

Appendix A: Inhibiting Tests

A. 1 Data on inhibiting tests for glucose on the growth of *L.plantarum*

		0.5% (m/v) glucose															
		OD										pH					
time (h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
09:12:00	0	0.10	-2.35	0.25	-1.41	0.25	-1.38	0.09	0.05	0.20	-1.71	5.94	5.93	5.93	0.01	0.00	5.93
10:12:00	1	0.13	-2.02	0.30	-1.19	0.16	-1.86	0.09	0.05	0.20	-1.69	5.86	5.60	5.81	0.14	0.08	5.76
11:12:00	2	0.27	-1.30	0.53	-0.63	0.34	-1.08	0.14	0.08	0.38	-1.00	5.68	5.31	5.62	0.20	0.11	5.54
12:11:00	3	0.59	-0.53	1.11	0.11	0.66	-0.42	0.29	0.17	0.79	-0.28	5.30	4.89	5.21	0.22	0.12	5.13
13:18:00	4	1.17	0.16	2.05	0.72	1.35	0.30	0.46	0.27	1.52	0.39	4.81	4.48	4.70	0.17	0.10	4.66
14:13:00	5	2.39	0.87	3.03	1.11	2.52	0.92	0.34	0.20	2.65	0.97	4.44	4.26	4.40	0.09	0.05	4.37
15:15:00	6	3.07	1.12	3.29	1.19	3.34	1.21	0.14	0.08	3.23	1.17	4.25	4.14	4.22	0.06	0.03	4.20
16:12:00	7	3.54	1.26	3.69	1.31	3.52	1.26	0.09	0.05	3.58	1.28	4.10	4.02	4.04	0.04	0.02	4.05
18:56:00	8	4.29	1.46	4.28	1.45	4.20	1.44	0.05	0.03	4.26	1.45	3.94	3.90	3.92	0.02	0.01	3.92
18:13:00	9	4.66	1.54	4.70	1.55	4.68	1.54	0.02	0.01	4.68	1.54	3.89	3.86	3.89	0.02	0.01	3.88
12:00:00	10	5.48	1.70	5.49	1.70	5.53	1.71	0.03	0.02	5.50	1.70	3.75	3.75	3.75	0.00	0.00	3.75
09:12:00	24	5.49	1.70	5.51	1.71	5.50	1.70	0.01	0.01	5.50	1.70	3.68	3.69	3.67	0.01	0.01	3.68
		2 % (m/v) glucose															
		OD										pH					
time (h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
09:12:00	0	0.18	-1.74	0.14	-1.94	0.12	-2.09	0.03	0.01	0.15	-1.93	5.64	5.63	5.64	0.01	0.00	5.64
10:12:00	1	0.20	-1.61	0.18	-1.70	0.16	-1.83	0.02	0.01	0.18	-1.72	5.59	5.56	5.06	0.30	0.17	5.40
11:12:00	2	0.32	-1.14	0.31	-1.18	0.30	-1.20	0.01	0.01	0.31	-1.18	5.42	5.41	5.41	0.01	0.00	5.41
12:11:00	3	0.65	-0.43	0.62	-0.48	0.62	-0.48	0.02	0.01	0.63	-0.47	5.09	5.10	5.08	0.01	0.01	5.09
13:18:00	4	1.20	0.18	1.19	0.17	1.19	0.17	0.01	0.00	1.19	0.18	4.69	4.67	4.69	0.01	0.01	4.68
14:13:00	5	2.17	0.77	2.12	0.75	2.22	0.80	0.05	0.03	2.17	0.77	4.39	4.40	4.39	0.01	0.00	4.39
15:15:00	6	3.40	1.22	3.24	1.18	3.13	1.14	0.14	0.08	3.26	1.18	4.07	4.09	4.07	0.01	0.01	4.08
16:12:00	7	3.80	1.34	3.78	1.33	3.80	1.34	0.01	0.01	3.79	1.33	3.95	3.96	3.95	0.01	0.00	3.95
18:56:00	8	5.05	1.62	4.34	1.47	4.26	1.45	0.43	0.25	4.55	1.51	3.91	3.90	3.91	0.01	0.00	3.91
18:13:00	9	5.73	1.75	5.85	1.77	5.89	1.77	0.08	0.05	5.82	1.76	3.86	3.87	3.88	0.01	0.01	3.87
12:00:00	10	5.67	1.74	5.78	1.75	5.80	1.76	0.07	0.04	5.75	1.75	3.80	3.79	3.79	0.01	0.00	3.79
09:12:00	24	5.59	1.72	5.60	1.72	5.60	1.72	0.01	0.00	5.60	1.72	3.66	3.67	3.67	0.01	0.00	3.67

		4 % (m/v) glucose															
		OD										pH					
time (h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
09:12:00	0	0.09	-2.36	0.14	-1.96	0.16	-1.85	0.03	0.02	0.13	-2.06	5.32	5.30	5.32	0.01	0.01	5.31
10:12:00	1	0.12	-2.10	0.16	-1.85	0.18	-1.70	0.03	0.02	0.15	-1.88	5.26	5.25	5.25	0.01	0.00	5.25
11:12:00	2	0.21	-1.55	0.26	-1.34	0.27	-1.29	0.03	0.02	0.25	-1.40	5.16	5.15	5.14	0.01	0.01	5.15
12:11:00	3	0.44	-0.83	0.46	-0.79	0.48	-0.73	0.02	0.01	0.46	-0.78	4.94	4.94	4.95	0.01	0.00	4.94
13:18:00	4	0.91	-0.09	0.88	-0.13	1.12	0.11	0.13	0.08	0.97	-0.04	4.63	4.64	4.63	0.01	0.00	4.63
14:13:00	5	1.48	0.39	1.44	0.36	1.41	0.34	0.04	0.02	1.44	0.37	4.40	4.38	4.39	0.01	0.01	4.39
15:15:00	6	2.57	0.94	2.51	0.92	2.47	0.90	0.05	0.03	2.52	0.92	4.06	4.07	4.06	0.01	0.00	4.06
16:12:00	7	3.19	1.16	2.97	1.09	3.12	1.14	0.11	0.06	3.09	1.13	3.96	3.96	3.94	0.01	0.01	3.95
18:56:00	8	4.04	1.40	3.01	1.10	3.21	1.17	0.55	0.32	3.42	1.22	3.91	3.91	3.90	0.01	0.00	3.91
18:13:00	9	4.39	1.48	4.39	1.48	4.38	1.48	0.01	0.00	4.39	1.48	3.86	3.84	3.84	0.01	0.01	3.85
12:00:00	10	4.44	1.49	4.46	1.50	4.47	1.50	0.02	0.01	4.46	1.49	3.78	3.77	3.76	0.01	0.01	3.77
09:12:00	24																
		control (standard MRS media with 2% (m/v) glucose)															
		OD										pH					
time (h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
09:12:00	0	0.28	-1.28	0.16	-1.85	0.17	-1.80	0.07	0.04	0.20	-1.64	6.04	6.02	6.01	0.02	0.01	6.02
10:12:00	1	0.17	-1.75	0.16	-1.81	0.18	-1.74	0.01	0.00	0.17	-1.77	5.89	5.91	5.92	0.02	0.01	5.91
11:12:00	2	0.34	-1.08	0.36	-1.03	0.35	-1.05	0.01	0.01	0.35	-1.05	5.71	5.70	5.70	0.01	0.00	5.70
12:11:00	3	0.68	-0.39	0.69	-0.38	0.69	-0.37	0.01	0.00	0.68	-0.38	5.28	5.28	5.26	0.01	0.01	5.27
13:18:00	4	1.34	0.29	1.34	0.29	1.36	0.31	0.01	0.01	1.35	0.30	4.81	4.78	4.76	0.03	0.01	4.78
14:13:00	5	2.46	0.90	2.49	0.91	2.54	0.93	0.04	0.02	2.50	0.91	4.44	4.43	4.44	0.01	0.00	4.44
15:15:00	6	3.15	1.15	3.13	1.14	3.02	1.11	0.07	0.04	3.10	1.13	4.24	4.23	4.23	0.01	0.00	4.23
16:12:00	7	3.55	1.27	3.49	1.25	3.56	1.27	0.04	0.02	3.53	1.26	4.12	4.11	4.12	0.01	0.00	4.12
18:56:00	8	4.23	1.44	4.18	1.43	4.00	1.39	0.12	0.07	4.14	1.42	3.95	3.95	3.98	0.02	0.01	3.96
18:13:00	9	3.19	1.16	4.61	1.53	4.30	1.46	0.75	0.43	4.03	1.38	3.90	3.90	3.92	0.01	0.01	3.91
12:00:00	10	4.74	1.56	4.67	1.54	4.72	1.55	0.04	0.02	4.71	1.55	3.71	3.71	3.72	0.01	0.00	3.71
09:12:00	24	5.10	1.63	5.00	1.61	5.00	1.61	0.06	0.03	5.03	1.62	3.60	3.61	3.61	0.01	0.00	3.61

A. 2 Data on inhibiting tests for inulin on the growth of *L.plantarum*

		0.5% (m/v) inulin															
		OD										pH					
time (h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:00:00	0	0.16	-1.86	0.15	-1.90	0.15	-1.88	0.02	0.01	0.15	-1.88	5.82	5.84	5.83	0.01	0.01	5.83
09:00:00	1	0.16	-1.81	0.18	-1.71	0.16	-1.83	0.06	0.04	0.17	-1.79	5.80	5.80	5.81	0.01	0.00	5.80
09:58:00	2	0.27	-1.29	0.29	-1.24	0.27	-1.30	0.03	0.02	0.28	-1.28	5.62	5.65	5.66	0.02	0.01	5.64
11:00:00	3	0.52	-0.66	0.52	-0.66	0.54	-0.62	0.02	0.01	0.52	-0.65	5.37	5.40	5.38	0.02	0.01	5.38
12:00:00	4	1.19	0.17	1.19	0.17	1.24	0.22	0.02	0.01	1.21	0.19	4.91	4.91	4.89	0.01	0.01	4.90
13:00:00	5	1.75	0.56	1.68	0.52	1.76	0.57	0.03	0.01	1.73	0.55	4.61	4.64	4.62	0.02	0.01	4.62
02:00:00	6	2.69	0.99	2.68	0.99	2.67	0.98	0.00	0.00	2.68	0.99	4.35	4.34	4.34	0.01	0.00	4.34
03:02:00	7	3.35	1.21	3.38	1.22	3.23	1.17	0.02	0.01	3.32	1.20	4.14	4.15	4.13	0.01	0.01	4.14
04:00:00	8	3.66	1.30	3.70	1.31	3.76	1.32	0.01	0.01	3.71	1.31	4.04	4.04	4.06	0.01	0.01	4.05
05:00:00	9	4.07	1.40	4.22	1.44	4.14	1.42	0.02	0.01	4.14	1.42	3.96	3.96	3.95	0.01	0.00	3.96
06:00:00	10	4.32	1.46	4.44	1.49	4.32	1.46	0.02	0.01	4.36	1.47	3.72	3.73	3.72	0.01	0.00	3.72
08:04:00	24	5.29	1.67	5.37	1.68	5.18	1.64	0.02	0.01	5.28	1.66	3.63	3.61	3.62	0.01	0.01	3.62

		2 % (m/v) inulin															
		OD										pH					
time (h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:00:00	0	0.16	-1.86	0.14	-1.99	0.15	-1.91	0.07	0.04	0.15	-1.92	5.73	5.76	5.73	0.02	0.01	5.74
09:00:00	1	0.17	-1.77	0.16	-1.84	0.18	-1.73	0.06	0.03	0.17	-1.78	5.76	5.78	5.78	0.01	0.01	5.77
09:58:00	2	0.31	-1.19	0.26	-1.34	0.26	-1.36	0.09	0.05	0.28	-1.29	5.56	5.59	5.56	0.02	0.01	5.57
11:00:00	3	0.49	-0.71	0.47	-0.75	0.51	-0.68	0.04	0.02	0.49	-0.71	5.31	5.34	4.86	0.27	0.16	5.17
12:00:00	4	1.14	0.13	1.08	0.08	1.06	0.06	0.04	0.02	1.09	0.09	4.88	4.89	4.90	0.01	0.01	4.89
13:00:00	5	1.46	0.38	1.46	0.38	1.46	0.38	0.00	0.00	1.46	0.38	4.61	4.62	4.62	0.01	0.00	4.62
02:00:00	6	2.28	0.82	2.28	0.82	2.31	0.84	0.01	0.00	2.29	0.83	4.33	4.34	4.32	0.01	0.01	4.33
03:02:00	7	3.09	1.13	3.11	1.13	3.23	1.17	0.02	0.01	3.14	1.15	4.14	4.14	4.15	0.01	0.00	4.14
04:00:00	8	3.34	1.21	3.29	1.19	3.32	1.20	0.01	0.00	3.32	1.20	4.05	4.05	4.06	0.01	0.00	4.05
05:00:00	9	3.76	1.32	3.73	1.32	3.77	1.33	0.01	0.00	3.75	1.32	3.97	3.96	3.97	0.01	0.00	3.97
06:00:00	10	4.11	1.41	4.00	1.39	4.14	1.42	0.02	0.01	4.08	1.41	3.81	3.82	3.81	0.01	0.00	3.81
08:04:00	24	5.27	1.66	5.18	1.64	5.14	1.64	0.01	0.01	5.20	1.65	3.60	3.58	3.57	0.02	0.01	3.58

		4% (m/v) inulin															
		OD										pH					
time (h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:00:00	0	0.14	-1.97	0.15	-1.93	0.14	-2.00	0.03	0.02	0.14	-1.96	5.63	5.64	5.61	0.02	0.01	5.63
09:00:00	1	0.17	-1.80	0.15	-1.93	0.15	-1.92	0.08	0.04	0.15	-1.88	5.62	5.72	5.67	0.05	0.03	5.67
09:58:00	2	0.26	-1.37	0.22	-1.50	0.24	-1.44	0.07	0.04	0.24	-1.44	5.45	5.44	5.45	0.01	0.00	5.45
11:00:00	3	0.45	-0.80	0.43	-0.84	0.44	-0.83	0.02	0.01	0.44	-0.82	5.22	5.25	5.22	0.02	0.01	5.23
12:00:00	4	0.91	-0.09	0.95	-0.05	0.90	-0.11	0.03	0.02	0.92	-0.08	4.82	4.83	4.82	0.01	0.00	4.82
13:00:00	5	1.32	0.28	1.28	0.25	1.34	0.29	0.02	0.01	1.31	0.27	4.59	4.60	4.59	0.01	0.00	4.59
02:00:00	6	2.09	0.74	2.10	0.74	2.12	0.75	0.01	0.00	2.10	0.74	4.34	4.34	4.34	0.00	0.00	4.34
03:02:00	7	2.76	1.02	2.73	1.00	2.73	1.00	0.01	0.00	2.74	1.01	4.14	4.15	4.15	0.01	0.00	4.15
04:00:00	8	3.15	1.15	3.23	1.17	3.11	1.13	0.02	0.01	3.16	1.15	4.04	4.06	4.05	0.01	0.01	4.05
05:00:00	9	3.76	1.32	3.75	1.32	3.80	1.34	0.01	0.00	3.77	1.33	3.98	3.98	3.96	0.01	0.01	3.97
06:00:00	10	4.23	1.44	4.26	1.45	4.27	1.45	0.00	0.00	4.25	1.45	3.84	3.85	3.84	0.01	0.00	3.84
08:04:00	24	5.12	1.63	5.21	1.65	5.12	1.63	0.01	0.01	5.15	1.64	3.55	3.56	3.56	0.01	0.00	3.56

		control (standard MRS media with 2% (m/v) glucose)															
		OD										pH					
time (h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:00:00	0	0.23	-1.47	0.15	-1.92	0.14	-2.00	0.29	0.17	0.17	-1.80	5.76	5.88	5.90	0.08	0.04	5.85
09:00:00	1	0.16	-1.84	0.16	-1.86	0.15	-1.90	0.03	0.02	0.16	-1.86	5.71	5.84	5.85	0.08	0.05	5.80
09:58:00	2	0.26	-1.34	0.28	-1.29	0.27	-1.31	0.03	0.02	0.27	-1.32	5.68	5.68	5.68	0.00	0.00	5.68
11:00:00	3	0.53	-0.64	0.52	-0.65	0.53	-0.63	0.01	0.00	0.53	-0.64	5.42	5.41	5.40	0.01	0.01	5.41
12:00:00	4	1.10	0.10	1.11	0.10	1.12	0.11	0.01	0.01	1.11	0.10	4.91	4.89	4.89	0.01	0.01	4.90
13:00:00	5	1.63	0.49	1.64	0.49	1.66	0.51	0.01	0.01	1.64	0.50	4.65	4.65	4.61	0.02	0.01	4.64
02:00:00	6	2.63	0.97	2.60	0.96	2.66	0.98	0.01	0.01	2.63	0.97	4.37	4.34	4.34	0.02	0.01	4.35
03:02:00	7	3.20	1.16	3.28	1.19	3.23	1.17	0.01	0.01	3.24	1.17	4.16	4.14	4.15	0.01	0.01	4.15
04:00:00	8	3.50	1.25	3.56	1.27	3.57	1.27	0.01	0.01	3.54	1.27	4.06	4.04	4.03	0.02	0.01	4.04
05:00:00	9	3.76	1.32	3.75	1.32	3.80	1.34	0.01	0.00	3.77	1.33	3.98	3.98	3.96	0.01	0.01	3.97
06:00:00	10	4.00	1.39	3.99	1.38	3.98	1.38	0.00	0.00	3.99	1.38	3.89	3.88	3.88	0.01	0.00	3.88
08:04:00	24	5.26	1.66	5.16	1.64	5.20	1.65	0.01	0.01	5.21	1.65	3.68	3.65	3.65	0.02	0.01	3.66

A. 3 Data on inhibiting tests for maltodextrin on the growth of *L.plantarum*

		0.5% (m/v) maltodextrin															
		OD										pH					
time (h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:00:00	0	0.09	-2.43	0.09	-2.42	0.09	-2.45	0.02	0.01	0.09	-2.43	6.05	6.03	6.04	0.01	0.01	6.04
09:00:00	1	0.13	-2.04	0.14	-1.97	0.14	-1.98	0.04	0.02	0.14	-2.00	5.98	5.96	5.96	0.01	0.01	5.97
10:00:00	2	0.21	-1.55	0.21	-1.56	0.22	-1.51	0.02	0.01	0.21	-1.54	5.82	5.81	5.80	0.01	0.01	5.81
11:00:00	3	0.52	-0.66	0.54	-0.62	0.54	-0.62	0.02	0.01	0.53	-0.63	5.50	5.47	5.46	0.02	0.01	5.48
12:00:00	4	0.76	-0.27	0.68	-0.39	0.69	-0.37	0.06	0.03	0.71	-0.34	5.04	5.03	5.02	0.01	0.01	5.03
13:00:00	5	1.54	0.43	0.52	-0.65	1.46	0.38	0.61	0.35	1.17	0.05	4.55	4.54	4.53	0.01	0.01	4.54
14:00:00	6	2.66	0.98	2.56	0.94	2.62	0.96	0.02	0.01	2.61	0.96	4.30	4.31	4.29	0.01	0.01	4.30
15:00:00	7	3.13	1.14	3.09	1.13	3.11	1.13	0.01	0.00	3.11	1.13	4.15	4.14	4.14	0.01	0.00	4.14
16:00:00	8	3.79	1.33	3.67	1.30	3.56	1.27	0.03	0.02	3.67	1.30	4.02	4.03	4.02	0.01	0.00	4.02
17:00:00	9	4.11	1.41	4.15	1.42	4.35	1.47	0.03	0.02	4.20	1.44	3.89	3.90	3.92	0.02	0.01	3.90
18:00:00	10	4.50	1.50	4.50	1.50	4.49	1.50	0.00	0.00	4.50	1.50	3.81	3.79	3.80	0.01	0.01	3.80
08:00:00	24	5.63	1.73	5.76	1.75	5.56	1.72	0.02	0.01	5.65	1.73	3.70	3.69	3.69	0.01	0.00	3.69

		2% (m/v) maltodextrin															
		OD										pH					
time (h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:00:00	0	0.15	-1.90	0.10	-2.26	0.13	-2.05	0.18	0.11	0.13	-2.07	5.99	5.98	5.99	0.01	0.00	5.99
09:00:00	1	0.15	-1.87	0.15	-1.93	0.16	-1.85	0.04	0.02	0.15	-1.88	5.99	6.00	5.97	0.02	0.01	5.99
10:00:00	2	0.23	-1.46	0.23	-1.48	0.23	-1.46	0.01	0.01	0.23	-1.47	5.78	5.76	5.77	0.01	0.01	5.77
11:00:00	3	0.51	-0.68	0.51	-0.67	0.53	-0.63	0.03	0.02	0.52	-0.66	5.42	5.43	5.41	0.01	0.01	5.42
12:00:00	4	0.99	-0.01	0.91	-0.09	0.98	-0.02	0.05	0.03	0.96	-0.04	5.01	4.99	4.98	0.02	0.01	4.99
13:00:00	5	2.12	0.75	2.14	0.76	2.01	0.70	0.03	0.02	2.09	0.74	4.52	4.51	4.51	0.01	0.00	4.51
14:00:00	6	2.85	1.05	2.77	1.02	2.66	0.98	0.03	0.02	2.76	1.01	4.29	4.27	4.28	0.01	0.01	4.28
15:00:00	7	3.29	1.19	3.23	1.17	3.20	1.16	0.01	0.01	3.24	1.18	4.13	4.13	4.12	0.01	0.00	4.13
16:00:00	8	4.06	1.40	3.99	1.38	3.84	1.35	0.03	0.02	3.96	1.38	4.02	4.01	4.02	0.01	0.00	4.02
17:00:00	9	4.84	1.58	4.69	1.55	4.60	1.53	0.03	0.01	4.71	1.55	3.92	3.91	3.94	0.02	0.01	3.92
18:00:00	10	4.70	1.55	4.68	1.54	4.64	1.53	0.01	0.00	4.67	1.54	3.83	3.83	3.84	0.01	0.00	3.83
08:00:00	24	4.75	1.56	4.71	1.55	4.64	1.53	0.01	0.01	4.70	1.55	3.68	3.68	3.68	0.00	0.00	3.68

		4% (m/v) maltodextrin															
		OD										pH					
time (h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:00:00	0	0.09	-2.41	0.14	-1.99	0.10	-2.31	0.22	0.13	0.11	-2.24	5.89	5.88	5.88	0.01	0.00	5.88
09:00:00	1	0.12	-2.16	0.14	-2.00	0.14	-2.00	0.09	0.05	0.13	-2.06	5.81	5.80	5.82	0.01	0.01	5.81
10:00:00	2	0.30	-1.21	0.22	-1.54	0.21	-1.58	0.20	0.12	0.24	-1.45	5.68	5.67	5.69	0.01	0.01	5.68
11:00:00	3	0.44	-0.82	0.47	-0.76	0.46	-0.78	0.03	0.02	0.46	-0.78	5.32	5.31	5.33	0.01	0.01	5.32
12:00:00	4	0.72	-0.33	0.82	-0.20	0.70	-0.36	0.08	0.05	0.75	-0.29	4.93	4.92	4.94	0.01	0.01	4.93
13:00:00	5	1.57	0.45	1.58	0.46	1.53	0.43	0.02	0.01	1.56	0.44	4.49	4.48	4.50	0.01	0.01	4.49
14:00:00	6	2.37	0.86	2.32	0.84	2.29	0.83	0.02	0.01	2.33	0.84	4.27	4.25	4.26	0.01	0.01	4.26
15:00:00	7	2.87	1.05	2.91	1.07	2.81	1.03	0.02	0.01	2.86	1.05	4.11	4.11	4.14	0.02	0.01	4.12
16:00:00	8	3.29	1.19	3.17	1.15	3.29	1.19	0.02	0.01	3.25	1.18	4.02	4.00	4.01	0.01	0.01	4.01
17:00:00	9	3.28	1.19	3.33	1.20	3.49	1.25	0.03	0.02	3.37	1.21	3.84	3.93	3.92	0.05	0.03	3.90
18:00:00	10	3.79	1.33	3.80	1.34	3.80	1.34	0.00	0.00	3.80	1.33	3.76	3.79	3.80	0.02	0.01	3.78
08:00:00	24	5.25	1.66	5.34	1.68	5.59	1.72	0.03	0.02	5.39	1.68	3.67	3.67	3.67	0.00	0.00	3.67

		control (standard MRS media with 2% (m/v) glucose)															
		OD										pH					
time (h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:00:00	0	0.11	-2.22	0.10	-2.34	0.14	-1.97	0.19	0.11	0.12	-2.18	6.06	6.08	6.05	0.02	0.01	6.06
09:00:00	1	0.15	-1.92	0.14	-2.00	0.16	-1.86	0.07	0.04	0.15	-1.93	5.99	6.00	5.97	0.02	0.01	5.99
10:00:00	2	0.27	-1.30	0.25	-1.39	0.29	-1.23	0.08	0.05	0.27	-1.31	5.84	5.87	5.83	0.02	0.01	5.85
11:00:00	3	0.57	-0.56	0.55	-0.61	0.60	-0.51	0.05	0.03	0.57	-0.56	5.52	5.56	5.50	0.03	0.02	5.53
12:00:00	4	1.38	0.32	1.28	0.25	1.42	0.35	0.05	0.03	1.36	0.31	5.06	5.10	5.05	0.03	0.02	5.07
13:00:00	5	2.47	0.90	2.38	0.87	2.42	0.88	0.02	0.01	2.42	0.89	4.57	4.59	4.55	0.02	0.01	4.57
14:00:00	6	3.51	1.26	3.43	1.23	3.52	1.26	0.01	0.01	3.49	1.25	4.32	4.32	4.29	0.02	0.01	4.31
15:00:00	7	3.99	1.38	4.00	1.39	3.97	1.38	0.00	0.00	3.99	1.38	4.17	4.17	4.16	0.01	0.00	4.17
16:00:00	8	4.49	1.50	4.65	1.54	4.69	1.55	0.02	0.01	4.61	1.53	4.03	4.02	4.03	0.01	0.00	4.03
17:00:00	9	5.04	1.62	4.93	1.60	5.03	1.62	0.01	0.01	5.00	1.61	3.92	3.90	3.91	0.01	0.01	3.91
18:00:00	10	5.12	1.63	5.11	1.63	5.10	1.63	0.00	0.00	5.11	1.63	3.80	3.82	3.80	0.01	0.01	3.81
08:00:00	24	5.00	1.61	5.08	1.63	5.09	1.63	0.01	0.01	5.06	1.62	3.62	3.62	3.62	0.00	0.00	3.62

A. 4 Data on inhibiting tests for sucrose on the growth of *L.plantarum*

		0.5% (m/v) sucrose															
		OD										pH					
time (h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:00:00	0	0.15	-1.90	0.17	-1.78	0.16	-1.86	0.06	0.04	0.16	-1.84	6.09	6.16	6.20	0.06	0.03	6.15
09:00:00	1	0.19	-1.67	0.20	-1.60	0.19	-1.67	0.04	0.02	0.19	-1.65	6.15	6.11	6.15	0.02	0.01	6.14
10:00:00	2	0.34	-1.07	0.35	-1.04	0.34	-1.09	0.03	0.01	0.34	-1.07	5.94	5.90	5.94	0.02	0.01	5.93
11:00:00	3	0.68	-0.39	0.66	-0.42	0.68	-0.39	0.02	0.01	0.67	-0.40	5.57	5.59	5.52	0.04	0.02	5.56
12:00:00	4	1.38	0.32	1.34	0.29	1.28	0.25	0.04	0.02	1.33	0.29	4.99	4.99	4.99	0.00	0.00	4.99
13:00:00	5	2.10	0.74	2.18	0.78	2.24	0.81	0.03	0.02	2.17	0.78	4.67	4.63	4.61	0.03	0.02	4.64
14:00:00	6	2.99	1.10	3.00	1.10	2.99	1.10	0.00	0.00	2.99	1.10	4.40	4.36	4.35	0.03	0.02	4.37
15:00:00	7	3.38	1.22	3.44	1.24	3.70	1.31	0.05	0.03	3.51	1.25	4.20	4.19	4.21	0.01	0.01	4.20
16:00:00	8	3.98	1.38	3.73	1.32	3.96	1.38	0.04	0.02	3.89	1.36	4.11	4.10	4.09	0.01	0.01	4.10
17:00:00	9	4.28	1.45	4.20	1.44	4.32	1.46	0.01	0.01	4.27	1.45	4.00	4.00	3.98	0.01	0.01	3.99
18:00:00	10	4.47	1.50	4.63	1.53	4.58	1.52	0.02	0.01	4.56	1.52	3.95	3.95	3.94	0.01	0.00	3.95
08:00:00	24	4.94	1.60	5.15	1.64	5.17	1.64	0.03	0.01	5.09	1.63	3.67	3.67	3.65	0.01	0.01	3.66

		2% (m/v) sucrose															
		OD										pH					
time (h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:00:00	0	0.13	-2.05	0.12	-2.12	0.14	-1.94	0.09	0.05	0.13	-2.04	6.21	6.24	6.14	0.05	0.03	6.20
09:00:00	1	0.15	-1.87	0.21	-1.58	0.18	-1.69	0.15	0.08	0.18	-1.71	6.14	6.15	6.04	0.06	0.04	6.11
10:00:00	2	0.29	-1.22	0.30	-1.21	0.33	-1.11	0.06	0.04	0.31	-1.18	5.96	5.96	5.87	0.05	0.03	5.93
11:00:00	3	0.60	-0.51	0.64	-0.44	0.66	-0.42	0.04	0.03	0.63	-0.46	5.59	5.59	5.48	0.06	0.04	5.55
12:00:00	4	1.20	0.18	1.27	0.24	1.27	0.24	0.03	0.02	1.25	0.22	5.03	4.95	4.95	0.05	0.03	4.98
13:00:00	5	2.00	0.69	2.19	0.78	1.97	0.68	0.06	0.03	2.05	0.72	4.63	4.56	4.60	0.04	0.02	4.60
14:00:00	6	2.84	1.04	2.85	1.05	2.74	1.01	0.02	0.01	2.81	1.03	4.36	4.32	4.36	0.02	0.01	4.35
15:00:00	7	3.48	1.25	3.51	1.26	3.34	1.21	0.03	0.02	3.44	1.24	4.22	4.17	4.21	0.03	0.02	4.20
16:00:00	8	4.08	1.41	3.72	1.31	3.52	1.26	0.07	0.04	3.77	1.33	4.11	4.06	4.10	0.03	0.02	4.09
17:00:00	9	4.02	1.39	4.16	1.43	3.88	1.36	0.03	0.02	4.02	1.39	3.99	3.97	3.99	0.01	0.01	3.98
18:00:00	10	4.36	1.47	4.40	1.48	4.09	1.41	0.04	0.02	4.28	1.45	3.94	3.93	3.95	0.01	0.01	3.94
08:00:00	24	4.88	1.59	4.85	1.58	4.70	1.55	0.02	0.01	4.81	1.57	3.65	3.65	3.65	0.00	0.00	3.65

		4% (m/v) sucrose															
		OD										pH					
time (h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:00:00	0	0.13	-2.06357	0.13	-2.06	0.14	-1.94	0.07	0.04	0.13	-2.02	6.19	6.19	6.12	0.04	0.02	6.17
09:00:00	1	0.15	-1.8708	0.21	-1.58	0.18	-1.69	0.15	0.08	0.18	-1.71	6.10	6.09	6.04	0.03	0.02	6.08
10:00:00	2	0.29	-1.22	0.29	-1.23	0.31	-1.17	0.03	0.02	0.30	-1.21	5.93	5.93	5.87	0.03	0.02	5.91
11:00:00	3	0.59	-0.52	0.55	-0.59	0.58	-0.55	0.04	0.02	0.57	-0.56	5.50	5.52	5.46	0.03	0.02	5.49
12:00:00	4	0.92	-0.08	0.53	-0.63	2.64	0.97	0.82	0.47	1.36	0.08	4.96	4.97	4.95	0.01	0.01	4.96
13:00:00	5	1.90	0.64	1.83	0.60	1.96	0.67	0.03	0.02	1.90	0.64	4.60	4.61	4.60	0.01	0.00	4.60
14:00:00	6	2.70	0.99	2.61	0.96	2.64	0.97	0.02	0.01	2.65	0.97	4.33	4.36	4.37	0.02	0.01	4.35
15:00:00	7	3.10	1.13	3.06	1.12	3.07	1.12	0.01	0.00	3.08	1.12	4.19	4.18	4.23	0.03	0.02	4.20
16:00:00	8	3.35	1.21	3.32	1.20	3.27	1.18	0.01	0.01	3.31	1.20	4.07	4.06	4.10	0.02	0.01	4.08
17:00:00	9	3.69	1.31	3.70	1.31	3.57	1.27	0.02	0.01	3.65	1.30	4.00	4.00	4.00	0.00	0.00	4.00
18:00:00	10	3.79	1.33	4.03	1.39	3.70	1.31	0.04	0.03	3.84	1.34	3.93	3.93	3.93	0.00	0.00	3.93
08:00:00	24	4.43	1.49	4.53	1.51	4.10	1.41	0.05	0.03	4.35	1.47	3.66	3.64	3.67	0.02	0.01	3.66

		control (standard MRS media with 2% (m/v) glucose)															
		OD										pH					
time (h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:00:00	0	0.14	-2.00	0.09	-2.41	0.09	-2.41	0.24	0.14	0.11	-2.27	6.25	6.27	6.24	0.02	0.01	6.25
09:00:00	1	0.17	-1.78	0.17	-1.77	0.17	-1.76	0.01	0.01	0.17	-1.77	6.11	6.14	6.16	0.03	0.01	6.14
10:00:00	2	0.33	-1.12	0.32	-1.15	0.34	-1.08	0.04	0.02	0.33	-1.12	5.94	5.98	5.96	0.02	0.01	5.96
11:00:00	3	0.63	-0.46	0.68	-0.39	0.68	-0.38	0.04	0.02	0.66	-0.41	5.54	5.46	5.53	0.04	0.03	5.51
12:00:00	4	1.87	0.63	1.87	0.63	1.88	0.63	0.00	0.00	1.87	0.63	5.00	4.99	4.96	0.02	0.01	4.98
13:00:00	5	2.07	0.73	2.05	0.72	2.09	0.74	0.01	0.01	2.07	0.73	4.61	4.63	4.61	0.01	0.01	4.62
14:00:00	6	2.93	1.08	2.94	1.08	2.91	1.07	0.01	0.00	2.93	1.07	4.39	4.35	4.35	0.02	0.01	4.36
15:00:00	7	3.44	1.24	3.42	1.23	3.59	1.28	0.03	0.02	3.48	1.25	4.21	4.20	4.10	0.06	0.04	4.17
16:00:00	8	3.64	1.29	3.56	1.27	3.66	1.30	0.01	0.01	3.62	1.29	4.1	4.1	4.1	0.00	0.00	4.10
17:00:00	9	4.12	1.42	4.49	1.50	3.98	1.38	0.06	0.04	4.20	1.43	4.02	4.00	3.98	0.02	0.01	4.00
18:00:00	10	4.23	1.442202	4.45	1.492904	4.17	1.43	0.03	0.02	4.28	1.45	3.96	3.94	3.94	0.01	0.01	3.95
08:00:00	24	4.91	1.59	5.10	1.63	5.07	1.62	0.02	0.01	5.03	1.61	3.62	3.64	3.64	0.01	0.01	3.63

Appendix B: Prebiotic Tests

B. 1 Data on prebiotic tests for glucose on the growth of *L.plantarum*

		0.5% (m/v) glucose															
		OD									pH						
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:32:00	0	0.17	-1.80	0.16	-1.85	0.19	-1.68	0.08	0.05	0.17	-1.78	5.52	5.49	5.50	0.02	0.01	5.50
09:30:00	1	0.19	-1.64	0.18	-1.74	0.19	-1.66	0.05	0.03	0.19	-1.68	5.45	5.46	5.46	0.01	0.00	5.46
10:31:00	2	0.32	-1.13	0.29	-1.23	0.32	-1.13	0.05	0.03	0.31	-1.16	5.36	5.37	5.37	0.01	0.00	5.37
11:32:00	3	0.54	-0.61	0.55	-0.60	0.59	-0.53	0.04	0.02	0.56	-0.58	5.19	5.19	5.18	0.01	0.00	5.19
12:32:00	4	1.33	0.29	1.28	0.25	1.22	0.20	0.04	0.02	1.28	0.24	4.85	4.88	4.84	0.02	0.01	4.86
13:31:00	5	2.00	0.69	1.94	0.66	2.07	0.73	0.03	0.02	2.00	0.69	4.63	4.62	4.61	0.01	0.01	4.62
14:36:00	6	2.72	1.00	2.79	1.03	2.82	1.04	0.02	0.01	2.78	1.02	4.32	4.30	4.29	0.02	0.01	4.30
15:32:00	7	3.56	1.27	3.46	1.24	3.23	1.17	0.05	0.03	3.42	1.23	4.22	4.22	4.23	0.01	0.00	4.22
16:36:00	8	3.26	1.18	3.39	1.22	3.37	1.21	0.02	0.01	3.34	1.21	4.25	4.24	4.25	0.01	0.00	4.25
17:36:00	9	3.39	1.22	3.16	1.15	3.16	1.15	0.04	0.02	3.24	1.17	4.23	4.25	4.22	0.02	0.01	4.23
18:31:00	10	3.18	1.16	3.08	1.12	3.14	1.14	0.02	0.01	3.13	1.14	4.25	4.26	4.27	0.01	0.01	4.26
08:32:00	24	2.18	0.78	2.24	0.81	2.04	0.71	0.05	0.03	2.15	0.77	4.39	4.44	4.42	0.03	0.01	4.42

		control (standard MRS media with 2% (m/v) glucose)															
		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:32:00	0	0.17	-1.77	0.16	-1.83	0.16	-1.83	0.04	0.02	0.16	-1.81	6.00	6.01	6.06	0.03	0.02	6.02
09:30:00	1	0.18	-1.72	0.18	-1.73	0.19	-1.67	0.03	0.02	0.18	-1.71	5.98	6.06	6.03	0.04	0.02	6.02
10:31:00	2	0.31	-1.17	0.30	-1.22	0.31	-1.16	0.03	0.02	0.31	-1.18	5.85	5.94	5.88	0.05	0.03	5.89
11:32:00	3	0.59	-0.53	0.55	-0.61	0.61	-0.49	0.06	0.03	0.58	-0.54	5.59	5.65	5.59	0.03	0.02	5.61
12:32:00	4	1.23	0.21	1.21	0.19	1.33	0.29	0.05	0.03	1.26	0.23	5.04	5.09	5.04	0.03	0.02	5.06
13:31:00	5	1.93	0.66	1.89	0.64	1.99	0.69	0.03	0.01	1.94	0.66	4.72	4.75	4.70	0.03	0.01	4.72
14:36:00	6	3.20	1.16	3.21	1.17	3.27	1.18	0.01	0.01	3.23	1.17	4.33	4.37	4.33	0.02	0.01	4.34
15:32:00	7	3.73	1.32	3.79	1.33	3.72	1.31	0.01	0.01	3.75	1.32	4.18	4.18	4.17	0.01	0.00	4.18
16:36:00	8	4.17	1.43	4.35	1.47	4.47	1.50	0.04	0.02	4.33	1.47	4.06	4.03	4.02	0.02	0.01	4.04
17:36:00	9	4.56	1.52	4.95	1.60	4.66	1.54	0.04	0.02	4.72	1.55	3.94	3.97	3.94	0.02	0.01	3.95
18:31:00	10	5.01	1.61	4.93	1.60	4.95	1.60	0.01	0.00	4.96	1.60	3.88	3.54	3.84	0.19	0.11	3.75
08:32:00	24	4.18	1.43	4.30	1.46	4.04	1.40	0.03	0.02	4.17	1.43	3.65	3.63	3.63	0.01	0.01	3.64
		4.0 % m/v glucose															
		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:32:00	0	0.23	-1.46	0.26	-1.36	0.29	0.00	0.00	0.38	0.26	-1.78	5.24	5.22	5.20	0.02	0.01	5.22
09:30:00	1	0.24	-1.41	0.26	-1.35	0.34	-1.09	0.17	0.10	0.28	-1.29	5.22	5.20	5.17	0.03	0.01	5.20
10:31:00	2	0.35	-1.05	0.36	-1.03	0.40	-0.92	0.07	0.04	0.37	-1.00	5.15	5.12	5.10	0.03	0.01	5.12
11:32:00	3	0.54	-0.61	0.55	-0.60	0.60	-0.50	0.06	0.03	0.57	-0.57	5.02	5.00	5.00	0.01	0.01	5.01
12:32:00	4	0.94	-0.06	1.02	0.02	1.05	0.05	0.06	0.03	1.00	0.00	5.02	5.00	5.00	0.01	0.01	5.01
13:31:00	5	1.71	0.54	1.68	0.52	1.64	0.49	0.02	0.01	1.68	0.52	4.56	4.55	4.55	0.01	0.00	4.55
14:36:00	6	2.25	0.81	2.38	0.87	2.60	0.96	0.07	0.04	2.41	0.88	4.28	4.27	4.26	0.01	0.01	4.27
15:32:00	7	3.06	1.12	3.14	1.14	3.06	1.12	0.01	0.01	3.09	1.13	4.14	4.10	4.11	0.02	0.01	4.12
16:36:00	8	6.00	1.79	3.68	1.30	3.81	1.34	0.27	0.16	4.50	1.48	3.98	3.96	3.98	0.01	0.01	3.97
17:36:00	9	4.17	1.43	4.36	1.47	4.55	1.52	0.04	0.03	4.36	1.47	3.88	3.88	3.88	0.00	0.00	3.88
18:31:00	10	4.61	1.53	4.57	1.52	4.67	1.54	0.01	0.01	4.62	1.53	3.78	3.77	3.80	0.02	0.01	3.78
08:32:00	24	5.64	1.73	5.06	1.62	5.66	1.73	0.06	0.04	5.45	1.69	3.46	3.44	3.45	0.01	0.01	3.45

B. 2 Data on prebiotic tests for inulin on the growth of *L.plantarum*

		0.5% (m/v) inulin															
		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:00:00	0	0.01	-4.27	0.06	-2.88	0.06	-2.75	0.84	0.49	0.04	-3.30	6.15	6.10	6.07	0.04	0.02	6.11
09:00:00	1	0.03	-3.41	0.08	-2.59	0.08	-2.49	0.51	0.29	0.06	-2.83	6.07	6.00	6.00	0.04	0.02	6.02
10:00:00	2	0.22	-1.53	0.28	-1.27	0.31	-1.19	0.18	0.10	0.27	-1.33	5.99	5.88	5.84	0.08	0.04	5.90
11:00:00	3	0.39	-0.93	0.55	-0.59	0.57	-0.56	0.21	0.12	0.51	-0.69	5.78	5.59	5.56	0.12	0.07	5.64
12:00:00	4	0.74	-0.30	0.85	-0.16	0.83	-0.19	0.07	0.04	0.81	-0.22	5.46	5.44	5.43	0.02	0.01	5.44
13:00:00	5	0.90	-0.11	0.87	-0.14	0.90	-0.11	0.02	0.01	0.89	-0.12	5.39	5.43	5.42	0.02	0.01	5.41
14:00:00	6	1.02	0.02	0.94	-0.06	0.92	-0.08	0.05	0.03	0.96	-0.04	5.37	5.42	5.40	0.03	0.01	5.40
15:00:00	7	0.94	-0.06	0.87	-0.14	0.80	-0.22	0.08	0.05	0.87	-0.14	5.38	5.41	5.40	0.02	0.01	5.40
16:00:00	8	1.11	0.10	0.90	-0.11	0.91	-0.09	0.12	0.07	0.97	-0.03	5.27	5.41	5.40	0.08	0.05	5.36
17:00:00	9	0.94	-0.06	0.87	-0.14	0.80	-0.22	0.08	0.05	0.87	-0.14	5.38	5.41	5.40	0.02	0.01	5.40
18:00:00	10	1.12	0.11	0.97	-0.03	0.96	-0.04	0.09	0.05	1.02	0.01	5.37	5.41	5.40	0.02	0.01	5.39
08:00:00	24	1.12	0.11	0.97	-0.03	0.96	-0.04	0.09	0.05	1.02	0.01	5.37	5.41	5.40	0.02	0.01	5.39

		2% (m/v) inulin															
		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:00:00	0	0.13	-2.06	0.13	-2.01	0.13	-2.07	0.03	0.02	0.13	-2.05	5.85	5.85	5.88	0.02	0.01	5.86
09:00:00	1	0.16	-1.86	0.17	-1.75	0.14	-1.94	0.09	0.05	0.16	-1.85	5.79	5.80	5.82	0.02	0.01	5.80
10:00:00	2	0.28	-1.28	0.28	-1.27	0.25	-1.38	0.06	0.04	0.27	-1.31	5.65	5.66	5.68	0.02	0.01	5.66
11:00:00	3	0.52	-0.66	0.55	-0.60	0.49	-0.71	0.05	0.03	0.52	-0.66	5.40	5.36	5.42	0.03	0.02	5.39
12:00:00	4	0.96	-0.04	0.97	-0.03	0.90	-0.11	0.04	0.02	0.94	-0.06	5.00	5.03	5.09	0.05	0.03	5.04
13:00:00	5	1.56	0.44	1.61	0.48	1.55	0.44	0.02	0.01	1.57	0.45	4.77	4.74	4.79	0.03	0.01	4.77
14:00:00	6	1.93	0.66	1.82	0.60	1.84	0.61	0.03	0.02	1.86	0.62	4.57	4.77	4.76	0.11	0.07	4.70
15:00:00	7	2.18	0.78	2.31	0.84	2.28	0.82	0.03	0.02	2.26	0.81	4.75	4.77	4.77	0.01	0.01	4.76
16:00:00	8	1.94	0.66	1.88	0.63	1.91	0.65	0.02	0.01	1.91	0.65	4.76	4.77	4.78	0.01	0.01	4.77
17:00:00	9	2.18	0.78	2.31	0.84	2.28	0.82	0.03	0.02	2.26	0.81	4.75	4.77	4.77	0.01	0.01	4.76
18:00:00	10	1.94	0.66	1.88	0.63	1.91	0.65	0.02	0.01	1.91	0.65	4.76	4.77	4.78	0.01	0.01	4.77
08:00:00	24	1.85	0.62	1.89	0.64	1.80	0.59	0.02	0.01	1.85	0.61	4.76	4.79	4.79	0.02	0.01	4.78

		4% (m/v) inulin															
		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:00:00	0	0.16	-1.86	0.16	-1.86	0.15	-1.92	0.04	0.02	0.15	-1.88	5.66	5.65	5.65	0.01	0.00	5.65
09:00:00	1	0.18	-1.70	0.19	-1.69	0.18	-1.73	0.02	0.01	0.18	-1.70	5.59	5.59	5.61	0.01	0.01	5.60
10:00:00	2	0.30	-1.20	0.32	-1.15	0.30	-1.20	0.03	0.02	0.31	-1.18	5.45	5.45	5.47	0.01	0.01	5.46
11:00:00	3	0.55	-0.61	0.55	-0.60	0.54	-0.61	0.01	0.00	0.55	-0.61	5.19	5.19	5.22	0.02	0.01	5.20
12:00:00	4	1.08	0.08	1.08	0.08	1.10	0.10	0.01	0.01	1.09	0.08	4.89	4.90	4.92	0.02	0.01	4.90
13:00:00	5	1.76	0.57	1.63	0.49	1.67	0.51	0.04	0.02	1.69	0.52	4.61	4.62	4.62	0.01	0.00	4.62
14:00:00	6	2.47	0.90	2.53	0.93	2.43	0.89	0.02	0.01	2.48	0.91	4.34	4.35	4.35	0.01	0.00	4.35
15:00:00	7	3.21	1.17	3.21	1.17	3.16	1.15	0.01	0.01	3.19	1.16	4.20	4.23	4.24	0.02	0.01	4.22
16:00:00	8	3.22	1.17	3.16	1.15	3.92	1.37	0.12	0.07	3.43	1.23	4.20	4.21	4.23	0.02	0.01	4.21
17:00:00	9	3.21	1.17	3.21	1.17	3.16	1.15	0.01	0.01	3.19	1.16	4.20	4.23	4.24	0.02	0.01	4.22
18:00:00	10	3.22	1.17	3.16	1.15	2.92	1.07	0.05	0.03	3.10	1.13	4.20	4.21	4.23	0.02	0.01	4.21
08:00:00	24	3.00	1.10	2.94	1.08	2.93	1.08	0.01	0.01	2.96	1.08	4.20	4.23	4.24	0.02	0.01	4.22

		control (standard MRS media with 2% (m/v) glucose)															
		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:00:00	0	0.14	-1.98	0.11	-2.22	0.10	-2.32	0.18	0.10	0.12	-2.17	5.63	5.66	5.69	0.03	0.02	5.66
09:00:00	1	0.15	-1.87	0.13	-2.07	0.12	-2.15	0.15	0.08	0.13	-2.03	5.58	5.58	5.62	0.02	0.01	5.59
10:00:00	2	0.26	-1.35	0.25	-1.39	0.26	-1.35	0.02	0.01	0.26	-1.36	5.45	5.45	5.49	0.02	0.01	5.46
11:00:00	3	0.52	-0.65	0.54	-0.62	0.51	-0.67	0.03	0.01	0.52	-0.65	5.20	5.20	5.22	0.01	0.01	5.21
12:00:00	4	1.16	0.15	1.28	0.25	1.12	0.11	0.07	0.04	1.19	0.17	4.87	4.88	4.88	0.01	0.00	4.88
13:00:00	5	1.59	0.46	1.70	0.53	1.57	0.45	0.04	0.02	1.62	0.48	4.58	4.46	4.58	0.07	0.04	4.54
14:00:00	6	2.58	0.95	2.71	1.00	2.75	1.01	0.03	0.02	2.68	0.99	4.31	4.29	4.30	0.01	0.01	4.30
15:00:00	7	3.28	1.19	3.28	1.19	3.24	1.18	0.01	0.00	3.27	1.18	4.12	4.09	4.10	0.02	0.01	4.10
16:00:00	8	3.64	1.29	3.76	1.32	3.60	1.28	0.02	0.01	3.67	1.30	4.03	4.02	4.01	0.01	0.01	4.02
17:00:00	9	3.28	1.19	3.28	1.19	3.24	1.18	0.01	0.00	3.27	1.18	4.12	4.09	4.10	0.02	0.01	4.10
18:00:00	10	3.64	1.29	3.76	1.32	3.60	1.28	0.02	0.01	3.67	1.30	4.03	4.02	4.01	0.01	0.01	4.02
08:00:00	24	3.88	1.36	3.90	1.36	3.82	1.34	0.01	0.01	3.87	1.35	3.98	3.96	3.94	0.02	0.01	3.96

B. 3 Data on prebiotic tests for maltodextrin on the growth of *L.plantarum*

		0.5% (m/v) maltodextrin															
		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
07:46:00	0	0.13	-2.06	0.14	-2.00	0.13	-2.06	0.03	0.02	0.13	-2.04	6.17	6.18	6.17	0.01	0.00	6.17
08:53:00	1	0.19	-1.68	0.18	-1.71	0.20	-1.62	0.05	0.03	0.19	-1.67	6.07	6.08	6.06	0.01	0.01	6.07
09:46:00	2	0.32	-1.14	0.32	-1.15	0.31	-1.16	0.01	0.01	0.32	-1.15	5.94	5.94	5.90	0.02	0.01	5.93
10:49:00	3	0.68	-0.39	0.70	-0.36	0.69	-0.37	0.01	0.01	0.69	-0.37	5.64	5.62	5.58	0.03	0.02	5.61
11:44:00	4	1.15	0.14	0.92	-0.08	1.05	0.05	0.11	0.06	1.04	0.04	5.40	5.41	5.39	0.01	0.01	5.40
12:51:00	5	1.23	0.21	1.30	0.26	1.32	0.28	0.04	0.02	1.28	0.25	5.25	5.24	5.24	0.01	0.00	5.24
13:49:00	6	1.81	0.59	1.74	0.55	1.61	0.48	0.06	0.03	1.72	0.54	5.01	4.99	5.00	0.01	0.01	5.00
14:50:00	7	2.52	0.92	2.39	0.87	2.35	0.85	0.04	0.02	2.42	0.88	4.75	4.72	4.74	0.02	0.01	4.74
15:58:00	8	3.33	1.20	3.38	1.22	3.08	1.12	0.05	0.03	3.26	1.18	4.51	4.49	4.51	0.01	0.01	4.50
17:00:00	9	3.88	1.36	3.68	1.30	3.73	1.32	0.03	0.02	3.76	1.33	4.40	4.44	4.42	0.02	0.01	4.42
17:50:00	10	4.07	1.40	3.95	1.37	3.92	1.37	0.02	0.01	3.98	1.38	4.42	4.46	4.46	0.02	0.01	4.45
07:49:00	24	3.79	1.33	3.57	1.27	3.41	1.23	0.05	0.03	3.59	1.28	4.50	4.50	4.49	0.01	0.00	4.50

		2% (m/v) maltodextrin															
		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
07:46:00	0	0.10	-2.28	0.12	-2.09	0.12	-2.16	0.10	0.06	0.11	-2.18	5.99	6.01	6.01	0.01	0.01	6.00
08:53:00	1	0.15	-1.88	0.16	-1.84	0.16	-1.82	0.03	0.02	0.16	-1.85	5.89	5.91	5.89	0.01	0.01	5.90
09:46:00	2	0.28	-1.29	0.27	-1.31	0.28	-1.26	0.03	0.01	0.28	-1.29	5.73	5.72	5.72	0.01	0.00	5.72
10:49:00	3	0.70	-0.36	0.64	-0.45	0.60	-0.51	0.08	0.04	0.65	-0.44	5.40	5.39	5.40	0.01	0.00	5.40
11:44:00	4	1.15	0.14	1.06	0.06	0.97	-0.03	0.09	0.05	1.06	0.06	5.07	5.04	5.06	0.02	0.01	5.06
12:51:00	5	1.78	0.58	1.80	0.59	1.71	0.54	0.03	0.02	1.76	0.57	4.74	4.74	4.74	0.00	0.00	4.74
13:49:00	6	2.11	0.75	1.92	0.65	1.97	0.68	0.05	0.03	2.00	0.69	4.63	4.57	4.60	0.03	0.02	4.60
14:50:00	7	2.56	0.94	2.66	0.98	2.55	0.94	0.02	0.01	2.59	0.95	4.43	4.39	4.43	0.02	0.01	4.42
15:58:00	8	3.32	1.20	3.32	1.20	3.33	1.20	0.00	0.00	3.32	1.20	4.26	4.21	4.25	0.03	0.02	4.24
17:00:00	9	3.79	1.33	3.73	1.32	3.53	1.26	0.04	0.02	3.68	1.30	4.12	4.08	4.12	0.02	0.01	4.11
17:50:00	10	4.15	1.42	4.11	1.41	3.88	1.36	0.04	0.02	4.05	1.40	4.06	4.03	4.06	0.02	0.01	4.05
07:49:00	24	3.77	1.33	3.74	1.32	3.78	1.33	0.01	0.00	3.76	1.33	3.77	3.74	3.78	0.02	0.01	3.76

		4% (m/v) maltodextrin															
		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
07:46:00	0	0.09	-2.36	0.09	-2.39	0.09	-2.44	0.04	0.02	0.09	-2.40	5.85	5.86	5.86	0.01	0.00	5.86
08:53:00	1	0.14	-1.94	0.13	-2.06	0.13	-2.06	0.07	0.04	0.13	-2.02	5.75	5.76	5.76	0.01	0.00	5.76
09:46:00	2	0.22	-1.51	0.21	-1.58	0.21	-1.55	0.04	0.02	0.21	-1.55	5.58	5.59	5.60	0.01	0.01	5.59
10:49:00	3	0.39	-0.94	0.46	-0.78	0.42	-0.87	0.08	0.05	0.42	-0.86	5.27	5.21	5.28	0.04	0.02	5.25
11:44:00	4	0.83	-0.19	0.89	-0.12	0.88	-0.13	0.04	0.02	0.87	-0.14	4.94	4.94	4.94	0.00	0.00	4.94
12:51:00	5	1.59	0.46	1.45	0.37	1.52	0.42	0.05	0.03	1.52	0.42	4.57	4.56	4.57	0.01	0.00	4.57
13:49:00	6	1.94	0.66	2.15	0.77	2.00	0.69	0.05	0.03	2.03	0.71	4.31	4.30	4.31	0.01	0.00	4.31
14:50:00	7	2.36	0.86	2.24	0.81	2.26	0.82	0.03	0.02	2.29	0.83	4.25	4.26	4.25	0.01	0.00	4.25
15:58:00	8	2.76	1.02	2.55	0.94	2.55	0.94	0.05	0.03	2.62	0.96	4.18	4.18	4.18	0.00	0.00	4.18
17:00:00	9	2.83	1.04	3.26	1.18	2.82	1.04	0.08	0.05	2.97	1.09	4.08	4.07	4.07	0.01	0.00	4.07
17:50:00	10	3.01	1.10	3.09	1.13	3.01	1.10	0.02	0.01	3.04	1.11	4.01	3.98	3.99	0.02	0.01	3.99
07:49:00	24	3.80	1.34	3.89	1.36	3.85	1.35	0.01	0.01	3.85	1.35	3.70	3.69	3.68	0.01	0.01	3.69

		control (standard MRS media with 2% (m/v) glucose)															
		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
07:46:00	0	0.13	-2.02	0.13	-2.01	0.14	-1.95	0.04	0.02	0.14	-2.00	5.95	6.00	6.01	0.03	0.02	5.99
08:53:00	1	0.21	-1.56	0.19	-1.64	0.20	-1.63	0.04	0.02	0.20	-1.61	5.85	5.89	5.89	0.02	0.01	5.88
09:46:00	2	0.36	-1.01	0.34	-1.09	0.34	-1.09	0.05	0.03	0.35	-1.06	5.65	5.70	5.71	0.03	0.02	5.69
10:49:00	3	0.93	-0.07	0.95	-0.05	0.87	-0.14	0.05	0.03	0.92	-0.09	5.21	5.26	5.29	0.04	0.02	5.25
11:44:00	4	1.67	0.51	1.47	0.39	1.44	0.36	0.08	0.05	1.53	0.42	4.82	4.86	4.87	0.03	0.02	4.85
12:51:00	5	2.82	1.04	2.60	0.96	2.70	0.99	0.04	0.02	2.71	1.00	4.42	4.43	4.44	0.01	0.01	4.43
13:49:00	6	3.67	1.30	3.43	1.23	3.47	1.24	0.04	0.02	3.52	1.26	4.21	4.22	4.23	0.01	0.01	4.22
14:50:00	7	3.83	1.34	3.75	1.32	3.87	1.35	0.02	0.01	3.82	1.34	4.07	4.09	4.09	0.01	0.01	4.08
15:58:00	8	4.42	1.49	4.21	1.44	4.32	1.46	0.02	0.01	4.32	1.46	3.96	3.97	3.97	0.01	0.00	3.97
17:00:00	9	4.75	1.56	4.72	1.55	4.60	1.53	0.02	0.01	4.69	1.55	3.87	3.89	3.90	0.02	0.01	3.89
17:50:00	10	5.08	1.63	4.93	1.60	4.96	1.60	0.02	0.01	4.99	1.61	3.83	3.84	3.84	0.01	0.00	3.84
11:19:00	24	5.15	1.64	5.21	1.65	5.27	1.66	0.01	0.01	5.21	1.65	3.67	3.63	3.67	0.02	0.01	3.66

B. 4 Data on prebiotic tests for sucrose on the growth of *L.plantarum*

		0.5% (m/v) sucrose															
		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave Ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:00:00	0	0.47	-0.76	0.18	-1.73	0.14	-2.00	0.65	0.38	0.26	-1.49	5.64	6.04	6.10	0.25	0.14	5.93
09:00:00	1	0.13	-2.01	0.14	-1.96	0.14	-1.96	0.03	0.02	0.14	-1.98	6.13	6.10	6.10	0.02	0.01	6.11
10:00:00	2	0.20	-1.61	0.20	-1.62	0.19	-1.64	0.02	0.01	0.20	-1.62	5.98	6.00	6.00	0.01	0.01	5.99
11:00:00	3	0.37	-0.99	0.36	-1.03	0.36	-1.02	0.02	0.01	0.36	-1.02	5.79	5.81	5.83	0.02	0.01	5.81
12:00:00	4	0.65	-0.44	0.65	-0.42	0.65	-0.43	0.01	0.00	0.65	-0.43	5.50	5.52	5.52	0.01	0.01	5.51
13:00:00	5	1.49	0.40	1.44	0.36	1.60	0.47	0.05	0.03	1.51	0.41	5.04	5.06	5.04	0.01	0.01	5.05
14:00:00	6	2.21	0.79	2.26	0.82	2.24	0.81	0.01	0.01	2.24	0.80	4.68	4.68	4.66	0.01	0.01	4.67
15:00:00	7	2.92	1.07	3.09	1.13	2.93	1.08	0.03	0.02	2.98	1.09	4.47	4.49	4.50	0.02	0.01	4.49
16:00:00	8	3.08	1.12	2.94	1.08	3.04	1.11	0.02	0.01	3.02	1.11	4.48	4.51	4.53	0.03	0.01	4.51
17:00:00	9	3.26	1.18	3.00	1.10	2.96	1.09	0.05	0.03	3.07	1.12	4.43	4.56	4.55	0.07	0.04	4.51
18:00:00	10	3.19	1.16	2.83	1.04	3.00	1.10	0.06	0.03	3.01	1.10	4.52	4.53	4.54	0.01	0.01	4.53
08:00:00	24	2.86	1.05	2.53	0.93	2.58	0.95	0.07	0.04	2.66	0.98	4.62	4.69	4.70	0.04	0.03	4.67

		2% (m/v) sucrose															
		OD										pH					
time(h)		Run A	ln(OD)	Run B	ln(OD)	Run C	ln(OD)	SD	SEM	Ave OD	Ave Ln(OD)	A	B	C	SD	SEM	AVE pH
08:00:00	0	0.12	-2.15	0.12	-2.15	0.11	-2.19	0.02	0.01	0.12	-2.16	6.11	6.11	6.11	0.00	0.00	6.11
09:00:00	1	0.12	-2.13	0.12	-2.15	0.13	-2.07	0.04	0.02	0.12	-2.12	6.08	6.08	6.06	0.01	0.01	6.07
10:00:00	2	0.19	-1.67	0.18	-1.69	0.19	-1.66	0.02	0.01	0.19	-1.67	5.98	5.99	5.98	0.01	0.00	5.98
11:00:00	3	0.38	-0.96	0.37	-1.00	0.37	-0.99	0.02	0.01	0.37	-0.98	5.74	5.76	5.73	0.02	0.01	5.74
12:00:00	4	0.65	-0.43	0.63	-0.47	0.67	-0.40	0.03	0.02	0.65	-0.43	5.43	5.47	5.41	0.03	0.02	5.44
13:00:00	5	1.47	0.39	1.49	0.40	1.51	0.41	0.01	0.01	1.49	0.40	4.98	4.96	4.94	0.02	0.01	4.96
14:00:00	6	2.25	0.81	2.07	0.73	2.16	0.77	0.04	0.02	2.16	0.77	4.62	4.66	4.60	0.03	0.02	4.63
15:00:00	7	3.13	1.14	3.07	1.12	3.05	1.12	0.01	0.01	3.08	1.13	4.36	4.35	4.33	0.02	0.01	4.35
16:00:00	8	4.14	1.42	4.16	1.43	4.08	1.41	0.01	0.01	4.13	1.42	4.19	4.19	4.15	0.02	0.01	4.18
17:00:00	9	4.53	1.51	4.72	1.55	4.89	1.59	0.04	0.02	4.71	1.55	4.06	4.06	4.06	0.00	0.00	4.06
18:00:00	10	4.54	1.51	4.45	1.49	4.54	1.51	0.01	0.01	4.51	1.51	4.00	3.97	3.95	0.03	0.01	3.97
08:00:00	24	5.70	1.74	5.70	1.74	5.54	1.71	0.02	0.01	5.65	1.73	3.64	3.65	3.61	0.02	0.01	3.63

		4% (m/v) sucrose															
		OD										pH					
time(h)		Run A	ln(OD)	Run B	ln(OD)	Run C	ln(OD)	SD	SEM	Ave OD	Ave Ln(OD)	A	B	C	SD	SEM	AVE pH
08:00:00	0	0.13	-2.04	0.11	-2.24	0.15	-1.92	0.16	0.09	0.13	-2.07	5.98	6.08	6.03	0.05	0.03	6.03
09:00:00	1	0.12	-2.15	0.12	-2.15	0.11	-2.22	0.04	0.02	0.11	-2.17	6.03	6.04	6.03	0.01	0.00	6.03
10:00:00	2	0.19	-1.69	0.18	-1.73	0.18	-1.73	0.02	0.01	0.18	-1.71	5.93	5.95	5.95	0.01	0.01	5.94
11:00:00	3	0.38	-0.97	0.38	-0.98	0.37	-0.99	0.01	0.01	0.37	-0.98	5.66	5.67	5.65	0.01	0.01	5.66
12:00:00	4	0.68	-0.39	0.67	-0.40	0.68	-0.39	0.01	0.01	0.67	-0.39	5.30	5.31	5.30	0.01	0.00	5.30
13:00:00	5	1.66	0.51	1.90	0.64	1.51	0.41	0.12	0.07	1.69	0.52	4.80	4.82	4.83	0.02	0.01	4.82
14:00:00	6	2.44	0.89	2.21	0.79	2.26	0.82	0.05	0.03	2.30	0.83	4.52	4.54	4.52	0.01	0.01	4.53
15:00:00	7	3.05	1.12	3.28	1.19	3.12	1.14	0.04	0.02	3.15	1.15	4.30	4.30	4.30	0.00	0.00	4.30
16:00:00	8	3.92	1.37	3.91	1.36	3.82	1.34	0.01	0.01	3.88	1.36	4.14	4.14	4.15	0.01	0.00	4.14
17:00:00	9	4.49	1.50	4.03	1.39	4.06	1.40	0.06	0.03	4.19	1.43	4.02	4.03	4.03	0.01	0.00	4.03
18:00:00	10	4.91	1.59	4.82	1.57	5.12	1.63	0.03	0.02	4.95	1.60	4.91	4.82	5.12	0.15	0.09	4.95
08:00:00	24	6.01	1.79	5.93	1.78	5.91	1.78	0.01	0.01	5.95	1.78	3.61	3.60	3.63	0.02	0.01	3.61

		control (standard MRS media with 2% (m/v) glucose)															
		OD										pH					
time(h)		Run A	ln(OD)	Run B	ln(OD)	Run C	ln(OD)	SD	SEM	Ave OD	Ave Ln(OD)	A	B	C	SD	SEM	AVE pH
08:00:00	0	0.10	-2.26	0.11	-2.25	0.12	-2.16	0.06	0.03	0.11	-2.23	5.98	5.97	6.02	0.03	0.02	5.99
09:00:00	1	0.11	-2.21	0.12	-2.10	0.12	-2.12	0.06	0.03	0.12	-2.14	5.94	5.92	5.97	0.03	0.01	5.94
10:00:00	2	0.20	-1.62	0.19	-1.65	0.20	-1.59	0.03	0.02	0.20	-1.62	5.83	5.83	5.87	0.02	0.01	5.84
11:00:00	3	0.42	-0.87	0.42	-0.88	0.43	-0.85	0.02	0.01	0.42	-0.87	5.57	5.56	5.58	0.01	0.01	5.57
12:00:00	4	0.78	-0.25	0.80	-0.23	0.81	-0.21	0.02	0.01	0.80	-0.23	5.39	5.37	5.37	0.01	0.01	5.38
13:00:00	5	1.78	0.58	1.69	0.52	1.80	0.59	0.03	0.02	1.76	0.56	4.73	4.77	4.70	0.04	0.02	4.73
14:00:00	6	2.70	0.99	2.52	0.92	2.68	0.99	0.04	0.02	2.63	0.97	4.45	4.42	4.41	0.02	0.01	4.43
15:00:00	7	3.38	1.22	3.21	1.17	3.27	1.18	0.03	0.02	3.29	1.19	4.24	4.26	4.27	0.02	0.01	4.26
16:00:00	8	3.56	1.27	3.70	1.31	3.63	1.29	0.02	0.01	3.63	1.29	4.11	4.14	4.16	0.03	0.01	4.14
17:00:00	9	4.04	1.40	4.03	1.39	4.00	1.39	0.01	0.00	4.02	1.39	4.04	4.07	4.05	0.02	0.01	4.05
18:00:00	10	4.50	1.50	4.44	1.49	4.29	1.46	0.02	0.01	4.41	1.48	3.98	3.95	3.95	0.02	0.01	3.96
08:00:00	24	5.02	1.61	5.34	1.56	5.02	1.61	0.03	0.02	5.13	1.63	3.61	3.67	3.63	0.03	0.02	3.64

Appendix C: Viability of freeze dried cells

C. 1 Viability of freeze dried cells after freeze drying

	Before Freeze Drying		After freeze drying			
	CFU/ml	Log CFU/ml	CFU/ml	Log CFU	% Viability	Log reduction
Water						
1	1.00E+09	9.00	1.50E+08	8.18	15.00	0.82
2	7.90E+08	8.90	2.50E+08	8.40	31.65	0.50
3	1.10E+09	9.04	2.50E+08	8.40	22.73	0.64
SD		0.07		0.13	83.30	0.16
SEM		0.04		0.07	48.09	0.09
Average	**9.63E+08**	**8.98**	**2.17E+08**	**8.32**	**22.49**	**0.66**
Inulin						
1	1.17E+09	9.07	1.36E+09	9.13	116.24	-0.07
2	1.55E+09	9.19	1.07E+09	9.03	69.03	0.16
3	1.45E+09	9.16	1.11E+09	9.05	76.55	0.12
SD		0.06		0.06	25.36	0.12
SEM		0.04		0.03	14.64	0.07
Average	**1.39E+09**	**9.14**	**1.18E+09**	**9.07**	**84.89**	**0.07**
Skimmed milk						
1	1.05E+09	9.02	9.30E+08	8.97	88.57	0.05
2	1.28E+09	9.11	8.90E+08	8.95	69.53	0.16
3	6.70E+08	8.83	9.10E+08	8.96	135.82	-0.13
SD		0.14		0.01	34.13	0.15
SEM		0.08		0.01	19.71	0.09
Average	**1.00E+09**	**8.98**	**9.10E+08**	**8.96**	**91.00**	**0.03**
Maltodextrin						
1	1.04E+09	9.02	3.71E+08	8.57	35.63	0.45
2	9.20E+08	8.96	3.17E+08	8.50	34.46	0.46
3	1.06E+09	9.03	2.37E+08	8.37	22.36	0.65
SD		0.03		0.10	7.35	0.11
SEM		0.02		0.06	4.24	0.07
Average	**1.01E+09**	**9.00**	**3.08E+08**	**8.48**	**30.61**	**0.51**
Sucrose						
1	9.10E+08	8.96	1.96E+08	8.29	21.54	0.67
2	1.33E+09	9.12	6.13E+08	8.79	46.09	0.34
3	8.80E+08	8.94	2.14E+08	8.33	24.26	0.62
SD		0.10		0.28	13.46	0.18
SEM		0.06		0.16	7.77	0.10
Average	**1.04E+09**	**9.01**	**3.41E+08**	**8.47**	**32.77**	**0.48**

C. 2 Viability of freeze dried cells in various drying media upon re-growth after 4 weeks storage at 4ºC and room temperature

	Before Freeze Drying		4 weeks							
			4C Temperature				Room Temperature			
	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml	% Viability	Log reduction	CFU/ml	Log CFU/ml	% Viability	Log reductio
Water										
1	1.08E+09	9.03	3.85E+07	7.59	3.56	1.45	6.50E+05	5.81	0.06	3.22
2	1.24E+09	9.09	5.70E+07	7.76	4.60	1.34	4.50E+05	5.65	0.04	3.44
3	1.08E+09	9.03	3.80E+07	7.58	3.52	1.45	6.50E+05	5.81	0.06	3.22
SD		0.03		0.10	0.61	0.07		0.09	0.01	0.13
SEM		0.02		0.06	0.35	0.04		0.05	0.01	0.07
Average	**1.13E+09**	**9.05**	**4.45E+07**	**7.64**	**3.93**	**1.41**	**5.83E+05**	**5.76**	**0.05**	**3.29**
Inulin										
1	1.04E+09	9.02	1.25E+07	7.10	1.20	1.92	4.50E+05	5.65	0.04	3.36
2	1.01E+09	9.00	1.30E+07	7.11	1.29	1.89	3.00E+05	5.48	0.03	3.53
3	1.24E+09	9.09	2.20E+07	7.34	1.77	1.75	4.00E+05	5.60	0.03	3.49
SD		0.05		0.14	0.31	0.09		0.09	0.01	0.09
SEM		0.03		0.08	0.18	0.05		0.05	0.00	0.05
Average	**1.10E+09**	**9.04**	**1.58E+07**	**7.18**	**1.44**	**1.84**	**3.83E+05**	**5.58**	**0.03**	**3.46**
Skimmed milk										
1	8.00E+08	8.90	5.30E+08	8.72	66.25	0.18	4.10E+08	8.61	51.25	0.29
2	6.70E+08	8.83	6.00E+08	8.78	89.55	0.05	4.60E+08	8.66	68.66	0.16
3	6.00E+08	8.78	7.15E+08	8.85	119.17	-0.08	4.35E+08	8.64	72.50	0.14
SD		0.06		0.07	26.52	0.13		0.02	11.32	0.08
SEM		0.04		0.04	15.31	0.07		0.01	6.54	0.05
Average	**6.90E+08**	**8.84**	**6.15E+08**	**8.79**	**89.13**	**0.05**	**4.35E+08**	**8.64**	**63.04**	**0.20**
Maltodextrin					-					
1	1.04E+09	9.02	1.29E+08	8.11	12.40	0.91	5.15E+07	7.71	4.95	1.31
2	9.20E+08	8.96	1.10E+08	8.04	11.96	0.92	3.85E+07	7.59	4.18	1.38
3	1.06E+09	9.03	3.77E+08	8.58	35.57	0.45	3.45E+07	7.54	3.25	1.49
SD		0.03		0.29	13.50	0.27		0.09	0.85	0.09
SEM		0.02		0.17	7.80	0.16		0.05	0.49	0.05
Average	**1.01E+09**	**9.00**	**2.05E+08**	**8.24**	**20.40**	**0.69**	**4.15E+07**	**7.61**	**4.12**	**1.39**
Sucrose										
1	9.10E+08	8.96	2.57E+08	8.41	28.24	0.55	3.00E+07	7.48	3.30	1.48
2	1.33E+09	9.12	4.51E+08	8.65	33.91	0.47	1.20E+07	7.08	0.90	2.04
3	8.80E+08	8.94	2.48E+08	8.39	28.13	0.55	1.25E+07	7.10	1.42	1.85
SD		0.10		0.15	3.31	0.05		0.22	0.82	0.29
SEM		0.06		0.08	1.91	0.03		0.13	2.10	0.16
Average	**1.04E+09**	**9.01**	**3.19E+08**	**8.49**	**30.63**	**0.51**	**1.82E+07**	**7.22**	**1.75**	**1.79**

C. 3 Viability of freeze dried cells in various drying media upon re-growth after 8 weeks storage at 4ºC and room temperature

	Before Freeze Drying		8 weeks							
			4C Temperature				Room Temperature			
	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml	% Viability	Log reduction	CFU/ml	Log CFU/ml	% Viability	Log reductio
Water										
1	1.08E+09	9.03	2.35E+07	7.37	2.18	1.66	8.00E+03	3.90	0.00	5.13
2	1.24E+09	9.09	4.25E+07	7.63	3.43	1.47	3.50E+04	4.54	0.00	4.55
3	1.08E+09	9.03	2.75E+07	7.44	2.55	1.59	4.90E+04	4.69	0.00	4.34
SD		0.03		0.13	0.64	0.10		0.42	0.00	0.41
SEM		0.02		0.08	0.37	0.06		0.24	0.00	0.24
Average	**1.13E+09**	**9.05**	**3.12E+07**	**7.48**	**2.75**	**1.57**	**3.07E+04**	**4.38**	**0.00**	**4.67**
Inulin										
1	1.04E+09	9.02	3.95E+05	5.60	0.04	3.42	0.00E+00	0.00	0.00	
2	1.01E+09	9.00	6.15E+05	5.79	0.06	3.22	0.00E+00	0.00	0.00	
3	1.24E+09	9.09	3.25E+05	5.51	0.03	3.58	0.00E+00	0.00	0.00	
SD		0.05		0.14	0.02	0.18		0.00	0.00	
SEM		0.03		0.08	0.01	0.11		0.00	0.00	
Average	**1.10E+09**	**9.04**	**4.45E+05**	**5.63**	**0.04**	**3.41**	**0.00E+00**	**0.00**	**0.00**	
Skimmed milk										
1	8.00E+08	8.90	6.40E+08	8.81	80.00	0.10	3.90E+08	8.59	48.75	0.31
2	6.70E+08	8.83	6.25E+08	8.80	93.28	0.03	5.00E+08	8.70	74.63	0.13
3	6.00E+08	8.78	6.55E+08	8.82	109.17	-0.04	4.95E+08	8.69	82.50	0.08
SD		0.06		0.01	14.60	0.07		0.06	17.66	0.12
SEM		0.04		0.01	8.43	0.04		0.04	10.19	0.07
Average	**6.90E+08**	**8.84**	**6.40E+08**	**8.81**	**92.75**	**0.03**	**4.62E+08**	**8.66**	**66.91**	**0.17**
Maltodextrin										
1	1.04E+09	9.02	4.80E+07	7.68	4.62	1.34	4.20E+05	5.62	0.04	3.39
2	9.20E+08	8.96	6.05E+07	7.78	6.58	1.18	9.95E+05	6.00	0.11	2.97
3	1.06E+09	9.03	1.83E+08	8.26	17.22	0.76	5.75E+05	5.76	0.05	3.27
SD		0.03		0.31	6.78	0.30		0.19	0.04	0.22
SEM		0.02		0.18	3.91	0.17		0.11	0.02	0.13
Average	**1.01E+09**	**9.00**	**9.70E+07**	**7.91**	**9.64**	**1.09**	**6.63E+05**	**5.79**	**0.07**	**3.21**
Sucrose										
1	9.10E+08	8.96	3.23E+08	8.51	35.49	0.45	5.50E+04	4.74	0.01	4.22
2	1.33E+09	9.12	3.16E+08	8.50	23.76	0.62	7.50E+04	4.88	0.01	4.25
3	8.80E+08	8.94	1.52E+08	8.18	17.22	0.76	2.25E+05	5.35	0.03	3.59
SD		0.10		0.19	9.26	0.16		0.32	0.01	0.37
SEM		0.06		0.11	5.35	0.09		0.19	0.01	0.21
Average	**1.04E+09**	**9.01**	**2.64E+08**	**8.40**	**25.34**	**0.61**	**1.18E+05**	**4.99**	**0.01**	**4.02**

C. 4 Viability of freeze dried cells in various drying media upon re-growth after 12 weeks storage at 4ºC and room temperature

	Before Freeze Drying		12 weeks							
			4C Temperature				Room Temperature			
	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml	% Viability	Log reduction	CFU/ml	Log CFU/ml	% Viability	Log reductio
Water										
1	1.08E+09	9.03	3.45E+06	6.54	0.32	2.50	2.50E+03	3.40	0.00	5.64
2	1.24E+09	9.09	9.35E+06	6.97	0.75	2.12	2.50E+03	3.40	0.00	5.70
3	1.08E+09	9.03	3.35E+06	6.53	0.31	2.51	2.50E+03	3.40	0.00	5.64
SD		0.03		0.25	0.25	0.22		0.00	0.00	0.03
SEM		0.02		0.15	0.15	0.13		0.00	0.00	0.02
Average	**1.13E+09**	**9.05**	**5.38E+06**	**6.68**	**0.48**	**2.38**	**2.50E+03**	**3.40**	**0.00**	**5.66**
Inulin										
1	1.04E+09	9.02	7.50E+03	3.88	0.00	5.14	1.15E+03	3.06	0.00	5.96
2	1.01E+09	9.00	2.50E+05	5.40	0.02	3.61	1.50E+03	3.18	0.00	5.83
3	1.24E+09	9.09	3.35E+06	6.53	0.27	2.57	1.62E+04	4.21	0.00	4.88
SD		0.05		1.33	0.15	1.29		0.63	0.00	0.59
SEM		0.03		0.77	0.09	0.75		0.37	0.00	0.34
Average	**1.10E+09**	**9.04**	**1.20E+06**	**5.27**	**0.11**	**3.77**	**6.28E+03**	**3.48**	**0.00**	**5.56**
Skimmed milk										
1	8.00E+08	8.90	6.35E+08	8.80	79.38	0.10	3.95E+08	8.60	49.38	0.31
2	6.70E+08	8.83	6.50E+08	8.81	97.01	0.01	4.35E+08	8.64	64.93	0.19
3	6.00E+08	8.78	6.00E+08	8.78	100.00	0.00	4.35E+08	8.64	72.50	0.14
SD		0.06		0.02	11.15	0.05		0.02	11.79	0.09
SEM		0.04		0.01	6.44	0.03		0.01	6.81	0.05
Average	**6.90E+08**	**8.84**	**6.28E+08**	**8.80**	**91.06**	**0.04**	**4.22E+08**	**8.62**	**61.11**	**0.21**
Maltodextrin										
1	1.04E+09	9.02	1.38E+08	8.14	13.22	0.88	1.90E+04	4.28	0.00	4.74
2	9.20E+08	8.96	1.55E+08	8.19	16.85	0.77	2.00E+05	5.30	0.02	3.66
3	1.06E+09	9.03	1.09E+08	8.04	10.28	0.99	1.60E+05	5.20	0.02	3.82
SD		0.03		0.08	3.29	0.11		0.56		0.58
SEM		0.02		0.04	1.90	0.06		0.33		0.34
Average	**1.01E+09**	**9.00**	**1.34E+08**	**8.12**	**13.29**	**0.88**	**1.26E+05**	**4.93**	**0.01**	**4.07**
Sucrose										
1	9.10E+08	8.96	4.29E+08	8.63	47.14	0.33	4.05E+04	4.61	0.00	4.35
2	1.33E+09	9.12	2.95E+08	8.47	22.18	0.65	1.47E+06	6.17	0.11	2.96
3	8.80E+08	8.94	3.04E+08	8.48	34.55	0.46	1.45E+04	4.16	0.00	4.78
SD		0.10		0.09	12.48	0.16		1.05		0.95
SEM		0.06		0.05	7.21	0.10		0.61		0.55
Average	**1.04E+09**	**9.01**	**3.43E+08**	**8.53**	**32.95**	**0.48**	**5.08E+05**	**4.98**	**0.05**	**4.03**

Appendix D: Propagation of freeze dried cells upon re-growth

D. 1 Propagation of freeze dried cells in water (control) upon re-growth

Before freeze drying

water (control)

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:40	0	0.51	-0.67	0.32	-1.16	0.31	-1.17	0.29	0.17	0.38	-1.00	6.07	6.10	6.09	0.02	0.01	6.09
09:40	1	0.43	-0.85	0.63	-0.46	0.44	-0.82	0.22	0.12	0.50	-0.71	5.88	5.91	5.87	0.02	0.01	5.89
10:40	2	1.00	0.00	1.12	0.11	1.02	0.02	0.06	0.03	1.05	0.04	5.40	5.35	5.35	0.03	0.02	5.37
11:40	3	1.78	0.58	1.60	0.47	1.45	0.37	0.10	0.06	1.61	0.47	4.92	4.85	4.87	0.04	0.02	4.88
12:40	4	3.43	1.23	2.97	1.09	2.93	1.08	0.09	0.05	3.11	1.13	4.47	4.42	4.45	0.03	0.01	4.45
13:40	5	3.59	1.28	3.68	1.30	3.46	1.24	0.03	0.02	3.58	1.27	4.21	4.23	4.27	0.03	0.02	4.24
14:40	6	3.85	1.35	4.28	1.45	4.12	1.42	0.05	0.03	4.08	1.41	4.10	4.07	4.06	0.02	0.01	4.08
15:40	7	4.65	1.54	4.59	1.52	4.48	1.50	0.02	0.01	4.57	1.52	4.01	3.98	3.97	0.02	0.01	3.99
16:40	8	4.80	1.57	4.97	1.60	4.87	1.58	0.02	0.01	4.88	1.59	3.93	3.93	3.90	0.02	0.01	3.92
17:40	9	5.20	1.65	5.20	1.65	5.56	1.72	0.04	0.02	5.32	1.67	3.86	3.88	3.92	0.03	0.02	3.89
18:40	10	5.45	1.70	5.32	1.67	5.05	1.62	0.04	0.02	5.27	1.66	3.83	3.81	3.82	0.01	0.01	3.82
08:40	24	4.70	1.55	4.94	1.60	4.85	1.58	0.03	0.01	4.83	1.57	3.77	3.76	3.76	0.01	0.00	3.76

After freeze drying

water (control)

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:40	0	0.34	-1.08	0.34	-1.09	0.37	-0.99	0.06	0.03	0.35	-1.05	5.70	5.68	5.69	0.01	0.01	5.69
09:40	1	0.35	-1.05	0.34	-1.08	0.39	-0.94	0.07	0.04	0.36	-1.02	5.67	5.69	5.64	0.03	0.01	5.67
10:40	2	0.44	-0.81	0.41	-0.89	0.47	-0.75	0.07	0.04	0.44	-0.82	5.48	5.55	5.48	0.04	0.02	5.50
11:40	3	0.58	-0.55	0.51	-0.67	0.61	-0.49	0.09	0.05	0.57	-0.57	5.38	5.46	5.37	0.05	0.03	5.40
12:40	4	0.51	-0.67	0.77	-0.26	0.75	-0.29	0.23	0.13	0.68	-0.41	5.21	5.05	5.20	0.09	0.05	5.15
13:40	5	1.20	0.18	1.30	0.26	1.39	0.33	0.07	0.04	1.30	0.26	4.84	4.69	4.67	0.09	0.05	4.73
14:40	6	2.26	0.82	2.24	0.81	2.30	0.83	0.01	0.01	2.27	0.82	4.52	4.44	4.39	0.07	0.04	4.45
15:40	7	2.66	0.98	2.78	1.02	2.85	1.05	0.03	0.02	2.76	1.02	4.32	4.22	4.21	0.06	0.04	4.25
16:40	8	4.33	1.47	3.90	1.36	3.97	1.38	0.06	0.03	4.07	1.40	4.14	4.04	4.06	0.05	0.03	4.08
17:40	9	4.18	1.43	4.14	1.42	4.36	1.47	0.03	0.02	4.23	1.44	3.99	3.98	3.94	0.03	0.02	3.97
18:40	10	4.96	1.60	4.80	1.57	4.76	1.56	0.02	0.01	4.84	1.58	3.87	3.92	3.88	0.03	0.02	3.89
08:40	24	5.12	1.63	4.99	1.61	5.01	1.61	0.01	0.01	5.04	1.62	3.67	3.71	3.65	0.03	0.02	3.68

4 weeks storage at 4℃

water (control)

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:30	0	0.39	-0.95	0.42	-0.87	0.36	-1.04	0.08	0.05	0.39	-0.95	6.19	6.18	6.21	0.02	0.01	6.19
09:30	1	0.39	-0.95	0.38	-0.98	0.35	-1.05	0.05	0.03	0.37	-0.99	6.19	6.17	6.21	0.02	0.01	6.19
10:30	2	0.66	-0.42	0.39	-0.95	0.39	-0.95	0.30	0.18	0.48	-0.77	6.17	6.17	6.20	0.02	0.01	6.18
11:30	3	0.46	-0.78	0.44	-0.83	0.40	-0.92	0.07	0.04	0.43	-0.84	6.08	6.10	6.13	0.03	0.01	6.10
12:30	4	0.60	-0.52	0.55	-0.60	0.49	-0.71	0.10	0.05	0.55	-0.61	5.90	5.94	5.99	0.05	0.03	5.94
13:30	5	0.79	-0.23	0.71	-0.34	0.64	-0.45	0.11	0.06	0.71	-0.34	5.60	4.72	5.80	0.57	0.33	5.37
14:30	6	1.11	0.10	1.02	0.02	0.94	-0.06	0.08	0.05	1.02	0.02	5.08	5.14	5.27	0.10	0.06	5.16
15:30	7	2.08	0.73	1.86	0.62	1.60	0.47	0.13	0.08	1.85	0.61	4.67	4.68	4.76	0.05	0.03	4.70
16:30	8	3.14	1.14	2.89	1.06	2.94	1.08	0.04	0.03	2.99	1.09	4.36	4.35	4.32	0.02	0.01	4.34
17:30	9	3.57	1.27	3.39	1.22	3.58	1.28	0.03	0.02	3.51	1.26	4.22	4.21	4.24	0.02	0.01	4.22
18:30	10	3.95	1.37	3.75	1.32	3.77	1.33	0.03	0.02	3.82	1.34	3.98	4.01	3.99	0.02	0.01	3.99
08:30	24	5.41	1.69	5.67	1.74	5.21	1.65	0.04	0.02	5.43	1.69	3.72	3.77	3.78	0.03	0.02	3.76

4 weeks storage at RT

water (control)

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:30	0	0.35	-1.06	0.38	-0.98	0.35	-1.04	0.04	0.02	0.36	-1.03	6.19	6.18	6.20	0.01	0.01	6.19
09:30	1	0.36	-1.03	0.37	-0.99	0.37	-1.00	0.02	0.01	0.37	-1.01	6.19	6.17	6.21	0.02	0.01	6.19
10:30	2	0.37	-1.00	0.39	-0.95	0.36	-1.04	0.04	0.02	0.37	-1.00	6.17	6.17	6.17	0.00	0.00	6.17
11:30	3	0.38	-0.96	0.37	-1.00	0.35	-1.06	0.05	0.03	0.37	-1.00	6.13	6.17	6.18	0.03	0.02	6.16
12:30	4	0.42	-0.86	0.37	-1.00	0.35	-1.05	0.10	0.06	0.38	-0.97	6.05	6.15	6.17	0.06	0.04	6.12
13:30	5	0.35	-1.05	0.38	-0.96	0.36	-1.02	0.04	0.02	0.36	-1.01	5.90	5.96	6.19	0.15	0.09	6.02
14:30	6	0.35	-1.05	0.38	-0.97	0.35	-1.05	0.05	0.03	0.36	-1.02	5.90	6.14	6.16	0.14	0.08	6.07
15:30	7	0.35	-1.05	0.39	-0.95	0.35	-1.06	0.06	0.04	0.36	-1.02	5.88	6.13	6.15	0.15	0.09	6.05
16:30	8	0.76	-0.28	0.45	-0.80	0.38	-0.98	0.36	0.21	0.53	-0.68	5.81	6.10	6.13	0.18	0.10	6.01
17:30	9	0.97	-0.03	0.57	-0.56	0.46	-0.79	0.39	0.22	0.67	-0.46	5.23	5.82	5.95	0.38	0.22	5.67
18:30	10	1.64	0.49	0.80	-0.22	0.55	-0.60	0.56	0.32	1.00	-0.11	5.16	5.79	5.90	0.40	0.23	5.62
08:30	24	5.23	1.65	5.20	1.65	5.19	1.65	0.00	0.00	5.21	1.65	3.89	3.91	3.87	0.02	0.01	3.89

8 weeks storage at 4℃

water (control)

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
09:16	0	0.38	-0.96	0.34	-1.09	0.39	-0.94	0.08	0.05	0.37	-1.00	6.51	6.49	6.53	0.02	0.01	6.51
10:16	1	0.38	-0.98	0.38	-0.96	0.39	-0.95	0.01	0.01	0.38	-0.97	6.46	6.43	6.46	0.02	0.01	6.45
11:16	2	0.42	-0.86	0.36	-1.01	0.39	-0.94	0.08	0.04	0.39	-0.94	6.44	6.42	6.48	0.03	0.02	6.45
12:16	3	0.45	-0.80	0.42	-0.86	0.42	-0.86	0.03	0.02	0.43	-0.84	6.44	6.43	6.49	0.03	0.02	6.45
13:16	4	0.52	-0.65	0.50	-0.69	0.47	-0.76	0.05	0.03	0.50	-0.70	6.24	6.29	6.37	0.07	0.04	6.30
14:16	5	0.67	-0.40	0.63	-0.47	0.58	-0.54	0.07	0.04	0.63	-0.47	5.92	6.00	6.15	0.12	0.07	6.02
15:16	6	1.47	0.39	1.40	0.34	1.20	0.18	0.11	0.06	1.36	0.30	5.47	5.45	5.68	0.13	0.07	5.53
16:16	7	1.57	0.45	1.74	0.55	1.48	0.39	0.08	0.05	1.60	0.47	5.00	4.98	5.02	0.02	0.01	5.00
17:16	8	2.55	0.94	2.74	1.01	2.38	0.87	0.07	0.04	2.56	0.94	4.62	4.55	4.66	0.06	0.03	4.61
18:16	9	3.09	1.13	3.11	1.13	3.00	1.10	0.02	0.01	3.07	1.12	4.12	4.00	4.00	0.07	0.04	4.04
19:16	10	3.43	1.23	3.61	1.28	3.33	1.20	0.04	0.02	3.46	1.24	4.00	3.99	3.98	0.01	0.01	3.99
09:16	24	5.15	1.64	5.14	1.64	5.11	1.63	0.00	0.00	5.13	1.64	3.72	3.69	3.69	0.02	0.01	3.70

8 weeks storage at RT

water (control)

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
09:16	0	0.34	-1.08	0.38	-0.96	0.36	-1.03	0.06	0.04	0.36	-1.02	6.53	6.53	6.54	0.01	0.00	6.53
10:16	1	0.36	-1.02	0.39	-0.93	0.35	-1.04	0.06	0.03	0.37	-1.00	6.44	6.46	6.46	0.01	0.01	6.45
11:16	2	0.36	-1.01	0.36	-1.03	0.33	-1.10	0.05	0.03	0.35	-1.05	6.51	6.55	6.54	0.02	0.01	6.53
12:16	3	0.35	-1.04	0.37	-0.99	0.36	-1.03	0.02	0.01	0.36	-1.02	6.53	6.54	6.53	0.01	0.00	6.53
13:16	4	0.39	-0.93	0.37	-0.99	0.36	-1.02	0.04	0.03	0.38	-0.98	6.49	6.53	6.37	0.08	0.05	6.46
14:16	5	0.55	-0.60	0.36	-1.02	0.33	-1.10	0.27	0.16	0.41	-0.91	6.15	6.50	6.51	0.21	0.12	6.39
15:16	6	0.62	-0.48	0.40	-0.92	0.35	-1.06	0.30	0.17	0.46	-0.82	6.09	6.46	6.50	0.23	0.13	6.35
16:16	7	0.89	-0.12	0.39	-0.94	0.35	-1.05	0.51	0.29	0.54	-0.70	5.78	6.31	6.55	0.39	0.23	6.21
17:16	8	1.64	0.49	0.29	-1.23	0.34	-1.07	0.95	0.55	0.76	-0.60	4.98	6.18	6.40	0.76	0.44	5.85
18:16	9	2.12	0.75	0.40	-0.92	0.36	-1.02	0.99	0.57	0.96	-0.40	4.44	6.02	6.33	1.01	0.59	5.60
19:16	10	3.21	1.17	0.42	-0.87	0.38	-0.97	1.20	0.70	1.34	-0.22	4.00	6.01	6.21	1.22	0.71	5.41
09:16	24	5.02	1.61	5.00	1.61	5.05	1.62	0.01	0.00	5.02	1.61	3.68	3.70	3.69	0.01	0.01	3.69

12 weeks storage at 4℃

water

time(h)		OD										pH					
		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
09:16	0	0.365	-1.01	0.357	-1.03	0.366	-1.01	0.01	0.01	0.36	-1.01	6.49	6.51	6.50	0.01	0.01	6.50
10:16	1	0.37	-0.99	0.38	-0.96	0.37	-0.99	0.02	0.01	0.37	-0.98	6.47	6.45	6.44	0.02	0.01	6.45
11:16	2	0.39	-0.94	0.40	-0.92	0.39	-0.94	0.01	0.01	0.39	-0.93	6.44	6.43	6.44	0.01	0.00	6.44
12:16	3	0.42	-0.86	0.42	-0.86	0.42	-0.86	0.00	0.00	0.42	-0.86	6.43	6.43	6.44	0.01	0.00	6.43
13:16	4	0.50	-0.70	0.52	-0.65	0.52	-0.65	0.03	0.02	0.51	-0.67	6.42	6.41	6.42	0.01	0.00	6.42
14:16	5	0.58	-0.54	0.59	-0.53	0.60	-0.51	0.02	0.01	0.59	-0.53	6.14	6.00	5.97	0.09	0.05	6.04
15:16	6	0.77	-0.26	0.79	-0.24	0.77	-0.26	0.01	0.01	0.78	-0.25	6.00	5.90	5.89	0.06	0.04	5.93
17:16	7	1.56	0.44	1.60	0.47	1.48	0.39	0.04	0.02	1.55	0.44	5.55	5.50	5.49	0.03	0.02	5.51
18:16	8	2.00	0.69	2.31	0.84	2.36	0.86	0.09	0.05	2.22	0.80	5.00	5.00	5.00	0.00	0.00	5.00
19:16	9	3.01	1.10	3.01	1.10	3.00	1.10	0.00	0.00	3.01	1.10	4.51	4.50	5.52	0.59	0.34	4.84
20:16	10	3.35	1.21	3.37	1.21	3.32	1.20	0.01	0.00	3.35	1.21	4.04	4.06	4.08	0.02	0.01	4.06
09:16	24	5.05	1.62	5.11	1.63	5.07	1.62	0.01	0.00	5.08	1.62	3.70	3.69	3.69	0.01	0.00	3.69

12 weeks storage at RT

water

time(h)		OD										pH					
		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
09:16	0.00	0.30	-1.20	0.33	-1.11	0.31	-1.17	0.05	0.03	0.31	-1.16	6.53	6.53	6.54	0.01	0.00	6.53
10:16	1.00	0.31	-1.17	0.33	-1.11	0.30	-1.20	0.05	0.03	0.31	-1.16	6.44	6.46	6.46	0.01	0.01	6.45
11:16	2.00	0.31	-1.17	0.32	-1.14	0.33	-1.10	0.04	0.02	0.32	-1.14	6.51	6.55	6.54	0.02	0.01	6.53
12:16	3.00	0.32	-1.13	0.33	-1.11	0.35	-1.05	0.04	0.02	0.33	-1.10	6.53	6.54	6.53	0.01	0.00	6.53
13:16	4.00	0.34	-1.08	0.34	-1.07	0.36	-1.02	0.03	0.02	0.35	-1.05	6.49	6.53	6.37	0.08	0.05	6.46
14:16	5.00	0.36	-1.02	0.37	-0.98	0.37	-1.01	0.02	0.01	0.37	-1.00	6.15	6.50	6.51	0.21	0.12	6.39
15:16	6.00	0.39	-0.95	0.40	-0.92	0.39	-0.94	0.01	0.01	0.39	-0.94	6.09	6.46	6.50	0.23	0.13	6.35
17:16	7.00	0.46	-0.78	0.43	-0.84	0.40	-0.92	0.07	0.04	0.43	-0.85	5.78	6.31	6.55	0.39	0.23	6.21
18:16	8.00	0.48	-0.73	0.40	-0.92	0.43	-0.84	0.09	0.05	0.44	-0.83	4.98	6.18	6.40	0.76	0.44	5.85
19:16	9.00	0.63	-0.46	0.40	-0.92	0.55	-0.60	0.23	0.13	0.53	-0.66	4.44	6.02	6.33	1.01	0.59	5.60
20:16	10.00	0.82	-0.20	0.69	-0.37	0.80	-0.22	0.09	0.05	0.77	-0.26	4.00	6.01	6.21	1.22	0.71	5.41
09:16	24.00	5.04	1.62	5.10	1.63	5.06	1.62	0.01	0.00	5.07	1.62	3.68	3.70	3.69	0.01	0.01	3.69

D. 2 Propagation of freeze dried cells in inulin upon re-growth

Before freeze drying																	
inulin																	
		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:40	0	0.26	-1.34	0.32	-1.16	0.31	-1.17	0.10	0.06	0.30	-1.22	6.04	6.01	6.07	0.03	0.02	6.04
09:40	1	0.36	-1.03	0.38	-0.97	0.40	-0.91	0.06	0.04	0.38	-0.97	5.84	5.81	5.82	0.02	0.01	5.82
10:40	2	1.00	0.00	0.95	-0.05	0.98	-0.02	0.03	0.01	0.98	-0.02	5.37	5.37	5.32	0.03	0.02	5.35
11:40	3	1.16	0.15	1.16	0.15	1.52	0.42	0.16	0.09	1.28	0.24	4.96	4.94	4.92	0.02	0.01	4.94
12:40	4	2.62	0.96	2.51	0.92	2.71	1.00	0.04	0.02	2.61	0.96	4.50	4.50	4.49	0.01	0.00	4.50
13:40	5	3.49	1.25	3.26	1.18	3.39	1.22	0.03	0.02	3.38	1.22	4.28	4.28	4.26	0.01	0.01	4.27
14:40	6	4.01	1.39	3.99	1.38	4.23	1.44	0.03	0.02	4.08	1.40	4.10	4.11	4.11	0.01	0.00	4.11
15:40	7	4.49	1.50	4.33	1.47	4.52	1.51	0.02	0.01	4.45	1.49	4.00	4.00	3.97	0.02	0.01	3.99
16:40	8	5.06	1.62	4.63	1.53	4.93	1.60	0.05	0.03	4.87	1.58	3.94	3.94	3.93	0.01	0.00	3.94
17:40	9	5.25	1.66	5.04	1.62	5.40	1.69	0.03	0.02	5.23	1.65	3.84	3.86	3.92	0.04	0.02	3.87
18:40	10	5.21	1.65	5.16	1.64	5.32	1.67	0.02	0.01	5.23	1.65	3.80	3.80	3.81	0.01	0.00	3.80
08:40	24	4.82	1.57	4.70	1.55	4.70	1.55	0.01	0.01	4.74	1.56	3.69	3.68	3.68	0.01	0.00	3.68
After freeze drying																	
inulin																	
		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:40	0	0.50	-0.69	0.31	-1.16	0.35	-1.04	0.24	0.14	0.39	-0.97	5.67	5.71	5.64	0.04	0.02	5.67
09:40	1	0.34	-1.09	0.32	-1.14	0.39	-0.95	0.10	0.06	0.35	-1.06	5.64	5.67	5.63	0.02	0.01	5.65
10:40	2	0.34	-1.08	0.36	-1.03	0.36	-1.02	0.03	0.02	0.35	-1.04	5.61	5.61	5.62	0.01	0.00	5.61
11:40	3	0.38	-0.96	0.43	-0.83	0.38	-0.97	0.08	0.04	0.40	-0.92	5.60	5.56	5.59	0.02	0.01	5.58
12:40	4	0.47	-0.76	0.64	-0.44	0.44	-0.83	0.21	0.12	0.51	-0.68	5.44	5.26	5.51	0.13	0.07	5.40
13:40	5	0.87	-0.14	1.09	0.09	0.84	-0.18	0.14	0.08	0.93	-0.08	5.20	4.94	5.38	0.22	0.13	5.17
14:40	6	1.12	0.11	1.84	0.61	0.61	0.61	0.29	0.17	1.19	0.44	4.84	4.59	5.04	0.23	0.13	4.82
15:40	7	1.64	0.49	2.44	0.89	1.41	0.34	0.28	0.16	1.83	0.58	4.60	4.34	4.86	0.26	0.15	4.60
16:40	8	2.96	1.09	3.65	1.29	2.33	0.85	0.22	0.13	2.98	1.08	4.32	4.17	4.54	0.19	0.11	4.34
17:40	9	3.47	1.24	4.18	1.43	3.04	1.11	0.16	0.09	3.56	1.26	4.12	4.02	4.25	0.12	0.07	4.13
18:40	10	4.16	1.43	4.76	1.56	3.76	1.32	0.12	0.07	4.23	1.44	4.16	4.06	3.76	0.21	0.12	3.99
08:40	24	4.89	1.59	4.73	1.55	4.88	1.59	0.02	0.01	4.83	1.58	3.87	3.71	3.68	0.10	0.06	3.75

4 weeks storage at 4℃

inulin

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:30	0	0.38	-0.98	0.34	-1.09	0.34	-1.08	0.06	0.04	0.35	-1.05	6.18	6.20	6.19	0.01	0.01	6.19
09:30	1	0.38	-0.97	0.33	-1.10	0.31	-1.16	0.09	0.05	0.34	-1.08	6.17	6.20	6.22	0.03	0.01	6.20
10:30	2	0.37	-0.99	0.34	-1.08	0.33	-1.12	0.06	0.04	0.35	-1.06	6.18	6.17	6.20	0.02	0.01	6.18
11:30	3	0.41	-0.89	0.34	-1.07	0.32	-1.15	0.13	0.08	0.36	-1.03	6.18	6.17	6.20	0.02	0.01	6.18
12:30	4	0.41	-0.88	0.36	-1.02	0.46	-0.78	0.12	0.07	0.41	-0.90	6.14	6.12	6.19	0.04	0.02	6.15
13:30	5	0.47	-0.77	0.38	-0.96	0.41	-0.90	0.10	0.06	0.42	-0.88	6.05	6.11	6.17	0.06	0.03	6.11
14:30	6	0.61	-0.49	0.33	-1.11	0.48	-0.73	0.31	0.18	0.47	-0.78	5.80	5.99	6.17	0.19	0.11	5.99
15:30	7	0.91	-0.10	0.35	-1.05	0.66	-0.42	0.49	0.28	0.64	-0.52	4.46	4.66	6.15	0.92	0.53	5.09
16:30	8	0.93	-0.07	0.39	-0.94	0.46	-0.78	0.46	0.27	0.59	-0.60	4.21	4.66	6.10	0.99	0.57	4.99
17:30	9	1.53	0.43	0.42	-0.87	0.46	-0.78	0.72	0.42	0.80	-0.41	4.16	4.37	5.78	0.88	0.51	4.77
18:30	10	1.92	0.65	0.47	-0.76	0.47	-0.76	0.81	0.47	0.95	-0.29	4.01	4.11	5.69	0.94	0.54	4.60
08:30	24	5.13	1.64	5.15	1.64	5.17	1.64	0.00	0.00	5.15	1.64	3.72	3.77	3.70	0.04	0.02	3.73

4 weeks storage at room temperature

inulin

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:30	0	0.36	-1.01	0.36	-1.02	0.43	-0.85	0.10	0.06	0.38	-0.96	6.19	6.21	6.15	0.03	0.02	6.18
09:30	1	0.37	-1.01	0.34	-1.09	0.39	-0.93	0.08	0.05	0.37	-1.01	6.18	6.23	6.14	0.05	0.03	6.18
10:30	2	0.37	-0.98	0.35	-1.04	0.40	-0.93	0.06	0.03	0.37	-0.98	6.14	6.20	6.12	0.04	0.02	6.15
11:30	3	0.37	-1.00	0.34	-1.08	0.39	-0.94	0.07	0.04	0.37	-1.01	6.17	6.22	6.15	0.04	0.02	6.18
12:30	4	0.37	-1.00	0.34	-1.08	0.41	-0.90	0.09	0.05	0.37	-0.99	6.17	6.22	6.13	0.05	0.03	6.17
13:30	5	0.46	-0.77	0.37	-0.99	0.35	-1.05	0.15	0.08	0.39	-0.94	6.15	6.15	6.17	0.01	0.01	6.16
14:30	6	0.37	-0.99	0.37	-0.99	0.36	-1.02	0.02	0.01	0.37	-1.00	6.14	6.21	6.17	0.04	0.02	6.17
15:30	7	0.35	-1.04	0.32	-1.14	0.32	-1.13	0.05	0.03	0.33	-1.10	6.15	6.18	6.16	0.02	0.01	6.16
16:30	8	0.37	-1.01	0.33	-1.12	0.35	-1.04	0.06	0.03	0.35	-1.06	6.19	6.17	6.23	0.03	0.02	6.20
17:30	9	0.38	-0.97	0.33	-1.10	0.38	-0.96	0.08	0.04	0.37	-1.01	6.15	6.17	6.20	0.03	0.01	6.17
18:30	10	0.39	-0.94	0.34	-1.08	0.49	-0.71	0.19	0.11	0.41	-0.91	6.14	6.15	6.16	0.01	0.01	6.15
08:30	24	4.68	1.54	4.57	1.52	4.51	1.51	0.02	0.01	4.59	1.52	4.00	3.89	3.95	0.06	0.03	3.95

8 weeks storage at 4℃

inulin

time(h)		OD run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	pH run A	run B	run C	SD	SEM	ave pH
09:16	0	0.37	-0.99	0.37	-1.00	0.37	-0.99	0.01	0.00	0.37	-0.99	6.46	6.45	6.51	0.03	0.02	6.47
10:16	1	0.37	-0.99	0.38	-0.96	0.35	-1.05	0.05	0.03	0.37	-1.00	6.39	6.40	6.45	0.03	0.02	6.41
11:16	2	0.36	-1.02	0.36	-1.02	0.35	-1.05	0.02	0.01	0.36	-1.03	6.47	6.49	6.50	0.02	0.01	6.49
12:16	3	0.37	-1.00	0.37	-1.00	0.35	-1.06	0.03	0.02	0.36	-1.02	6.46	6.46	6.45	0.01	0.00	6.46
13:16	4	0.37	-1.00	0.36	-1.02	0.35	-1.06	0.03	0.02	0.36	-1.02	6.47	6.46	6.45	0.01	0.01	6.46
14:16	5	0.37	-0.99	0.35	-1.06	0.31	-1.17	0.09	0.05	0.34	-1.07	6.42	6.44	6.44	0.01	0.01	6.43
15:16	6	0.37	-1.01	0.44	-0.83	0.37	-1.01	0.10	0.06	0.39	-0.95	6.38	6.42	6.45	0.04	0.02	6.42
16:16	7	0.43	-0.85	0.34	-1.08	0.41	-0.90	0.12	0.07	0.39	-0.94	6.27	6.37	6.44	0.09	0.05	6.36
17:16	8	0.49	-0.71	0.40	-0.92	0.41	-0.89	0.12	0.07	0.43	-0.84	6.19	6.33	6.43	0.12	0.07	6.32
18:16	9	0.50	-0.69	0.42	-0.87	0.41	-0.89	0.11	0.06	0.44	-0.82	6.11	6.13	6.20	0.05	0.03	6.15
19:16	10	0.62	-0.48	0.50	-0.69	0.49	-0.71	0.13	0.08	0.54	-0.63	6.00	6.11	6.18	0.09	0.05	6.10
09:16	24	5.53	1.71	5.51	1.71	5.54	1.71	0.00	0.00	5.53	1.71	3.69	3.70	3.76	0.04	0.02	3.72

8 weeks storage at room temperature

inulin

time(h)		OD run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	pH run A	run B	run C	SD	SEM	ave pH
09:16	0	0.40	-0.93	0.37	-1.01	0.34	-1.08	0.08	0.04	0.37	-1.01	6.47	6.51	6.53	0.03	0.02	6.50
10:16	1	0.35	-1.05	0.32	-1.13	0.32	-1.13	0.05	0.03	0.33	-1.10	6.45	6.45	6.48	0.02	0.01	6.46
11:16	2	0.32	-1.13	0.32	-1.13	0.32	-1.15	0.01	0.01	0.32	-1.14	6.47	6.48	6.50	0.02	0.01	6.48
12:16	3	0.34	-1.08	0.39	-0.94	0.34	-1.08	0.08	0.05	0.36	-1.04	6.47	6.48	6.52	0.03	0.02	6.49
13:16	4	0.34	-1.09	0.38	-0.96	0.33	-1.12	0.09	0.05	0.35	-1.06	6.49	6.50	6.53	0.02	0.01	6.51
14:16	5	0.32	-1.14	0.31	-1.18	0.30	-1.22	0.04	0.02	0.31	-1.18	6.43	6.48	6.49	0.03	0.02	6.47
15:16	6	0.36	-1.04	0.32	-1.14	0.33	-1.11	0.05	0.03	0.33	-1.10	6.46	6.46	6.53	0.04	0.02	6.48
16:16	7	0.35	-1.05	0.30	-1.20	0.30	-1.20	0.09	0.05	0.32	-1.15	6.45	6.44	6.50	0.03	0.02	6.46
17:16	8	0.30	-1.19	0.29	-1.24	0.29	-1.25	0.03	0.02	0.29	-1.23	6.41	6.43	6.49	0.04	0.02	6.44
18:16	9	0.31	-1.17	0.33	-1.11	0.32	-1.14	0.03	0.02	0.32	-1.14	6.39	6.39	6.40	0.01	0.00	6.39
19:16	10	0.38	-0.97	0.37	-0.99	0.36	-1.02	0.03	0.02	0.37	-0.99	6.37	6.38	6.38	0.01	0.00	6.38
09:16	24	4.90	1.59	4.97	1.60	5.00	1.61	0.01	0.01	4.96	1.60	3.71	3.70	3.69	0.01	0.01	3.70

12 weeks storage at 4℃																	
inulin																	
		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
09:16	0	0.324	-1.13	0.344	-1.07	0.343	-1.07	0.03	0.02	0.34	-1.09	6.44	6.46	6.47	0.02	0.01	6.46
10:16	1	0.34	-1.07	0.33	-1.11	0.35	-1.06	0.03	0.02	0.34	-1.08	6.44	6.45	6.47	0.02	0.01	6.45
11:16	2	0.34	-1.07	0.35	-1.06	0.35	-1.06	0.00	0.00	0.35	-1.06	6.44	6.45	6.46	0.01	0.01	6.45
12:16	3	0.35	-1.06	0.35	-1.06	0.35	-1.06	0.00	0.00	0.35	-1.06	6.43	6.46	6.45	0.02	0.01	6.45
13:16	4	0.35	-1.06	0.35	-1.06	0.37	-1.01	0.03	0.02	0.35	-1.04	6.43	6.46	6.45	0.02	0.01	6.45
14:16	5	0.35	-1.04	0.35	-1.05	0.38	-0.97	0.04	0.03	0.36	-1.02	6.42	6.45	6.44	0.02	0.01	6.44
15:16	6	0.36	-1.04	0.35	-1.04	0.37	-1.01	0.02	0.01	0.36	-1.03	6.40	6.45	6.45	0.03	0.02	6.43
17:16	7	0.36	-1.01	0.34	-1.08	0.37	-0.98	0.05	0.03	0.36	-1.03	6.39	6.40	6.44	0.03	0.02	6.41
18:16	8	0.36	-1.02	0.36	-1.04	0.38	-0.97	0.03	0.02	0.36	-1.01	6.20	6.35	6.36	0.09	0.05	6.30
19:16	9	0.36	-1.01	0.36	-1.02	0.39	-0.94	0.04	0.02	0.37	-0.99	6.17	6.20	6.20	0.02	0.01	6.19
20:16	10	0.37	-1.01	0.36	-1.02	0.49	-0.71	0.17	0.10	0.41	-0.91	6.00	6.15	6.16	0.09	0.05	6.10
09:16	24	5.40	1.69	5.36	1.68	5.51	1.71	0.01	0.01	5.42	1.69	3.71	3.68	3.69	0.02	0.01	3.69
12 weeks storage at RT																	
inulin																	
		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
09:16	0	0.33	-1.11	0.32	-1.14	0.335	-1.09	0.02	0.01	0.33	-1.11	6.48	6.52	6.55	0.04	0.02	6.52
10:16	1	0.33	-1.12	0.34	-1.09	0.33	-1.10	0.02	0.01	0.33	-1.10	6.46	6.49	6.50	0.02	0.01	6.48
11:16	2	0.32	-1.13	0.33	-1.11	0.32	-1.15	0.02	0.01	0.32	-1.13	6.49	6.49	6.50	0.01	0.00	6.49
12:16	3	0.34	-1.08	0.34	-1.07	0.34	-1.08	0.01	0.01	0.34	-1.08	6.49	6.49	6.51	0.01	0.01	6.50
13:16	4	0.33	-1.11	0.34	-1.08	0.33	-1.12	0.02	0.01	0.33	-1.10	6.49	6.49	6.51	0.01	0.01	6.50
14:16	5	0.33	-1.10	0.33	-1.10	0.31	-1.17	0.04	0.02	0.33	-1.12	6.49	6.49	6.50	0.01	0.00	6.49
15:16	6	0.33	-1.11	0.34	-1.07	0.31	-1.16	0.04	0.03	0.33	-1.11	6.49	6.48	6.50	0.01	0.01	6.49
17:16	7	0.34	-1.07	0.34	-1.08	0.32	-1.14	0.04	0.02	0.33	-1.09	6.48	6.47	6.49	0.01	0.01	6.48
18:16	8	0.35	-1.06	0.35	-1.06	0.35	-1.04	0.01	0.01	0.35	-1.06	6.46	6.46	6.49	0.02	0.01	6.47
19:16	9	0.35	-1.06	0.36	-1.04	0.34	-1.07	0.02	0.01	0.35	-1.05	6.40	6.41	6.45	0.03	0.02	6.42
20:16	10	0.36	-1.02	0.37	-0.99	0.36	-1.02	0.02	0.01	0.36	-1.01	6.38	6.40	6.41	0.02	0.01	6.40
09:16	24	4.84	1.58	4.92	1.59	4.95	1.60	0.01	0.01	4.90	1.59	3.69	3.71	3.72	0.02	0.01	3.71

Before freeze drying

skimmed milk

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:40	0	0.34	-1.09	0.34	-1.09	0.40	-0.92	0.10	0.06	0.36	-1.04	6.07	6.08	6.06	0.01	0.01	6.07
09:40	1	0.46	-0.78	0.44	-0.81	0.47	-0.76	0.03	0.02	0.46	-0.78	5.83	5.86	5.80	0.03	0.02	5.83
10:40	2	1.05	0.05	1.01	0.01	1.09	0.09	0.04	0.02	1.05	0.05	5.33	5.37	5.31	0.03	0.02	5.34
11:40	3	1.55	0.44	1.54	0.43	1.52	0.42	0.01	0.01	1.54	0.43	4.82	4.84	4.80	0.02	0.01	4.82
12:40	4	2.23	0.80	2.43	0.89	3.12	1.14	0.17	0.10	2.59	0.94	4.46	4.48	4.45	0.02	0.01	4.46
13:40	5	2.37	0.86	3.75	1.32	3.17	1.15	0.23	0.13	3.10	1.11	4.29	4.29	4.27	0.01	0.01	4.28
14:40	6	3.87	1.35	4.40	1.48	3.49	1.25	0.12	0.07	3.92	1.36	4.15	4.18	4.15	0.02	0.01	4.16
15:40	7	3.37	1.21	3.87	1.35	3.97	1.38	0.09	0.05	3.74	1.32	4.06	4.06	4.08	0.01	0.01	4.07
16:40	8	4.79	1.57	4.48	1.50	4.59	1.52	0.03	0.02	4.62	1.53	3.98	3.97	3.97	0.01	0.00	3.97
17:40	9	5.00	1.61	4.77	1.56	5.92	1.78	0.11	0.07	5.23	1.65	3.85	3.86	3.93	0.04	0.03	3.88
18:40	10	5.13	1.64	5.29	1.67	4.74	1.56	0.06	0.03	5.05	1.62	3.87	3.86	3.89	0.02	0.01	3.87
08:40	24	4.94	1.60	4.85	1.58	4.88	1.59	0.01	0.01	4.89	1.59	3.76	3.77	3.77	0.01	0.00	3.77

After freeze drying

skimmed milk

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:40	0	0.35	-1.05	0.38	-0.96	0.45	-0.80	0.13	0.07	0.39	-0.94	5.65	5.65	5.68	0.02	0.01	5.66
09:40	1	0.39	-0.95	0.43	-0.85	0.45	-0.79	0.08	0.05	0.42	-0.86	5.55	5.58	5.54	0.02	0.01	5.56
10:40	2	0.64	-0.45	0.70	-0.36	0.74	-0.30	0.08	0.04	0.69	-0.37	5.13	5.08	5.00	0.07	0.04	5.07
11:40	3	1.07	0.07	1.53	0.43	1.35	0.30	0.18	0.10	1.32	0.26	4.92	4.88	4.81	0.06	0.03	4.87
12:40	4	2.04	0.71	2.24	0.81	2.32	0.84	0.07	0.04	2.20	0.79	4.51	4.46	4.45	0.03	0.02	4.47
13:40	5	2.49	0.91	2.41	0.88	2.48	0.91	0.02	0.01	2.46	0.90	4.33	4.29	4.27	0.03	0.02	4.30
14:40	6	3.13	1.14	3.92	1.37	3.09	1.13	0.13	0.08	3.38	1.21	4.16	4.12	4.12	0.02	0.01	4.13
15:40	7	3.59	1.28	3.64	1.29	3.53	1.26	0.02	0.01	3.59	1.28	4.04	4.02	4.02	0.01	0.01	4.03
16:40	8	4.24	1.44	4.99	1.61	5.10	1.63	0.10	0.06	4.78	1.56	3.94	3.93	3.91	0.02	0.01	3.93
17:40	9	4.78	1.56	4.89	1.59	4.96	1.60	0.02	0.01	4.88	1.58	3.87	3.84	3.84	0.02	0.01	3.85
18:40	10	5.25	1.66	5.51	1.71	5.43	1.69	0.02	0.01	5.40	1.69	3.82	3.82	3.82	0.00	0.00	3.82
08:40	24	5.21	1.65	5.06	1.62	5.13	1.64	0.01	0.01	5.13	1.64	3.65	3.66	3.69	0.02	0.01	3.67

4 weeks storage at 4℃

skimmed milk

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:30	0	0.40	-0.92	0.46	-0.77	0.45	-0.80	0.08	0.05	0.44	-0.83	6.20	6.15	6.23	0.04	0.02	6.19
09:30	1	0.44	-0.81	0.49	-0.71	0.47	-0.76	0.05	0.03	0.47	-0.76	6.00	6.09	6.03	0.05	0.03	6.04
10:30	2	0.61	-0.49	0.67	-0.41	0.66	-0.42	0.04	0.03	0.65	-0.44	5.79	5.71	5.68	0.06	0.03	5.73
11:30	3	1.12	0.11	1.26	0.23	1.23	0.21	0.06	0.04	1.20	0.18	5.15	5.22	5.09	0.07	0.04	5.15
12:30	4	2.71	1.00	2.98	1.09	3.15	1.15	0.08	0.04	2.95	1.08	4.76	4.69	4.64	0.06	0.03	4.70
13:30	5	3.07	1.12	3.47	1.24	3.87	1.35	0.12	0.07	3.47	1.24	4.53	4.47	4.45	0.04	0.02	4.48
14:30	6	3.22	1.17	3.63	1.29	4.88	1.59	0.21	0.12	3.91	1.35	4.31	4.29	4.27	0.02	0.01	4.29
15:30	7	4.25	1.45	5.41	1.69	5.60	1.72	0.15	0.09	5.09	1.62	4.19	4.17	4.14	0.03	0.01	4.17
16:30	8	5.48	1.70	4.63	1.53	4.07	1.40	0.15	0.09	4.73	1.55	4.11	4.10	4.09	0.01	0.01	4.10
17:30	9	4.37	1.47	5.38	1.68	6.17	1.82	0.17	0.10	5.31	1.66	4.01	4.03	4.02	0.01	0.01	4.02
18:30	10	4.23	1.44	5.28	1.66	5.15	1.64	0.12	0.07	4.89	1.58	3.88	3.89	3.88	0.01	0.00	3.88
08:30	24	4.78	1.56	4.99	1.61	5.02	1.61	0.03	0.02	4.93	1.60	3.70	3.69	3.68	0.01	0.01	3.69

4 weeks storage at room temperature

skimmed milk

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:30	0	0.44	-0.83	0.45	-0.80	0.44	-0.81	0.02	0.01	0.44	-0.82	6.01	6.05	6.05	0.02	0.01	6.04
09:30	1	0.55	-0.59	0.44	-0.83	0.54	-0.61	0.13	0.08	0.51	-0.68	5.92	6.00	6.04	0.06	0.04	5.99
10:30	2	0.95	-0.05	0.77	-0.26	0.92	-0.08	0.11	0.07	0.88	-0.13	5.46	5.67	5.49	0.11	0.07	5.54
11:30	3	1.55	0.44	1.75	0.56	1.77	0.57	0.07	0.04	1.69	0.52	4.85	4.97	4.85	0.07	0.04	4.89
12:30	4	3.07	1.12	3.57	1.27	3.87	1.35	0.12	0.07	3.50	1.25	4.76	4.69	4.64	0.06	0.03	4.70
13:30	5	3.89	1.36	3.63	1.29	3.99	1.38	0.05	0.03	3.84	1.34	4.53	4.47	4.45	0.04	0.02	4.48
14:30	6	4.01	1.39	3.69	1.31	4.02	1.39	0.05	0.03	3.91	1.36	4.22	4.22	4.23	0.01	0.00	4.22
15:30	7	4.10	1.41	3.78	1.33	4.60	1.53	0.10	0.06	4.16	1.42	4.15	4.14	4.14	0.01	0.00	4.14
16:30	8	4.15	1.42	4.14	1.42	4.14	1.42	0.00	0.00	4.14	1.42	4.07	4.10	4.08	0.02	0.01	4.08
17:30	9	4.37	1.47	5.38	1.68	4.91	1.59	0.10	0.06	4.89	1.58	4.02	4.00	4.02	0.01	0.01	4.01
18:30	10	5.05	1.62	4.82	1.57	5.47	1.70	0.06	0.04	5.11	1.63	3.99	3.91	3.97	0.04	0.02	3.96
08:30	24	5.41	1.69	5.45	1.70	5.44	1.69	0.00	0.00	5.43	1.69	3.67	3.71	3.69	0.02	0.01	3.69

8 weeks storage at 4℃

skimmed milk

time(h)		OD run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	pH run A	run B	run C	SD	SEM	ave pH
09:16	0	0.45	-0.81	0.41	-0.88	0.43	-0.84	0.04	0.02	0.43	-0.84	6.45	6.45	6.42	0.02	0.01	6.44
10:16	1	0.53	-0.63	0.54	-0.61	0.50	-0.69	0.04	0.02	0.53	-0.64	6.21	6.25	6.20	0.03	0.02	6.22
11:16	2	0.81	-0.21	0.72	-0.33	0.86	-0.15	0.09	0.05	0.80	-0.23	5.74	5.77	5.74	0.02	0.01	5.75
12:16	3	1.76	0.57	1.68	0.52	1.61	0.48	0.04	0.03	1.68	0.52	5.03	5.09	5.07	0.03	0.02	5.06
13:16	4	3.01	1.10	3.00	1.10	2.99	1.10	0.00	0.00	3.00	1.10	4.49	4.49	4.48	0.01	0.00	4.49
14:16	5	3.76	1.32	3.47	1.24	3.48	1.25	0.05	0.03	3.57	1.27	4.33	4.45	4.37	0.06	0.04	4.38
15:16	6	4.11	1.41	3.93	1.37	4.99	1.61	0.13	0.07	4.34	1.46	4.19	4.18	4.19	0.01	0.00	4.19
16:16	7	3.90	1.36	4.29	1.46	4.77	1.56	0.10	0.06	4.32	1.46	4.11	4.12	4.11	0.01	0.00	4.11
17:16	8	4.23	1.44	4.10	1.41	4.01	1.39	0.03	0.02	4.11	1.41	4.03	4.00	4.00	0.02	0.01	4.01
18:16	9	4.31	1.46	4.30	1.46	4.67	1.54	0.05	0.03	4.43	1.49	3.98	3.97	3.98	0.01	0.00	3.98
19:16	10	4.51	1.51	4.49	1.50	4.70	1.55	0.03	0.01	4.57	1.52	3.90	3.87	3.88	0.02	0.01	3.88
09:16	24	5.45	1.70	5.43	1.69	5.44	1.69	0.00	0.00	5.44	1.69	3.72	3.70	3.73	0.02	0.01	3.72

8 weeks storage at room temperature

skimmed milk

time(h)		OD run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	pH run A	run B	run C	SD	SEM	ave pH
09:16	0	0.39	-0.95	0.44	-0.82	0.45	-0.80	0.08	0.05	0.43	-0.86	6.50	6.46	6.47	0.02	0.01	6.48
10:16	1	0.45	-0.80	0.53	-0.64	0.53	-0.63	0.09	0.05	0.50	-0.69	6.32	6.22	6.27	0.05	0.03	6.27
11:16	2	0.50	-0.69	0.62	-0.48	0.76	-0.27	0.21	0.12	0.63	-0.48	6.00	6.01	5.85	0.09	0.05	5.95
12:16	3	1.18	0.17	1.26	0.23	1.45	0.37	0.11	0.06	1.30	0.26	5.38	5.27	5.29	0.06	0.03	5.31
13:16	4	2.18	0.78	2.68	0.99	2.46	0.90	0.10	0.06	2.44	0.89	4.74	4.68	4.61	0.07	0.04	4.68
14:16	5	3.45	1.24	3.45	1.24	3.41	1.23	0.01	0.00	3.44	1.23	4.37	4.45	4.37	0.05	0.03	4.40
15:16	6	3.61	1.28	3.77	1.33	3.82	1.34	0.03	0.02	3.73	1.32	4.32	4.28	4.28	0.02	0.01	4.29
16:16	7	3.72	1.31	3.93	1.37	3.83	1.34	0.03	0.02	3.83	1.34	4.20	4.21	4.19	0.01	0.01	4.20
17:16	8	3.62	1.29	4.14	1.42	3.85	1.35	0.07	0.04	3.87	1.35	4.14	4.00	4.09	0.07	0.04	4.08
18:16	9	3.78	1.33	4.17	1.43	3.88	1.36	0.05	0.03	3.94	1.37	3.98	3.99	3.99	0.01	0.00	3.99
19:16	10	4.00	1.39	4.22	1.44	4.10	1.41	0.03	0.02	4.11	1.41	3.90	3.89	3.90	0.01	0.00	3.90
09:16	24	5.21	1.65	5.20	1.65	5.20	1.65	0.00	0.00	5.20	1.65	3.69	3.71	3.70	0.01	0.01	3.70

12 weeks storage at 4℃

skimmed milk

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:30	0	0.447	-0.81	0.413	-0.88	0.43	-0.84	0.04	0.02	0.43	-0.84	6.46	6.43	6.41	0.03	0.01	6.43
09:30	1	0.53	-0.63	0.54	-0.61	0.50	-0.69	0.04	0.02	0.53	-0.64	6.27	6.30	6.20	0.05	0.03	6.26
10:30	2	0.81	-0.21	0.72	-0.33	0.86	-0.15	0.09	0.05	0.80	-0.23	5.76	5.77	5.76	0.01	0.00	5.76
11:30	3	1.76	0.57	1.68	0.52	1.61	0.48	0.04	0.03	1.68	0.52	5.00	5.06	5.08	0.04	0.02	5.05
12:30	4	3.01	1.10	3.00	1.10	2.99	1.10	0.00	0.00	3.00	1.10	4.65	4.64	4.66	0.01	0.01	4.65
13:30	5	3.76	1.32	3.47	1.24	3.48	1.25	0.05	0.03	3.57	1.27	4.36	4.40	4.42	0.03	0.02	4.39
14:30	6	4.11	1.41	3.93	1.37	4.99	1.61	0.13	0.07	4.34	1.46	4.21	4.24	4.22	0.02	0.01	4.22
15:30	7	3.90	1.36	4.29	1.46	4.77	1.56	0.10	0.06	4.32	1.46	4.15	4.14	4.16	0.01	0.01	4.15
16:30	8	4.23	1.44	4.10	1.41	4.01	1.39	0.03	0.02	4.11	1.41	4.05	4.02	4.04	0.02	0.01	4.04
17:30	9	4.31	1.46	4.30	1.46	4.67	1.54	0.05	0.03	4.43	1.49	4.00	4.00	4.02	0.01	0.01	4.01
18:30	10	4.51	1.51	4.49	1.50	4.70	1.55	0.03	0.01	4.57	1.52	3.96	3.80	3.90	0.08	0.05	3.89
09:30	24	5.45	1.70	5.43	1.69	5.44	1.69	0.00	0.00	5.44	1.69	3.69	3.70	3.71	0.01	0.01	3.70

12 weeks storage at RT

skimmed milk

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
09:16	0	0.386	-0.95	0.44	-0.82	0.451	-0.80	0.08	0.05	0.43	-0.86	6.51	6.50	6.49	0.01	0.01	6.50
10:16	1	0.45	-0.80	0.53	-0.64	0.53	-0.63	0.09	0.05	0.50	-0.69	6.35	6.30	6.30	0.03	0.02	6.32
11:16	2	0.50	-0.69	0.62	-0.48	0.76	-0.27	0.21	0.12	0.63	-0.48	6.02	6.01	6.00	0.01	0.01	6.01
12:16	3	1.18	0.17	1.26	0.23	1.45	0.37	0.11	0.06	1.30	0.26	5.55	5.57	5.57	0.01	0.01	5.56
13:16	4	2.18	0.78	2.68	0.99	2.46	0.90	0.10	0.06	2.44	0.89	4.91	4.93	4.92	0.01	0.01	4.92
14:16	5	3.45	1.24	3.45	1.24	3.41	1.23	0.01	0.00	3.44	1.23	4.51	4.50	4.50	0.01	0.00	4.50
15:16	6	3.61	1.28	3.77	1.33	3.82	1.34	0.03	0.02	3.73	1.32	4.40	4.41	4.40	0.01	0.00	4.40
17:16	7	3.72	1.31	3.93	1.37	3.83	1.34	0.03	0.02	3.83	1.34	4.35	4.32	4.30	0.03	0.01	4.32
18:16	8	3.62	1.29	4.14	1.42	3.85	1.35	0.07	0.04	3.87	1.35	4.22	4.24	4.22	0.01	0.01	4.23
19:16	9	3.78	1.33	4.17	1.43	3.88	1.36	0.05	0.03	3.94	1.37	4.00	4.03	4.04	0.02	0.01	4.02
20:16	10	4.00	1.39	4.22	1.44	4.10	1.41	0.03	0.02	4.11	1.41	3.96	3.98	3.99	0.02	0.01	3.98
09:16	24	5.21	1.65	5.20	1.65	5.20	1.65	0.00	0.00	5.20	1.65	3.74	3.73	3.70	0.02	0.01	3.72

D. 4 Propagation of freeze dried cells in maltodexrin upon re-growth

Before freeze drying

maltodextrin

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:40	0	0.93	-0.08	0.37	-0.99	0.33	-1.11	0.57	0.33	0.54	-0.73	5.99	6.08	6.10	0.06	0.03	6.06
09:40	1	0.71	-0.34	0.42	-0.86	0.41	-0.90	0.31	0.18	0.51	-0.70	5.71	5.94	5.92	0.13	0.07	5.86
10:40	2	1.09	0.08	0.74	-0.31	0.71	-0.35	0.24	0.14	0.84	-0.19	5.14	5.51	5.54	0.22	0.13	5.40
11:40	3	2.17	0.77	1.36	0.31	1.45	0.37	0.25	0.15	1.66	0.48	4.59	4.96	4.97	0.22	0.13	4.84
12:40	4	2.67	0.98	1.88	0.63	1.89	0.64	0.20	0.12	2.15	0.75	4.46	4.54	4.58	0.06	0.04	4.53
13:40	5	3.85	1.35	3.28	1.19	3.22	1.17	0.10	0.06	3.45	1.24	4.16	4.28	4.38	0.11	0.06	4.27
14:40	6	4.30	1.46	3.73	1.32	3.68	1.30	0.09	0.05	3.90	1.36	4.04	4.14	4.12	0.05	0.03	4.10
15:40	7	4.69	1.55	4.24	1.44	4.18	1.43	0.06	0.04	4.37	1.47	3.94	4.02	4.05	0.06	0.03	4.00
16:40	8	5.31	1.67	4.66	1.54	4.94	1.60	0.07	0.04	4.97	1.60	3.91	3.94	3.96	0.03	0.01	3.94
17:40	9	5.34	1.68	4.98	1.61	4.95	1.60	0.04	0.02	5.09	1.63	3.81	3.91	3.93	0.06	0.04	3.88
18:40	10	5.35	1.68	5.25	1.66	5.10	1.63	0.02	0.01	5.23	1.65	3.80	3.84	3.84	0.02	0.01	3.83
08:40	24	5.45	1.70	5.56	1.72	5.32	1.67	0.02	0.01	5.44	1.69	3.66	3.65	3.64	0.01	0.01	3.65

After freeze drying

maltodextrin

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:40	0	0.13	-2.07	0.38	-0.98	0.35	-1.05	0.61	0.35	0.28	-1.37	6.11	6.07	6.12	0.03	0.02	6.10
09:40	1	0.13	-2.06	0.39	-0.95	0.34	-1.07	0.61	0.35	0.29	-1.36	6.08	6.02	6.04	0.03	0.02	6.05
10:40	2	0.20	-1.62	0.42	-0.87	0.42	-0.87	0.44	0.25	0.35	-1.12	6.03	5.90	5.94	0.07	0.04	5.96
11:40	3	0.20	-1.62	0.70	-0.36	0.61	-0.49	0.69	0.40	0.50	-0.82	6.00	5.60	5.62	0.23	0.13	5.74
12:40	4	0.33	-1.11	1.29	0.25	1.21	0.19	0.77	0.44	0.94	-0.22	5.83	5.20	5.33	0.33	0.19	5.45
13:40	5	0.64	-0.45	1.94	0.66	1.70	0.53	0.61	0.35	1.43	0.25	5.76	4.76	4.81	0.56	0.33	5.11
14:40	6	1.07	0.07	2.76	1.02	2.04	0.71	0.48	0.28	1.96	0.60	5.17	4.39	4.45	0.43	0.25	4.67
15:40	7	1.55	0.44	3.38	1.22	3.17	1.15	0.43	0.25	2.70	0.94	4.82	4.26	4.27	0.32	0.19	4.45
16:40	8	2.48	0.91	3.72	1.31	3.66	1.30	0.23	0.13	3.29	1.17	4.47	4.12	4.17	0.19	0.11	4.25
17:40	9	3.08	1.12	4.24	1.44	4.07	1.40	0.17	0.10	3.80	1.32	4.29	4.00	4.03	0.16	0.09	4.11
18:40	10	3.67	1.30	4.45	1.49	4.29	1.46	0.10	0.06	4.14	1.42	4.15	3.94	3.94	0.12	0.07	4.01
08:40	24	5.06	1.62	5.09	1.63	4.89	1.59	0.02	0.01	5.01	1.61	3.68	3.68	3.69	0.01	0.00	3.68

4 weeks storage at 4℃

maltodextrin

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
09:16	0	0.32	-1.15	0.33	-1.10	0.28	-1.29	0.10	0.06	0.31	-1.18	5.82	5.84	5.82	0.01	0.01	5.83
10:16	1	0.35	-1.06	0.30	-1.21	0.40	-0.91	0.15	0.09	0.35	-1.06	5.81	5.78	5.82	0.02	0.01	5.80
11:16	2	0.35	-1.06	0.39	-0.95	0.32	-1.15	0.10	0.06	0.35	-1.05	5.76	5.76	5.76	0.00	0.00	5.76
12:16	3	0.42	-0.88	0.47	-0.76	0.42	-0.87	0.07	0.04	0.43	-0.83	5.68	5.64	5.67	0.02	0.01	5.66
13:16	4	0.59	-0.53	0.60	-0.52	0.60	-0.52	0.01	0.01	0.59	-0.52	5.48	5.42	5.41	0.04	0.02	5.44
14:16	5	1.66	0.51	1.03	0.03	1.20	0.18	0.24	0.14	1.30	0.24	5.17	5.05	5.07	0.06	0.04	5.10
15:16	6	2.67	0.98	2.76	1.02	2.63	0.97	0.02	0.01	2.69	0.99	4.76	4.74	4.67	0.05	0.03	4.72
16:16	7	2.94	1.08	2.89	1.06	3.02	1.11	0.02	0.01	2.95	1.08	4.37	4.36	4.32	0.03	0.02	4.35
17:16	8	3.15	1.15	3.21	1.17	3.65	1.29	0.08	0.05	3.34	1.20	4.26	4.24	4.20	0.03	0.02	4.23
18:16	9	4.10	1.41	4.15	1.42	4.34	1.47	0.03	0.02	4.20	1.43	4.11	4.10	4.07	0.02	0.01	4.09
19:16	10	5.07	1.62	4.50	1.50	4.61	1.53	0.06	0.04	4.73	1.55	4.02	3.95	4.00	0.04	0.02	3.99
09:16	24	5.93	1.78	5.63	1.73	5.93	1.78	0.03	0.02	5.83	1.76	3.63	3.64	3.63	0.01	0.00	3.63

4 weeks storage at room temperature

maltodextrin

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
09:16	0	0.44	-0.83	0.20	-1.60	0.16	-1.84	0.53	0.30	0.27	-1.43	5.85	5.83	5.82	0.02	0.01	5.83
10:16	1	0.43	-0.85	0.43	-0.85	0.17	-1.78	0.54	0.31	0.34	-1.16	5.84	5.84	5.81	0.02	0.01	5.83
11:16	2	0.42	-0.86	0.45	-0.81	0.22	-1.51	0.39	0.23	0.36	-1.06	5.80	5.76	5.79	0.02	0.01	5.78
12:16	3	0.45	-0.80	0.48	-0.74	0.23	-1.48	0.41	0.24	0.38	-1.01	5.66	5.71	5.75	0.05	0.03	5.71
13:16	4	0.55	-0.60	0.55	-0.60	0.27	-1.32	0.42	0.24	0.46	-0.84	5.62	5.61	5.71	0.06	0.03	5.65
14:16	5	0.62	-0.47	0.64	-0.45	0.35	-1.06	0.35	0.20	0.54	-0.66	5.48	5.43	5.65	0.12	0.07	5.52
15:16	6	1.03	0.03	1.01	0.01	0.44	-0.83	0.49	0.28	0.83	-0.26	5.17	5.13	5.84	0.40	0.23	5.38
16:16	7	1.48	0.39	1.47	0.39	0.72	-0.33	0.41	0.24	1.22	0.15	4.73	4.70	5.09	0.22	0.13	4.84
17:16	8	2.02	0.70	1.93	0.66	1.06	0.06	0.36	0.21	1.67	0.47	4.59	4.62	4.94	0.19	0.11	4.72
18:16	9	2.65	0.97	2.67	0.98	1.68	0.52	0.27	0.15	2.33	0.83	4.39	4.36	4.65	0.16	0.09	4.47
19:16	10	3.27	1.18	3.40	1.22	2.28	0.82	0.22	0.13	2.98	1.08	4.24	4.23	4.44	0.12	0.07	4.30
09:16	24	5.76	1.75	6.17	1.82	4.89	1.59	0.12	0.07	5.61	1.72	3.65	3.63	3.67	0.02	0.01	3.65

8 weeks storage at 4℃

maltodextrin

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
09:16	0	0.29	-1.23	0.34	-1.08	0.34	-1.07	0.09	0.05	0.33	-1.13	6.15	6.13	6.17	0.02	0.01	6.15
10:16	1	0.31	-1.17	0.33	-1.10	0.34	-1.08	0.05	0.03	0.33	-1.12	6.13	6.09	6.10	0.02	0.01	6.11
11:16	2	0.33	-1.11	0.38	-0.97	0.28	-1.28	0.16	0.09	0.33	-1.12	6.04	6.02	5.95	0.05	0.03	6.00
12:16	3	0.56	-0.58	0.54	-0.62	0.62	-0.48	0.07	0.04	0.57	-0.56	5.70	5.74	5.60	0.07	0.04	5.68
13:16	4	1.34	0.29	0.86	-0.15	1.11	0.10	0.22	0.13	1.10	0.08	5.29	5.31	5.32	0.02	0.01	5.31
14:16	5	1.57	0.45	1.40	0.34	1.81	0.59	0.13	0.07	1.59	0.46	5.01	5.05	5.04	0.02	0.01	5.03
15:16	6	1.72	0.54	1.65	0.50	1.99	0.69	0.10	0.06	1.79	0.58	4.89	4.90	4.88	0.01	0.01	4.89
16:16	7	2.50	0.92	2.26	0.82	2.52	0.92	0.06	0.04	2.43	0.89	4.61	4.64	4.57	0.04	0.02	4.61
17:16	8	2.86	1.05	2.88	1.06	2.78	1.02	0.02	0.01	2.84	1.04	4.43	4.44	4.44	0.01	0.00	4.44
18:16	9	3.00	1.10	2.98	1.09	2.78	1.02	0.04	0.02	2.92	1.07	4.37	4.40	4.39	0.02	0.01	4.39
19:16	10	3.13	1.14	3.02	1.11	3.03	1.11	0.02	0.01	3.06	1.12	4.32	4.33	4.30	0.02	0.01	4.32
10:16	24	4.66	1.54	4.57	1.52	4.77	1.56	0.02	0.01	4.67	1.54	3.99	4.01	4.00	0.01	0.01	4.00

8 weeks storage at room temperature

maltodextrin

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
09:16	0	0.29	-1.24	0.34	-1.09	0.33	-1.11	0.08	0.05	0.32	-1.14	6.22	6.20	6.16	0.03	0.02	6.19
10:16	1	0.28	-1.28	0.31	-1.16	0.24	-1.44	0.14	0.08	0.28	-1.29	6.21	6.22	6.16	0.03	0.02	6.20
11:16	2	0.28	-1.28	0.38	-0.97	0.33	-1.11	0.16	0.09	0.33	-1.12	6.20	6.15	6.11	0.05	0.03	6.15
12:16	3	0.32	-1.16	0.37	-1.01	0.37	-1.01	0.09	0.05	0.35	-1.06	6.15	6.16	6.14	0.01	0.01	6.15
13:16	4	0.32	-1.14	0.38	-0.96	0.38	-0.98	0.10	0.06	0.36	-1.03	6.15	6.12	6.11	0.02	0.01	6.13
14:16	5	0.32	-1.13	0.34	-1.09	0.40	-0.92	0.11	0.06	0.35	-1.05	5.98	6.11	6.11	0.08	0.04	6.07
15:16	6	0.24	-1.45	0.33	-1.11	0.33	-1.10	0.20	0.11	0.30	-1.22	5.65	6.02	6.09	0.24	0.14	5.92
16:16	7	0.24	-1.42	0.36	-1.02	0.39	-0.94	0.26	0.15	0.33	-1.13	5.21	6.01	6.05	0.47	0.27	5.76
17:16	8	0.24	-1.41	0.46	-0.78	0.32	-1.14	0.32	0.18	0.34	-1.11	4.98	5.93	6.04	0.72	0.42	5.46
18:16	9	0.27	-1.32	0.59	-0.53	0.35	-1.05	0.40	0.23	0.40	-0.97	4.65	5.70	6.03	0.72	0.42	5.46
19:16	10	0.35	-1.04	0.61	-0.49	0.48	-0.74	0.27	0.16	0.48	-0.76	4.49	5.36	6.01	0.76	0.44	5.29
09:16	24	5.35	1.68	5.37	1.68	5.61	1.72	0.03	0.02	5.44	1.69	4.10	4.09	4.15	0.03	0.02	4.11

12 weeks storage at 4℃																	
maltodextrin																	
		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:30	0	0.33	-1.12	0.35	-1.06	0.35	-1.06	0.03	0.02	0.34	-1.08	6.20	6.22	6.22	0.01	0.01	6.21
09:30	1	0.32	-1.15	0.33	-1.11	0.35	-1.05	0.05	0.03	0.33	-1.11	6.17	6.19	6.20	0.02	0.01	6.19
10:30	2	0.32	-1.13	0.37	-1.00	0.37	-1.01	0.07	0.04	0.35	-1.05	6.12	6.12	6.12	0.00	0.00	6.12
11:30	3	0.36	-1.01	0.42	-0.86	0.41	-0.89	0.08	0.05	0.40	-0.92	6.09	6.09	6.09	0.00	0.00	6.09
12:30	4	0.46	-0.77	0.52	-0.66	0.51	-0.68	0.06	0.03	0.50	-0.70	5.96	5.90	5.94	0.03	0.02	5.93
13:30	5	0.64	-0.45	0.72	-0.33	0.70	-0.36	0.06	0.04	0.69	-0.38	5.70	5.60	5.66	0.05	0.03	5.65
14:30	6	0.86	-0.15	1.07	0.07	0.88	-0.13	0.12	0.07	0.94	-0.07	5.31	5.18	5.28	0.07	0.04	5.26
15:30	7	1.57	0.45	1.65	0.50	1.55	0.44	0.03	0.02	1.59	0.46	4.95	4.81	4.88	0.07	0.04	4.88
16:30	8	2.00	0.69	2.44	0.89	2.28	0.82	0.10	0.06	2.24	0.80	4.69	4.58	4.64	0.06	0.03	4.64
17:30	9	2.56	0.94	2.88	1.06	2.93	1.08	0.07	0.04	2.79	1.02	4.50	4.41	4.45	0.05	0.03	4.45
18:30	10	2.85	1.05	3.24	1.18	3.79	1.33	0.14	0.08	3.29	1.19	4.38	4.28	4.31	0.05	0.03	4.32
08:30	24	5.10	1.63	4.99	1.61	5.00	1.61	0.01	0.01	5.03	1.62	3.69	3.68	3.66	0.02	0.01	3.68
12 weeks storage at RT																	
maltodextrin																	
		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:30	0	0.32	-1.15	0.31	-1.17	0.34	-1.07	0.05	0.03	0.32	-1.13	6.21	6.19	6.14	0.04	0.02	6.18
09:30	1	0.32	-1.15	0.35	-1.06	0.35	-1.06	0.05	0.03	0.34	-1.09	6.16	6.20	6.12	0.04	0.02	6.16
10:30	2	0.39	-0.93	0.41	-0.90	0.35	-1.05	0.08	0.05	0.38	-0.96	6.17	6.14	6.08	0.05	0.03	6.13
11:30	3	0.37	-1.00	0.35	-1.04	0.37	-1.01	0.02	0.01	0.36	-1.02	6.16	6.11	6.04	0.06	0.03	6.10
12:30	4	0.31	-1.16	0.35	-1.04	0.37	-0.99	0.09	0.05	0.35	-1.06	6.15	6.16	6.05	0.06	0.04	6.12
13:30	5	0.32	-1.14	0.36	-1.04	0.37	-0.99	0.08	0.04	0.35	-1.05	6.16	6.02	6.05	0.07	0.04	6.08
14:30	6	0.23	-1.47	0.31	-1.18	0.39	-0.95	0.26	0.15	0.31	-1.20	6.14	6.14	5.99	0.09	0.05	6.09
15:30	7	0.30	-1.19	0.34	-1.08	0.43	-0.86	0.17	0.10	0.36	-1.04	6.11	6.11	5.92	0.11	0.06	6.05
16:30	8	0.30	-1.19	0.35	-1.05	0.48	-0.74	0.23	0.13	0.38	-0.99	6.10	6.09	5.86	0.14	0.08	6.02
17:30	9	0.31	-1.17	0.32	-1.16	0.54	-0.62	0.31	0.18	0.39	-0.98	6.12	6.08	5.70	0.23	0.13	5.97
18:30	10	0.58	-0.54	0.43	-0.85	0.57	-0.56	0.17	0.10	0.53	-0.65	6.06	6.03	5.54	0.29	0.17	5.88
08:30	24	5.12	1.63	5.10	1.63	5.33	1.67	0.02	0.01	5.18	1.65	3.87	3.88	3.85	0.02	0.01	3.87

D. 5 Propagation of freeze dried cells in sucrose upon re-growth

Before freeze drying

sucrose

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:40	0	0.17	-1.78	0.22	-1.52	0.34	-1.08	0.35	0.20	0.24	-1.46	6.11	6.08	6.09	0.02	0.01	6.09
09:40	1	0.22	-1.53	0.26	-1.35	0.42	-0.87	0.34	0.20	0.30	-1.25	5.95	5.88	5.87	0.04	0.03	5.90
10:40	2	0.34	-1.09	0.47	-0.77	0.70	-0.36	0.37	0.21	0.50	-0.74	5.45	5.46	5.45	0.01	0.00	5.45
11:40	3	0.47	-0.76	0.81	-0.21	1.19	0.17	0.47	0.27	0.82	-0.26	4.94	4.94	4.91	0.02	0.01	4.93
12:40	4	0.83	-0.19	1.17	0.16	1.85	0.62	0.40	0.23	1.28	0.20	4.56	4.52	4.54	0.02	0.01	4.54
13:40	5	2.26	0.82	2.70	0.99	3.22	1.17	0.18	0.10	2.73	0.99	4.27	4.28	4.30	0.02	0.01	4.28
14:40	6	3.03	1.11	3.30	1.19	3.60	1.28	0.09	0.05	3.31	1.19	4.16	4.13	4.13	0.02	0.01	4.14
15:40	7	3.58	1.28	3.93	1.37	4.38	1.48	0.10	0.06	3.96	1.37	4.01	4.01	4.04	0.02	0.01	4.02
16:40	8	4.34	1.47	4.29	1.46	4.75	1.56	0.06	0.03	4.46	1.49	3.96	3.96	3.95	0.01	0.00	3.96
17:40	9	4.44	1.49	4.80	1.57	4.81	1.57	0.05	0.03	4.68	1.54	3.91	3.89	3.90	0.01	0.01	3.90
18:40	10	4.79	1.57	5.09	1.63	4.98	1.61	0.03	0.02	4.95	1.60	3.85	3.84	3.85	0.01	0.00	3.85
08:40	24	5.20	1.65	5.33	1.67	5.51	1.71	0.03	0.02	5.35	1.68	3.62	3.65	3.67	0.03	0.01	3.65

After freeze drying

sucrose

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:40	0	0.35	-1.05	0.38	-0.97	0.38	-0.96	0.05	0.03	0.37	-1.00	6.05	6.03	6.05	0.01	0.01	6.04
09:40	1	0.35	-1.05	0.39	-0.95	0.38	-0.97	0.05	0.03	0.37	-0.99	6.01	5.94	5.97	0.04	0.02	5.97
10:40	2	0.52	-0.66	0.62	-0.48	0.55	-0.60	0.09	0.05	0.56	-0.58	5.94	5.78	5.79	0.09	0.05	5.84
11:40	3	0.79	-0.23	0.86	-0.15	0.84	-0.17	0.04	0.02	0.83	-0.18	5.43	5.35	5.41	0.04	0.02	5.40
12:40	4	1.60	0.47	1.72	0.54	1.62	0.48	0.04	0.02	1.65	0.50	4.99	4.93	4.97	0.03	0.02	4.96
13:40	5	2.39	0.87	2.52	0.92	2.40	0.88	0.03	0.02	2.44	0.89	4.57	4.50	4.53	0.04	0.02	4.53
14:40	6	3.13	1.14	3.32	1.20	3.21	1.17	0.03	0.02	3.22	1.17	4.32	4.47	4.33	0.08	0.05	4.37
15:40	7	3.56	1.27	3.64	1.29	3.62	1.29	0.01	0.01	3.61	1.28	4.19	4.15	4.19	0.02	0.01	4.18
16:40	8	4.01	1.39	4.02	1.39	4.06	1.40	0.01	0.00	4.03	1.39	4.07	4.05	4.05	0.01	0.01	4.06
17:40	9	4.32	1.46	4.38	1.48	4.30	1.46	0.01	0.01	4.33	1.47	3.97	3.96	3.97	0.01	0.00	3.97
18:40	10	4.08	1.41	4.11	1.41	4.13	1.42	0.01	0.00	4.11	1.41	3.90	3.91	3.89	0.01	0.01	3.90
08:40	24	5.25	1.66	5.18	1.64	5.29	1.67	0.01	0.01	5.24	1.66	3.62	3.64	3.63	0.01	0.01	3.63

4 weeks storage at 4℃

sucrose

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
09:16	0	0.30	-1.19	0.31	-1.17	0.30	-1.19	0.01	0.01	0.31	-1.19	5.84	5.79	5.78	0.03	0.02	5.80
10:16	1	0.45	-0.79	0.36	-1.02	0.36	-1.02	0.13	0.07	0.39	-0.94	5.82	5.75	5.72	0.05	0.03	5.76
11:16	2	0.21	-1.58	0.38	-0.96	0.45	-0.79	0.41	0.24	0.35	-1.11	5.77	5.66	5.62	0.08	0.04	5.68
12:16	3	0.31	-1.18	0.55	-0.60	0.61	-0.50	0.37	0.21	0.49	-0.76	5.65	5.48	5.41	0.12	0.07	5.51
13:16	4	0.53	-0.64	1.03	0.03	1.20	0.18	0.44	0.25	0.92	-0.14	5.40	5.13	5.07	0.18	0.10	5.20
14:16	5	1.08	0.08	1.55	0.44	1.19	0.18	0.19	0.11	1.27	0.23	5.04	4.69	4.68	0.21	0.12	4.80
15:16	6	1.86	0.62	2.46	0.90	2.75	1.01	0.20	0.12	2.36	0.84	4.65	4.43	4.40	0.14	0.08	4.49
16:16	7	2.72	1.00	2.99	1.10	3.65	1.29	0.15	0.09	3.12	1.13	4.31	4.18	4.13	0.09	0.05	4.21
17:16	8	3.46	1.24	4.15	1.42	4.75	1.56	0.16	0.09	4.12	1.41	4.20	4.07	4.04	0.09	0.05	4.10
18:16	9	4.46	1.50	4.78	1.56	4.79	1.57	0.04	0.02	4.68	1.54	4.07	4.01	3.99	0.04	0.02	4.02
19:16	10	5.07	1.62	4.94	1.60	5.04	1.62	0.01	0.01	5.02	1.61	3.94	3.88	3.82	0.06	0.03	3.88
09:16	24	6.05	1.80	5.88	1.77	3.55	1.27	0.30	0.17	5.16	1.61	3.61	3.58	3.55	0.03	0.02	3.58

4 weeks storage at room temperature

sucrose

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
09:16	0	0.31	-1.17	0.30	-1.22	0.33	-1.12	0.05	0.03	0.31	-1.17	5.81	5.84	5.84	0.02	0.01	5.83
10:16	1	0.31	-1.18	0.29	-1.26	0.30	-1.19	0.04	0.02	0.30	-1.21	5.81	5.84	5.85	0.02	0.01	5.83
11:16	2	0.31	-1.17	0.31	-1.17	0.28	-1.27	0.06	0.03	0.30	-1.20	5.79	5.81	5.83	0.02	0.01	5.81
12:16	3	0.34	-1.09	0.31	-1.17	0.29	-1.24	0.08	0.04	0.31	-1.17	5.77	5.80	5.81	0.02	0.01	5.79
13:16	4	0.35	-1.06	0.33	-1.11	0.31	-1.19	0.07	0.04	0.33	-1.12	5.75	5.78	5.81	0.03	0.02	5.78
14:16	5	0.36	-1.02	0.34	-1.08	0.32	-1.14	0.06	0.03	0.34	-1.08	5.71	5.76	5.76	0.03	0.02	5.74
15:16	6	0.41	-0.90	0.38	-0.98	0.37	-1.00	0.05	0.03	0.38	-0.96	5.66	5.71	5.70	0.03	0.02	5.69
16:16	7	0.49	-0.71	0.45	-0.80	0.45	-0.80	0.06	0.03	0.46	-0.77	5.45	5.56	5.48	0.06	0.03	5.50
17:16	8	0.62	-0.49	0.54	-0.62	0.58	-0.54	0.07	0.04	0.58	-0.55	5.33	5.45	4.39	0.58	0.34	5.06
18:16	9	1.10	0.10	0.84	-0.17	0.88	-0.13	0.14	0.08	0.94	-0.07	5.07	5.20	5.13	0.07	0.04	5.13
19:16	10	1.50	0.41	1.21	0.19	1.39	0.33	0.11	0.06	1.37	0.31	4.79	4.92	4.88	0.07	0.04	4.86
09:16	24	6.00	1.79	5.94	1.78	5.91	1.78	0.01	0.00	5.95	1.78	3.65	3.65	3.67	0.01	0.01	3.66

8 weeks storage at 4℃

sucrose

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
09:16	0	0.31	-1.16	0.42	-0.87	0.34	-1.07	0.15	0.09	0.36	-1.03	6.13	6.15	6.11	0.02	0.01	6.13
10:16	1	0.39	-0.95	0.41	-0.89	0.40	-0.92	0.03	0.02	0.40	-0.92	6.04	6.01	6.02	0.02	0.01	6.02
11:16	2	0.51	-0.68	0.54	-0.63	0.52	-0.65	0.03	0.02	0.52	-0.65	5.80	5.82	5.81	0.01	0.01	5.81
12:16	3	0.98	-0.02	1.03	0.03	1.06	0.06	0.04	0.02	1.02	0.02	5.22	5.21	5.27	0.03	0.02	5.23
13:16	4	2.18	0.78	2.38	0.87	2.11	0.75	0.06	0.04	2.22	0.80	4.83	4.88	4.87	0.03	0.02	4.86
14:16	5	2.26	0.82	2.65	0.97	2.66	0.98	0.09	0.05	2.52	0.92	4.71	4.70	4.70	0.01	0.00	4.70
15:16	6	2.40	0.88	2.98	1.09	2.68	0.99	0.11	0.06	2.69	0.98	4.55	4.53	4.52	0.02	0.01	4.53
16:16	7	2.97	1.09	3.06	1.12	3.40	1.22	0.07	0.04	3.14	1.14	4.47	4.46	4.45	0.01	0.01	4.46
17:16	8	4.41	1.48	3.15	1.15	3.20	1.16	0.19	0.11	3.59	1.26	4.31	4.30	4.31	0.01	0.00	4.26
18:16	9	3.04	1.11	3.33	1.20	3.22	1.17	0.05	0.03	3.20	1.16	4.25	4.26	4.28	0.02	0.01	4.24
19:16	10	3.14	1.14	3.13	1.14	3.23	1.17	0.02	0.01	3.17	1.15	4.22	4.24	4.26	0.02	0.01	4.24
09:16	24	4.94	1.60	4.98	1.61	4.98	1.61	0.00	0.00	4.97	1.60	4.02	3.99	4.03	0.02	0.01	4.01

8 weeks storage at room temperature

sucrose

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
09:16	0	0.33	-1.12	0.30	-1.22	0.32	-1.14	0.05	0.03	0.31	-1.16	6.12	6.23	6.19	0.06	0.03	6.18
10:16	1	0.33	-1.11	0.30	-1.20	0.27	-1.30	0.09	0.05	0.30	-1.20	6.15	6.24	6.10	0.07	0.04	6.16
11:16	2	0.31	-1.16	0.28	-1.28	0.32	-1.14	0.07	0.04	0.30	-1.19	6.14	6.25	6.08	0.09	0.05	6.16
12:16	3	0.34	-1.08	0.30	-1.21	0.34	-1.08	0.08	0.04	0.33	-1.12	6.13	6.25	6.08	0.09	0.05	6.15
13:16	4	0.29	-1.24	0.32	-1.14	0.35	-1.04	0.10	0.06	0.32	-1.14	6.13	6.24	6.03	0.11	0.06	6.13
14:16	5	0.35	-1.06	0.29	-1.24	0.35	-1.04	0.11	0.06	0.33	-1.11	6.13	6.23	6.03	0.10	0.06	6.13
15:16	6	0.32	-1.13	0.21	-1.57	0.29	-1.23	0.23	0.13	0.27	-1.31	6.12	6.22	6.03	0.10	0.05	6.12
16:16	7	0.29	-1.22	0.18	-1.71	0.30	-1.19	0.29	0.17	0.26	-1.38	6.12	6.22	6.02	0.10	0.06	6.12
17:16	8	0.30	-1.20	0.17	-1.77	0.33	-1.11	0.36	0.21	0.27	-1.36	6.10	6.18	6.00	0.09	0.05	6.09
18:16	9	0.35	-1.05	0.16	-1.81	0.40	-0.91	0.48	0.28	0.31	-1.26	6.08	6.11	5.98	0.07	0.04	6.06
19:16	10	0.53	-0.63	0.48	-0.74	0.61	-0.50	0.12	0.07	0.54	-0.62	5.85	6.45	5.69	0.40	0.23	6.00
09:16	24	5.14	1.64	5.31	1.67	5.02	1.61	0.03	0.02	5.16	1.64	3.96	3.97	3.98	0.01	2.29	3.97

12 weeks storage at 4℃

sucrose

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:30	0	0.33	-1.11	0.37	-1.00	0.39	-0.95	0.08	0.05	0.36	-1.02	6.19	6.16	6.11	0.04	0.02	6.15
09:30	1	0.33	-1.12	0.41	-0.90	0.39	-0.94	0.12	0.07	0.37	-0.99	6.16	6.08	6.03	0.07	0.04	6.09
10:30	2	0.39	-0.95	0.42	-0.86	0.45	-0.81	0.07	0.04	0.42	-0.87	6.04	5.98	5.93	0.06	0.03	5.98
11:30	3	0.50	-0.70	0.56	-0.58	0.56	-0.59	0.06	0.04	0.54	-0.62	5.87	5.83	5.80	0.04	0.02	5.83
12:30	4	0.74	-0.30	0.77	-0.26	0.75	-0.29	0.02	0.01	0.75	-0.28	5.50	5.52	5.50	0.01	0.01	5.51
13:30	5	1.29	0.25	1.15	0.14	1.12	0.11	0.08	0.04	1.19	0.17	5.08	5.11	5.08	0.02	0.01	5.10
14:30	6	1.99	0.69	1.89	0.64	1.97	0.68	0.03	0.02	1.95	0.67	4.70	4.73	4.74	0.02	0.01	4.49
15:30	7	2.73	1.00	2.57	0.94	2.79	1.03	0.04	0.02	2.70	0.99	4.48	4.49	4.50	0.01	0.01	4.37
16:30	8	2.00	0.69	3.70	1.31	3.04	1.11	0.31	0.18	2.91	1.04	4.35	4.38	4.37	0.02	0.01	4.23
17:30	9	3.72	1.31	3.41	1.23	3.30	1.19	0.06	0.04	3.48	1.24	4.22	4.22	4.26	0.02	0.01	4.23
18:30	10	5.89	1.77	5.00	1.61	4.40	1.48	0.15	0.08	5.10	1.62	4.15	4.13	4.16	0.02	0.01	4.15
08:30	24	5.54	1.71	5.21	1.65	5.20	1.65	0.04	0.02	5.32	1.67	3.66	3.67	3.67	0.01	0.00	3.67

12 weeks storage at RT

sucrose

		OD										pH					
time(h)		run A	ln(OD)	run B	ln(OD)	run C	ln(OD)	SD	SEM	ave OD	ave ln(OD)	run A	run B	run C	SD	SEM	ave pH
08:30	0	0.28	-1.28	0.37	-1.01	0.29	-1.23	0.15	0.08	0.31	-1.18	6.18	6.17	6.31	0.08	0.05	6.22
09:30	1	0.28	-1.28	0.37	-1.00	0.29	-1.24	0.15	0.09	0.31	-1.17	6.23	6.13	6.26	0.07	0.04	6.21
10:30	2	0.27	-1.31	0.36	-1.02	0.28	-1.28	0.16	0.09	0.30	-1.21	6.17	6.13	6.25	0.06	0.04	6.18
11:30	3	0.28	-1.28	0.37	-1.01	0.28	-1.28	0.16	0.09	0.31	-1.19	6.17	6.14	6.27	0.07	0.04	6.19
12:30	4	0.28	-1.28	0.36	-1.03	0.28	-1.28	0.14	0.08	0.30	-1.20	6.18	6.13	6.27	0.07	0.04	6.19
13:30	5	0.28	-1.26	0.36	-1.02	0.27	-1.29	0.15	0.09	0.31	-1.19	6.19	6.13	6.28	0.08	0.04	6.20
14:30	6	0.28	-1.26	0.35	-1.04	0.26	-1.35	0.16	0.09	0.30	-1.22	6.20	6.11	6.28	0.09	0.05	6.20
15:30	7	0.30	-1.21	0.34	-1.07	0.28	-1.26	0.10	0.06	0.31	-1.18	6.14	6.12	6.28	0.09	0.05	6.18
16:30	8	0.35	-1.05	0.35	-1.04	0.35	-1.04	0.00	0.00	0.35	-1.05	6.07	6.10	6.27	0.11	0.06	6.15
17:30	9	0.42	-0.86	0.39	-0.93	0.26	-1.35	0.26	0.15	0.36	-1.05	5.97	6.08	6.25	0.14	0.08	6.10
18:30	10	0.58	-0.54	0.43	-0.85	0.27	-1.32	0.39	0.22	0.43	-0.90	5.76	5.98	6.24	0.24	0.14	5.99
08:30	24	4.99	1.61	5.02	1.61	5.01	1.61	0.00	0.00	5.01	1.61	3.78	3.88	3.80	0.05	0.03	3.82