Structural and Functional Properties of Probiotic Strains as Affected by Multi-Stress Adaptation Process and Subsequent Freezing

BY

Thobeka Nomzamo Dlangalala

Submitted in partial fulfilment of the requirements for the degree Magister Scientiae

In the Faculty of Natural and Agricultural Sciences, Department of Biochemistry, Genetics and Microbiology, University of Pretoria, Pretoria, South Africa

December 2019

Supervisor: Prof MS Thantsha

Co-supervisor: Dr MG Mathipa

DECLARATION

I declare that the dissertation '**Structural and Functional Properties of Probiotic Strains as Affected by Multi-Stress Adaptation Process and Subsequent Freezing**' which I hereby submit for the degree of Magister Scientiae at the University of Pretoria is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature 

Table Contents

Acknowledgements viii

Conference contributions ix

List of Abbreviations x

List of Tables xii

List of Figures xiii

Dedication xiv

Summary 1

Introduction 4

References: 7

CHAPTER 1: LITERATURE REVIEW

1.1 Gastrointestinal Microflora 11

1.2 Probiotics 12

1.2.1 History and Definitions 12

1.2.2 Probiotic Organisms 13

1.2.2.1 Lactobacilli 13

1.2.2.2 Bifidobacteria 14

1.2.3 Properties of Probiotic Bacteria 15

1.2.4 Health Benefits of Probiotics 16

1.2.4.1 Lactose intolerance 17

1.2.4.2 Antibiotic Associated Diarrhoea 17

1.2.4.3 Anti-carcinogenicity 18

1.2.4.4 Probiotics and mental health 18

1.2.5 Mechanisms of Action 19

1.2.5.1 Competitive exclusion of pathogens 19

1.2.5.2 Production of antimicrobial compounds 20

1.2.5.3 Improved Barrier Function 20
1.2.5.4 Enhancement of Host Immune Function 21
1.2.6 Adverse effects of Probiotics 21
1.2.7 Precautions when using probiotics 22
1.3 Application of Probiotics 23
1.3.1 Food Industry 23
1.3.1.1 Dairy products 23
1.3.1.2 Non Dairy Probiotic Foods 24
1.4 Threats to probiotic viability 25
1.4.1 Stress Conditions that Threaten Probiotic Survival 26
1.4.1.1 Technological stress 26
1.4.1.2 Gastrointestinal Stress Factor 29
1.5 Ways to Enhance Probiotic Survival 30
1.5.1 Food Additives 30
1.5.2 Cell Immobilization 30
1.5.3 Microencapsulation 31
1.5.4 Stress Adaptation 32
1.6 Conclusion 33
1.7 References: 34

CHAPTER 2: MORPHOLOGICAL AND FUNCTIONAL PROPERTIES OF *LACTOBACILLI* AND *BIFIDOBACTERIUM* STRAINS AS INFLUENCED BY ACID-BILE-TEMPERATURE STRESS ADAPTATION PROCESS

2.1 Abstract 45
2.2 Introduction 46
2.3 Materials and Methods 48
2.3.1 Bacterial Cultures 48
2.3.2 Stress Adaptation of Probiotics 48
2.3.2.1 Acid Adaptation 48

2.3.2.2 Bile Adaptation 49
2.3.2.3 Temperature Adaptation 49
2.3.3 Acid Tolerance 49
2.3.4 Bile Tolerance 50
2.3.5 Bile Salt Hydrolase Activity 50
2.3.6 Antibiotic Susceptibility 50
2.3.7 Antimicrobial Activity Assay 51
2.3.8 Scanning Electron Microscopy (SEM) 51
2.3.9 Auto-aggregation 51
2.3.10 Cell Surface Hydrophobicity 52
2.3.11 Statistical Analysis 52
2.4 Results 53
2.4.1 Acid- bile and temperature adaptation 53
2.4.2 Acid Tolerance 55
2.4.3 Bile Tolerance 57
2.4.4 Bile Salt Hydrolase Activity 58
2.4.5 Antimicrobial Activity 59
2.4.6 Antibiotic Susceptibility 59
2.4.7 Scanning Electron Microscopy 59
2.4.8 Auto-aggregation 61
2.4.9 Cell Surface Hydrophobicity 62
2.5 Discussion 63
2.6 Conclusions 68
2.7 References: 70

CHAPTER 3: SURVIVAL OF MULTI_STRESS ADAPTED *LACTOBACILLUS PLANTARUM* IN DIFFERENT FOOD MATRICES AND IN SIMULATED GASTROINTESTINAL FLUIDS

3.1 Abstract: 76

3.2 Introduction 77

3.3 Materials and Methods 79

3.3.1 Preparation of Yoghurt 79

3.3.2 Selection of fruit and vegetable juice 79

3.3.3 Preparation of *L. plantarum* 80

3.3.4 Assessment of *L. plantarum* viability in yoghurt and juices during storage 80

3.3.5 Enumeration of *L. plantarum* over six weeks of storage 80

3.3.6 pH and ^{o}Brix measurements 80

3.3.7 Effect of food on the survival of *L. plantarum* in simulated GIT conditions. 81

3.3.7.1 Preparation of probiotic 81

3.3.7.2 Survival of *L. plantarum* in different foods under simulated gastrointestinal conditions 81

3.3.8 Statistical Analysis 82

3.4 Results 83

3.4.1 Survival of *L. plantarum* stored in different food matrices 83

3.4.2 Physico-chemical properties of foods containing *L. plantarum* 85

3.4.3 Changes in the ^{0}Brix content of juices containing *L. plantarum* 87

3.4.4 Gastrointestinal survival of non- old- and freshly adapted *L. plantarum* within different foods 89

3.5 Discussion 92

3.6 Conclusions 96

3.7 References 97

CHAPTER 4: GENERAL CONCLUSIONS AND FUTURE RECOMMENDATIONS

4.1 General Conclusions 102

4.2 Recommendations for future work 104

Acknowledgements

Firstly, I would like to thank my Lord and Saviour, Jesus, for being my Rock, my Source of Strength and my constant help during these past few years. He is the reason I've made it to the finish line.

To my supervisors, Prof MS Thantsha, thank you for patiently guiding and imparting wisdom to me. I have experienced much growth under your supervision and will use what you have taught me in every area of my life going forward. Dr Moloko G Mathipa, thank you for selflessly giving of your time to mentor, advise and to teach me and also for being a listening ear when things appeared to be falling apart.

Thank you to Eudri Venter and Erna van Wilpe at the Laboratory for Microscopy and Microanalysis for all their assistance with electron microscopy.

To my parents, thank you for patiently bearing with me and for your support throughout this time. Every sacrifice that you have made for me throughout my life has brought me to this point here today. My siblings, Nhlalo and Lwa, your messages of encouragement kept me going and have not gone unnoticed.

To my friends, there are too many of you to mention by name but you were the people who saw me going through the ups and downs, uplifted me in prayer, listened to me rejoice or cry about every victory and failure throughout this degree. I cannot thank you enough for all that you have done for me during these past few years.

To my fellow lab mates, thank you for making my studies that much more enjoyable, for sharing in the late nights and the terror of lab meetings. It really has been a privilege working with you all.

Lastly, thank you to the National Research Foundation (NRF) and the University of Pretoria for providing me with funding.

Conference contributions

Dlangalala, T. N, Mathipa M.G, Thantsha M.S. **‘**Structural and functional properties of probiotic strains as affected by the multi- stress adaptation process and subsequent freezing’**,** 23rd Biennial International Congress and Exhibition- SAAFoST 2019, Birchwood Hotel and Conference Centre, Johannesburg, 1-4 September 2019, (Poster Presentation).

List of Abbreviations

ANOVA	: Analysis of Variance
BSH	: Bile salt hydrolase
C	: Chloramphenicol
CFU/ml	: Colony Forming Units per Millilitre
CSH	: Cell Surface Hydrophobicity
Cys- HCl	: L- cysteine Hydrochloric Acid
DNA	: Deoxyribonucleic acid
E	: Erythromycin
FA	: Freshly-adapted
FC	: Fusidic acid
FDA	: Food and Drug Administration
GC	: Glycine: Cytosine content
GIT	: Gastrointestinal Tract
GRAS	: Generally Regarded As Safe
HCl	: Hydrochloric acid
IBS	: Irritable Bowel Syndrome
LAB	: Lactic Acid Bacteria
LB	: Luria Bertani
LSD	: Least Significant Difference
MRS	: De Man Rogosa Sharpe
MDD	: Major Depressive Disorder

NA : Non-adapted

NaCl : Sodium Chloride

NaOH : Sodium Hydroxide

NK : Natural Killer Cells

NO : Novobiocin

NRF : National Research Foundation

OA : Old-adapted

OD : Optical Density

OX : Oxacillin

PBS : Phosphate Buffered Saline

PG : Penicillin G

QPS : Qualified Presumption of Safety

RNA : Ribonucleic Acid

rpm : Revolutions Per Minute

S : Streptomycin

SD : Standard Deviation

SEM : Scanning Electron Microscopy

SGF : Simulated Gastric Fluid

SIF : Simulated Intestinal Fluid

spp. : Species

T : Tetracyclin

w/v : Weight per Volume

WHO : World Health Organization

List of Tables

Table 2. 1 Bile salt hydrolase activity of probiotic strains. The presence of opaque halos around punctured holes indicated bile salt hydrolase activity 58

Table 2. 2 The zones of inhibition of the adapted, non- adapted and freshly adapted strains resulting from different antibiotics. NA: Non-adapted, OA: Old-adapted and FA: Freshly adapted. 61

Table 2. 3 Auto-aggregation percentages of probiotic strains over a six hour period as determined by spectrophotometry.....61

List of Figures

Figure 1.1: Summary of the benefits associated with consumption of probiotics (Tripathi and Giri 2014). 17

Figure 2. 1: Survival of probiotics after exposure to sub-lethal stresses acid (pH 2), bile (2%) and temperature (55^{o}C). 54

Figure 2. 2: Survival of non-, old- and freshly adapted probiotic cells in pH 2 (A), 2.5 (B) and 3 (C) over a duration of 180 minutes. 56

Figure 2. 3: Survival of probiotic strains in different concentrations (A: 0.3%, B: 0.5%) of bile salts over time. 57

Figure 2. 4: Antimicrobial activity of cell free supernatants of probiotics against pathogens *E. coli* (A) and *S. aureus* (B) depicted as zones of inhibition in millimetres. 59

Figure 2. 5: Scanning electromicrographs of *B. bifidum* LMG 11041, *B. longum* Bb46, *B. longum* LMG 13197 as non-adapted (a, d & g), freshly-adapted (b, e & h), and (c, f & i) old-adapted cultures, respectively. 59

Figure 2. 6: Scanning electromicrograph of *L. acidophilus* and *L. plantarum*, as non-adapted (a & d), freshly-adapted (b & e), and old-adapted (c & f) cultures, respectively. 60

Figure 2. 7: Cell surface hydrophobicity of non-, old- and freshly- adapted *Lactobacilli* and *Bifidobacterial* species. 62

Figure 3. 1: Survival of non-adapted and multi- stress adapted *L. plantarum* in food matrices (Yoghurt (A), carrot (B) and cranberry juice (C)) over six weeks. 84

Figure 3. 2: pH changes in yoghurt (A), carrot (B) and cranberry juice (C) containing *L. plantarum* (non-, old- and freshly adapted) stored at 4^{o}C for six weeks.. 86

Figure 3. 3: Shows the changes in ^{0}Brix content of carrot (A) and cranberry juice (B) containing non- and multi-stress adapted *L. plantarum* stored at 4^{o}C for 6 weeks.. 88

Figure 3. 4: Survival of *L. plantarum* in yoghurt (A), carrot (B) and cranberry (C) during exposure to simulated gastrointestinal conditions for 8 hours. 91

Dedication

This dissertation is dedicated to Bruk Tessera who has been a confidant, friend, constructive critic, cheerleader and a constant source of support and encouragement. Your support has been monumental in me completing my studies, thank you.

Summary

Structural and functional properties of probiotic strains as affected by multi-stress adaptation process and subsequent freezing

Student : **Thobeka Nomzamo Dlangalala**

Supervisor : **Prof MS Thantsha**

Co-supervisor : **Dr MG Mathipa**

Department : **Biochemistry, Genetics and Microbiology**

Degree : **MSc (Microbiology)**

Consumers have become more aware of the importance of consuming foods and products that boost health. This is one of the reasons why the probiotic industry has been booming for the past several decades. Clinical studies have shown that when consumed in sufficient numbers, probiotics are capable of exerting certain beneficial characteristics on the host. However, their shortfall is their sensitivity to environmental stress factors, which alter their physiological state and thereby hinder their viability and functionality. The reality is, probiotics must endure various technological and gastrointestinal (GIT) stress factors before they arrive at their active site in the intestines. This therefore means that robust strains capable of withstanding threats to viability and functionality during production and after ingestion are a must. Moreover, understanding which food matrices facilitate viability during storage and in the conditions of the GIT can help in developing probiotic products that deliver on their claims. Much research has been conducted focused on producing stress tolerant probiotic strains. For example, by exposing cells to a sub-lethal dose of stress, this produces a stress response that later allows them to survive a more lethal dose of the same stress, a process known as stress adaptation. Adaptation to one stress is known to provide protection against other stresses, this is called cross protection. Research has also shown that adapting cells to multiple stress factors is better than adaptation to just one. Strains that undergo this type of manipulation must be assessed once more for retention of their probiotic properties.

Taking that into consideration, the current study aimed to determine whether long term storage of multi-stress adapted (acid, bile and temperature) strains altered their functional and structural properties when compared to non-adapted and freshly adapted cells. In the first experimental

chapter, five probiotic strains (*Bifidobacterium bifidum* LMG 11041, *Bifidobacterium. longum* LMG 13197, *Bifidobacterium longum* Bb46, *Lactobacillus acidophilus* LA14 150B and *Lactobacillus plantarum*) were sequentially adapted to acid, bile and temperature. The results show that after exposure to each stress factor, the strains not only survived better, but were capable of proliferating. Investigation into acid and bile tolerance showed insignificant differences in the strains' ability to withstand acidic conditions $p>0.05$ However, bile resistance was better for the non-adapted cells compared to their adapted counterparts. Results from bile salt hydrolase (BSH) assay showed that only freshly-adapted cells and non-adapted *L. acidophilus* could hydrolyse bile salts. However, this did not enable these cells to survive bile exposure better than the cells that tested negative for BSH. The bile resistance was therefore attributed to other stress response genes. The freshly-adapted cells could, however, be beneficial for reducing serum blood cholesterol, which has been linked to BSH activity. The tests for antimicrobial activity showed that inhibition by old-adapted *L. plantarum* was significantly lower than non- and freshly adapted counterparts ($p<0.05$). The antibiotic sensitivity profile of the cells remained largely unchanged except for *B. longum* Bb46 and *L. plantarum,* which developed sensitivity following fresh adaptation and long-term storage. Additionally, the auto-aggregation percentages were reduced by the fresh stress adaptation as well as long term storage. Moreover, scanning electron microscopy (SEM) revealed that cold storage following stress adaptation changed the morphology of strains and there were significant changes that occurred in cell surface hydrophobicity (CSH) of the strains as a result of fresh adaptation and long term storage ($p<0.05$). Auto-aggregation, cell morphology and CSH are properties linked to adhesion to epithelial cells. It is likely therefore that adherence would also be affected by stress adaptation and subsequent cold storage.

Since the alteration of probiotic properties by long-term storage was unique for each strain studies that look at each strain individually were necessary. Furthermore, in many cases foods are the vehicles used to deliver probiotic products to the body. So, investigating how different food matrices affect survival of probiotics in the product and in GIT conditions is an important aspect to consider. Multi-stress adapted *L. plantarum* was used for the second experimental chapter of the study. Its survival during storage in yoghurt, carrot and cranberry juice was determined, followed by assessing its GIT survival within the same foods. A decline in viability was observed for all cells in all three foods by the end of storage. This was attributed to be as a result of the low pH maintained by these foods throughout storage. Both adapted cultures survived better in the foods compared to non-adapted cells. However, in simulated GIT, the

survival of *L. plantarum* was better for freshly adapted, followed by old-adapted and lowest for non-adapted cells. It was evident that process of adaptation improved stability of the cells in the foods during storage but the results also show that the long-term cold storage negatively affected viability in simulated GIT conditions. In terms of the matrices themselves carrot juice had the highest number of surviving cells, followed by yoghurt and cranberry juice had the least numbers.

To our knowledge there has been no work done looking at how stress-adaptation of probiotics is affected by storage over extended periods of time. The results demonstrate how particular probiotic properties are changed in certain strains following long term storage. This had further implications for *L. plantarum* in simulated GIT conditions in which survival was lowered in the old-adapted cells.

This study is relevant for both the probiotic food industry and consumers alike. The study showed how storage can negatively affect the properties of certain strains. Therefore, industries that use pre-stress treatment as a means to boost viability should explore different methods of storage that do not disrupt functionality and viability. Furthermore, these industries should incorporate the probiotic strains in food matrices that facilitate the survival of probiotics. As this study showed, carrot juice resulted in the highest number of surviving cells after storage. This information would also be useful to consumers as they would be able to make informed decisions about which probiotic foods will deliver the highest number of cells. This also benefits the probiotic manufacturers because satisfied customers will drive the continued growth of the industry.

Introduction

Bifidobacteria and lactic acid bacteria (LAB) are constituents of the intestinal microbiota of humans and animals. They maintain gut health by the preservation of intestinal microbiota and modulation of the immune system (Sanz 2007; Mathipa and Thantsha 2015). Consumption of microorganisms with an intention to promote health was first recorded by Eli Metchnikoff, who noted that Bulgarian peasants who consumed fermented products containing LAB lived longer (Culligan, Hill and Sleator 2009a). These health promoting microorganisms are commonly referred to as probiotics, which are defined as "Live microorganisms which when administered in adequate amounts confer a health benefits on host" (FAO/WHO 2001). The past two decades have seen a renewed interest in the clinical relevance of these microorganisms and many have been suggested to treat and prevent gastrointestinal infections and/or disorders (Culligan, Hill and Sleator 2009a; Tejero-Sariñena *et al.* 2012).

There are certain criteria that have to be met by strains before they can be developed for probiotic use. These include among others that they must be safe for human consumption and furthermore, should possess traits such as suitability for large scale production and incorporation into foods, that enable them to be exploited for technological processes (Saarela *et al.* 2000; Gueimonde *et al.* 2013; Amund 2016). Additionally, probiotics require functional abilities that aid in gastrointestinal survival. Therefore, they should possess acid and bile resistance, demonstrate the ability to exclude pathogenic microorganisms, adhere to intestinal epithelial cells and modulate the immune system (Ouwehand and Salminen 1998; Saarela *et al.* 2000).

Traditionally, probiotics are taken in capsules or incorporated into dairy products. Due to their perception as being healthy, fermented and non- fermented dairy products have been the leading carrier product for probiotics (Heller 2001). Recently, changing dietary lifestyles such as vegetarianisms, and allergic reactions to milk protein and lactose, have driven the development of non-dairy probiotic product alternatives manufactured from cereals, vegetables and fruit (Tripathi and Giri 2014; Amund 2016; Misselwitz *et al.* 2019). To achieve desired effects, probiotic products must contain a minimum level of $10^7/10^6$ CFU/ml of microorganism at the time of consumption or by the end of shelf life (Corcoran *et al.* 2005). Moreover, to acquire beneficial effects a recommended daily intake of probiotic product at 10^8-10^9 CFU/ml is required (Sanz 2007; Amund 2016).

The production of probiotic products threatens their viability as the strains are exposed to lethal levels of acid, oxygen, temperature extremes, low water activity and the presence of the chemicals (Sanz 2007; Sánchez *et al.* 2012). These threats are present at all stages of production and throughout storage of the final product. They can greatly influence the stability and probiotic characteristics of the product (Sánchez *et al.* 2012). Additionally, following consumption, the probiotics must contend with the lethal conditions of acid, bile and digestive enzymes found in the gastrointestinal tract (GIT) (Corcoran *et al.* 2008; Sánchez *et al.* 2012; Mathipa and Thantsha 2015). The concern is that many probiotic strains are sensitive to environmental stress and rapidly decline during manufacture, storage and GIT transit. Therefore, the quality of the final product is greatly diminished as many of their benefits are linked to the number of probiotic microorganisms present (Sánchez *et al.* 2012).

It is crucial then that viability and functional properties are sufficiently conserved within the product following production, storage and passage through the GIT (Corcoran *et al.* 2008). Choice of food carrier has been implicated in enhancing GIT survival of probiotics. Dairy, for instance, has a high buffering capacity and protective effect thought to be due to its high fat content and milk proteins (Corcoran *et al.* 2008; Amund 2016). The food matrices may also contain prebiotics, which are defined as, "a selectively fermented ingredient that results in specific changes in composition and/or activity of the gastrointestinal microbiota, thus conferring benefits(s). These can positively influence probiotic functionality (Gibson *et al.* 2010; Ranadheera, Prasanna and Vidanarachchi 2014). Fruit and vegetables are rich in vitamins, minerals, antioxidants and fibres that can be exploited by probiotics for their growth, which can make them ideal matrices. Furthermore, fruit and vegetables naturally contain prebiotics that aids in probiotic survival (Ranadheera, Prasanna and Vidanarachchi 2014; Tripathi and Giri 2014; Kumar, Vijayendra and Reddy 2015).

A different method used to improve cell viability is through manipulation of the physiological state of the probiotics. This is done by exploiting stress responses present in probiotics, through stress pre-treatment (Sanz 2007). This process allows stress responses to be elicited by exposing the microorganism to sub-lethal doses of the stress factor so that they are then able to overcome the subsequent lethal stresses encountered during manufacture and within the GIT (Champagne and Gardner 2008; Sánchez *et al.* 2012). Researchers have also demonstrated that exposure to one type of stress can offer resilience to a different type of stress downstream, a mechanism known as cross-protection. Much of the early research into stress adaptation focused on a single type of stress at a time (Ventura *et al.* 2004; Sánchez *et al.* 2005, 2007).

More recent work has found that adaptation to multiple stresses can offer superior protection (Mathipa and Thantsha 2015). However, following stress adaptation strains must be reassessed for functional properties as this process can negatively or positively impact probiotic traits (Ruiz, Margolles and Sánchez 2013a). There is limited knowledge on how multi stress adapted probiotics are affected by long periods of cold storage. This is an important aspect to consider since many probiotics are stored under these conditions. Taken together, this study expands on the findings by Mathipa and Thantsha (2015) and aims to determine how pre-adaptation of probiotics to multiple stress factors followed by long-term cold storage affect their functional properties and survival in food matrices and under simulated gastrointestinal conditions.

The specific objects were:

- To determine the effect of multi-stress adaptation process and subsequent storage on functional properties of probiotics
- To determine the effect of multi-stress adaptation on viability of probiotic cultures in different food matrices.
- To investigate the survival of multi-stress adapted *Lactobacillus plantarum* in food matrices and the effect of these matrices on its survival in simulated gastrointestinal conditions.

References

Amund OD. Exploring the relationship between exposure to technological and gastrointestinal stress and probiotic functional properties of *Lactobacilli* and *Bifidobacteria*. *Can J Microbiol* 2016;**62**:715–25.

Champagne CP, Gardner N. Effect of storage in a fruit drink on subsequent survival of probiotic *Lactobacilli* to gastro-intestinal stresses. *Food Res Int* 2008;**41**:539–43.

Corcoran B, Stanton C, Fitzgerald G *et al.* Life under stress: The probiotic stress response and how it may be manipulated. *Curr Pharm Des* 2008;**14**:1382–99.

Corcoran BM, Stanton C, Fitzgerald GF *et al.* Survival of probiotic *Lactobacilli* is enhanced in acidic environments in the presence of metabolizable sugars. *Appl Environ Microbiol* 2005;**71**:3060–7.

Culligan EP, Hill C, Sleator RD. Probiotics and gastrointestinal disease: successes, problems and future prospects. *Gut Pathog* 2009;**1**:19.

FAO/WHO. Evaluation of health an nutitional properties of powdered milk and lactic acid bacteria. 2001.

Gueimonde M, Sánchez B, G. de los Reyes-Gavilán C *et al.* Antibiotic resistance in probiotic bacteria. *Front Microbiol* 2013;**4**:202.

Gibson GR, Scott KP, Rastall RA et al. Dietary prebiotics: current status and new definition. *Food Sci Technol Bull* 2010;**7**:1-16.

Heller KJ. Probiotic bacteria in fermented foods: product characteristics and starter organisms. *Am J Clin Nutr* 2001;**73**:374s-379s.

Kumar VB, Vijayendra SVN, Reddy OVS. Trends in dairy and non-dairy probiotic products - a review. *J Food Sci Technol* 2015;**52**:6112–24.

Mathipa M, Thantsha M. Cocktails of probiotics pre-adapted to multiple stress factors are more robust under simulated gastrointestinal conditions than their parental counterparts and exhibit enhanced antagonistic capabilities against *Escherichia coli* and *Staphylococcus aureus*. *Gut Pathog* 2015;**7**:5.

Misselwitz B, Butter M, Verbeke *et al.* Update on lactose malabsorption and intolerance: Pathogenesis, diagnoses and clinical management.*Gut* 2019;**68**:2080-2091.

Ouwehand AC, Salminen SJ. The health effects of cultured milk products with viable and non-viable bacteria. *Int Dairy J* 1998;**8**:749–58.

Ranadheera CS, Prasanna PHB, Vidanarachchi JK. *Fruit Juices : Types, Nutritional Composition and Health Benefits*. Elder KE (ed.). New York: Nova Science Publishers, 2014.

Ruiz L, Margolles A, Sánchez B. Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium*. *Front Microbiol* 2013;**4**:396.

Saarela M, Mogensen G, Fondén R *et al.* Probiotic bacteria: safety, functional and technological properties. *J Biotechnol* 2000;**84**:197–215.

Sánchez B, Champomier-Vergès M-C, Anglade P *et al.* Proteomic analysis of global changes in protein expression during bile salt exposure of *Bifidobacterium longum* NCIMB 8809. *J Bacteriol* 2005;**187**:5799–808.

Sánchez B, Champomier-Vergès M-C, Collado M del C *et al.* Low-pH adaptation and the acid tolerance response of *Bifidobacterium longum* biotype longum. *Appl Environ Microbiol* 2007;**73**:6450–9.

Sánchez B, Ruiz L, Gueimonde M *et al.* Toward improving technological and functional properties of probiotics in foods. *Trends Food Sci Technol* 2012;**26**:56–63.

Sanz Y. Ecological and functional implications of the acid-adaptation ability of *Bifidobacterium*: A way of selecting improved probiotic strains. *Int Dairy J* 2007;**17**:1284–9.

Tejero-Sariñena S, Barlow J, Costabile A *et al.* In vitro evaluation of the antimicrobial activity of a range of probiotics against pathogens: Evidence for the effects of organic acids. *Anaerobe* 2012;**18**:530–8.

Tripathi MK, Giri SK. Probiotic functional foods: Survival of probiotics during processing and storage. *J Funct Foods* 2014;**9**:225–41.

Ventura M, Canchaya C, Zink R *et al.* Characterization of the groEL and groES Loci in

Bifidobacterium breve UCC 2003: Genetic, Transcriptional, and phylogenetic analyses. *Appl Environ Microbiol* 2004;**70**:6197–209.

Chapter 1

Review of Literature

1.1 Gastrointestinal Microflora

The mucosal surface of the human gastrointestinal tract (GIT) is expansive ranging between 200-300 m^2. It is home to over 400 bacterial species and subspecies (Hao and Lee 2004). At birth the GIT is sterile but becomes progressively inhabited by environmental microbes immediately thereafter. Usually, the first colonizers are facultative anaerobes *Enterococci* and *Lactobacilli* (Gill 2003; Tojo *et al.* 2014). Following establishment, the intestinal microflora remains stable throughout life and at this time it has made a gradual transition to strict anaerobes such as *Bifidobacterium, Bacteroides* and *Clostridium* (Gill 2003; Tojo *et al.* 2014). Two kinds of GIT microflora can be encountered, autochthonous and allochthonous flora. The first are those that are indigenous to the GIT and are found colonizing different parts and the latter are transient and under normal circumstances cannot colonize any part of the GIT (Savage 1977). Different regions of the GIT are colonized to varying degrees by specific microorganisms. The factors that determine colonization are pH, peristalsis, diet, nutrient availability and bacterial adhesion to name a few (Hao and Lee 2004; Bäumler and Sperandio 2016). Consequently, the stomach and the initial two thirds of the small intestine, which experience peristalsis and high acidity, have a lower number of microorganisms. The abundance of intestinal microflora is therefore found in the distal small intestine and in the colon where there is an estimated 10^7-10^8 bacteria/ml of intestinal contents (Hao and Lee 2004). The gut microflora varies in individuals at the genus and species level, however, a conservation is seen at a phylum level with *Firmicutes* and *Bacteroidetes* dominating (Bäumler and Sperandio 2016).

The intestinal microflora ferments non-digestible dietary components in the large intestine which produces short chain fatty acids that are useful to both the bacteria and the host cells. Additionally, the microflora uses various mechanisms to regulate energy expenditure, glucose homeostasis and satiety within the host (Flint *et al.* 2012; Tojo *et al.* 2014). Moreover, by occupying adherence sites, the intestinal microflora can prevent foodborne pathogens from establishing and causing disease (Bäumler and Sperandio 2016). They also play a role in maintaining host immunity and could be linked to certain cognitive functions of the brain (Flint *et al.* 2012; Hsiao *et al.* 2013).

Therefore, it is imperative that the intestinal balance is maintained. However, external factors like diet, illness, stress, antibiotic and radiation therapy can disrupt this balance. Leaving the

host susceptible to infection, immuno-inflammatory and autoimmune disease (Gill 2003; Bäumler and Sperandio 2016). Research shows that supplementation with probiotics can lead to the maintenance and restoration of microbial balance in the intestines (Gill 2003; Flint *et al.* 2012).

1.2 Probiotics

1.2.1 History and Definitions

The use of probiotic strains dates back to the Old Testament, where Abraham was said to consume fermented milk. Other evidence from wall paintings in 2500 BC suggest that Sumarians fermented milk by inoculation (Fuller 1992). Whether this was practised for the inferred health benefits or for the sake of preserving food is still unknown. However, there is no doubt that the intake of fermented milk has seen a steady increase over the centuries, and is currently at an all-time high. Metchnikoff and his colleagues were the pioneers of probiotic studies. In 1907, they found that the species currently known as *Lactobacillus delbrőkii* subspecies *bulgaricus* was involved in the fermentation of sour milk and consumption thereof contributed to health and longevity. It was only in 1920 that researchers from Yale university found that this bacterium could not withstand the harsh environments found in the gut, however, later studies revealed that other members from the group of lactic acid bacteria (LAB) could survive the human gut (Ljungh and Wadström 2009). These findings showed a promising future for the consumption of bacteria for health benefits. Nonetheless, it was not until the 1930's when Dr Minoru Shirota isolated the first commercialised probiotic strain (*Lactobacillus casei* strain Shirota) that probiotic research began to gain momentum (Tojo *et al.* 2014).

The term probiotic is Greek in its origin, meaning, "for life" and with the rise in the use and interest of these bacteria so have the attempts to define them (Kechagia *et al.* 2013). Over the past few decades there have been many efforts to define probiotics, usually in terms of their mechanisms of action. The first use of the term was by Lilly and Stillwell in (1965) who described probiotics as "substances produced by one microorganism to stimulate the growth of another". Later, Parker, in (1974) described probiotics as "organisms and substances that contribute to intestinal health". Owing to the ambiguity of the term "substances" which could also have been referring to antibiotics the definitions was improved upon by Fuller in 1989

(Fooks, Fuller and Gibson 1999). He defined them as “live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance”. The definition was further expanded in (1998) by Salminen who called them “live microbial food ingredients that have a beneficial effect on human health”. Currently, the accepted definition for probiotics is by the food and drug administration/world health organization (FAO/WHO) and they are defined as “live microorganism which when administered in adequate number confers health benefit to the host”. The definition is still being used today because of the emphasis placed on the adequate amounts required to benefit from the microorganisms (Blum *et al.* 2002).

1.2.2 Probiotic Organisms

Probiotics are found over a wide range of bacterial genera including; *Enterococcus*, *Propionibacterium*, *Escherichia*, *Streptococcus*, *Wesselia* and *Lactococcus*. Fungal species such *Saccharomyces boulardii* are also used as probiotics. However, the most commonly used strains commercially are those belonging to species *Lactobacillus* and *Bifidobacterium*, these genera are prominent inhabitants of the GIT microflora and have long been used for human health and are generally regarded as safe (GRAS) (Rolfe 2000; Kolida, Saulnier and Gibson 2006; Tripathi and Giri 2014).

1.2.2.1 *Lactobacilli*

The genus *Lactobacillus* consists of gram positive, aero-tolerant, non-spore forming rods with a genome that has a low GC content. During carbohydrate fermentation, *Lactobacilli* produce lactic acid as a major by-product, because of this property they also falls into a broader group called the lactic acid bacteria (Halasz 2009; Papadimitriou *et al.* 2016). This particular genus has fastidious nutrition requirements needing not only carbohydrates in their diets but also amino acids, nucleic acids, fatty acids, esters and vitamins in order to grow (Tharmaraj and Shah 2003). This diverse genus contains more than 12 phylogenetic groups, boasting more than 201 described species making it the largest genus in the LAB group (Zotta, Parente and Ricciardi 2017).

Lactobacilli can be found occupying a wide range of ecological niches including soil, water and sewage. These bacteria also form part of the normal microbiota of live hosts such as plants, animals and human beings. In humans, they occupy the oral (10^3-10^4 CFU/ml) and intestinal

cavities (10^4-10^8 CFU/ml) in addition to being the predominant members of the vaginal microflora (Ma *et al.* 2013). Although, their presence in the intestine only occurs at varying degrees it is critical for homeostasis and has been linked to the alleviation of many gastrointestinal disorders. Moreover, their presence in the intestine prevents infection from pathogens through different mechanisms. For these reasons live cultures of these bacteria are ingested in fermented foods or in supplement form and they are regarded as probiotic (Nissen *et al.* 2009; Ma *et al.* 2013).

In the food industry *Lactobacilli* are used as starter cultures for many fermented foods, from dairy products like cheese and milk to meats and fresh produce. The reason that many species from this genus are utilized in food production and human health is because they were classified as (Generally Regarded As Safe; US, FDA) and given a QPS (qualified presumption of safety) status by the European Food Safety Authority (De Angelis and Gobbetti 2004; Zotta, Parente and Ricciardi 2017). Some species of *Lactobacilli* used industrially as probiotics or food cultures are: *Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus fermentum* (Zotta, Parente and Ricciardi 2017).

1.2.2.2 Bifidobacteria

*Bifidobacteri*a is genus of gram positive, non-motile, non-sporulating, non-gas forming bacteria with a high GC content. Members of this genus belong to the family *Bifidobacteriaceae* and phylum *Actinobacteria*. They were first discovered by Henri Tisser who isolated them from the faeces of breast fed babies and named them *Bacillus bifidum*. Tisser further described them as rods that curve or those that split to form what is now known as their characteristic y-shape. For a great part of the 20th century they were classified as *Lactobacillus*. It was only in recent times that they were re-classified as their own genus. To date there are more than 72 recognised (sub) species of Bifidobacteria which have been primarily isolated from animal GIT, human intestines and oral cavity and the hindgut of insects (Poupard, Husain and Norris 1973; Bottacini *et al.* 2014; Lugli *et al.* 2019).

Within the densely populated microbial ecosystem found in the GIT, *Bifidobacteria* are the dominant bacteria with 25% of the cultivatable faecal bacteria of adults belonging to this group. Furthermore, they are the most abundant bacterial group in the GIT of infants that are both naturally delivered and breastfed. In these infants they constitute up to 80% of the GIT microbiota (Picard *et al.* 2005; Bottacini *et al.* 2014). Their presence is not limited to the human

GIT only. They also colonise the gut of other mammals as well as birds and insects (Kopency *et al*. 2010). Moreover, they occupy other ecological niches including fermented milk and sewage although they are more commonly found within the GIT of animals (Botticini *et al*. 2014).

Particular strains belonging to this group are used as probiotics. This was true even in ancient times when they were being consumed in fermented milk for their health benefits (Sanz 2007; Toja *et al.* 2014). Just as in the case of *Lactobacilli*, *Bifidibacteria* have also been given GRAS status. Currently, *Bifidobacteria* are mostly carried in fermented milk products, although, other suitable carriers are under investigation (Sanz 2007). It should be noted that unlike *Lactobacilli*, *Bifidobacteria* are more susceptible to environmental stresses leading to more loss in cell viability during production of probiotic products (Corcoran *et al.* 2008).

1.2.3 Properties of Probiotic Bacteria

There are several characteristics that bacteria should possess before being referred to as probiotic. In order to survive passage through the GIT, probiotics should be resistant to the conditions found in this environment, which include low pH and presence of bile salts. Therefore, probiotics administered orally should be both bile and acid tolerant (Fuller 1989; Bezkorovainy 2001; Kechagia *et al.* 2013). They must also remain viable in high numbers as stipulated in their description. Their functionality depends on their viability without which properties such as adherence to mucosal and epithelial surfaces, reduction of gut permeability and immunomodulation would be affected (Ouwehand, Tolkko and Salminen 2001; Kechagia *et al.* 2013). It should be noted that other studies have found that probiotic viability is not always necessary for efficacy or for health benefits to be derived. In these cases only good growth is required in the initial production stages and probiotics need not retain viability during storage (Ouwehand and Salminen 1998; Lahtinen, Davis and Ouwehand 2012).

A critical functional property that all other benefits hinge upon is the ability of probiotics to temporarily colonize the intestinal tract (du Toit *et al.* 2013). This is facilitated by bacterial aggregation. Additionally, protection against pathogens can be achieved through a barrier formed by auto-aggregation or co-aggregating with a pathogen. Auto-aggregation also results in the release of beneficial substances into the host. Both of these abilities are linked to

adherence to the intestinal epithelial which can further be enhanced by presence of exopolysaccharides found on the outer membrane of probiotic cells (Li *et al.* 2015).

In addition to functional properties, probiotics should be safe to use and for this reason are often of human origin. (Ouwehand, Tolkko and Salminen 2001). They should not be pathogenic in any way or possess any antibiotic resistance genes that can be passed on to pathogenic organisms (Saarela *et al.* 2000). Furthermore, commercial probiotic bacteria should display good technological properties such as the stability during storage, retain high viability as well as the ability to handle large scale cultivation (Saarela *et al.* 2000). Moreover, their presence in a food product should not alter any sensory properties, produce unwanted odours or unpleasant tastes (Saarela *et al.* 2000; Kolida, Saulnier and Gibson 2006).

1.2.4 Health Benefits of Probiotics

Probiotics have long been incorporated into fermented foods and their positive effects on human health have been duly noted. They have been reported to prevent and treat certain types of diarrhoea, possess anti-carcinogenic activities, alleviate lactose intolerance reduce symptoms of gastrointestinal infections and decrease cholesterol levels. The ability to provide these benefits is mainly derived from their maintenance of intestinal microflora (Figure 1.1). However, to derive the health benefits from the probiotics they must be consumed in sufficient numbers. Currently there is no consensus as to what that amount is but the recommended value is between 10^7-10^8 CFU/ml, taken daily to derive the maximum benefits from the bacteria. This high number ensures that enough cells reach target site after encountering the harsh conditions of the GIT. They should be taken on a regular basis because these bacteria only colonize the gut transiently. It's also worth noting that the benefits listed below do not apply to all probiotics as specific benefits differ on a strain to strain basis, therefore to derive mechanism of action, efficacy and safety each strain should be assessed individually (Talwalkar and Kailasapathy 2004a; Urbańska and Szajewska 2014).

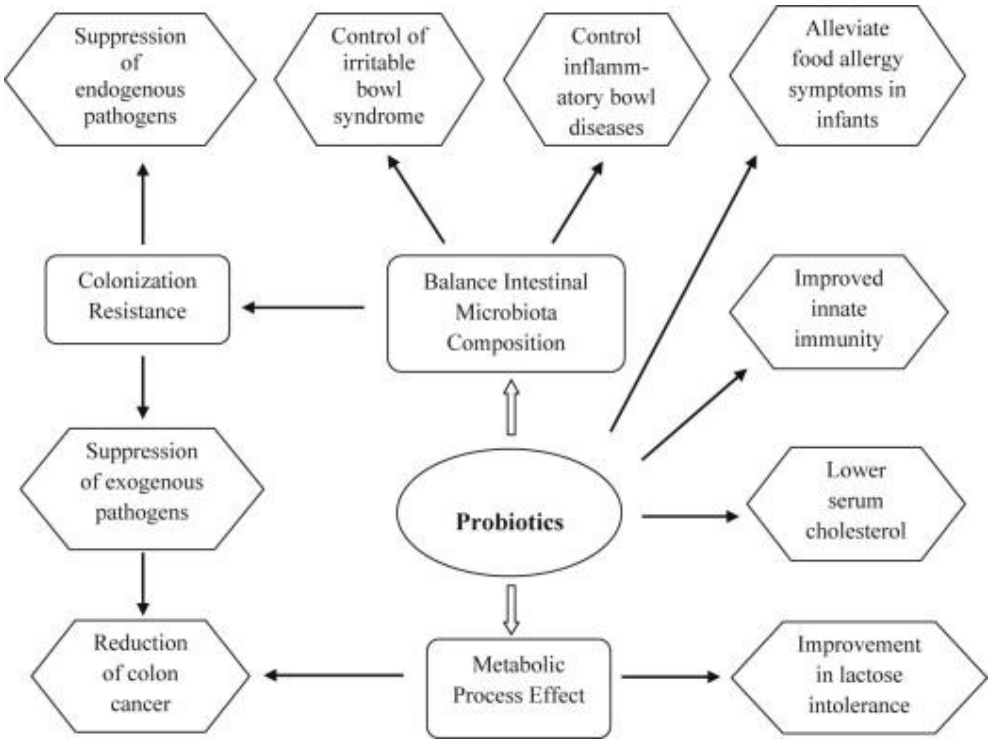

Figure 1.1: Summary of the benefits associated with consumption of probiotics (Tripathi and Giri 2014).

1.2.4.1 Lactose intolerance

Lactose intolerance is a condition in which individuals lack the enzyme beta-galactosidase, meaning they are unable to hydrolyse lactose into its smaller constituents, glucose and galactose. Once the un-degraded lactose reaches the large intestine the residential bacteria begin to break it down resulting in diarrhoea. Other symptoms of this condition include abdominal discomfort and flatulence usually following the consumption of milk or milk products. Probiotics such as *Lactobacillus* and *Bifidobacteria* which possess beta-galactose activity are usually added to dairy products to help in alleviating symptoms of lactose intolerance (Levri *et al.* 2005; de Vrese *et al.* 2011).

1.2.4.2 Antibiotic Associated Diarrhoea

The use of antibiotics for treatment of ailments often results in mild to severe cases of diarrhoea because the growth of the normal microflora is suppressed while pathogenic strains have the

opportunity to grow. The various types of diarrhoea that result can vary in severity, the worst form being that caused by *Clostridium difficile*. In this case, the cytotoxic strains of this bacterium, which usually appear after antibiotic treatment, disrupt the mucosal layer. The symptoms usually include; diarrhoea, fever, distention and gone untreated can lead to perforation or toxic megacolon. Studies have shown that this type of diarrhoea can be treated by administering probiotic bacteria such as *Lactobacillus rhamnosus* and *S. boulardii* (Kechagia *et al.* 2013). Additionally, other reports show that with regular use probiotics reduce the chances of acquiring antibiotic-associated diarrhoea (McFarland 2006; Blaabjerg, Artzi and Aabenhus 2017). The evidence also suggests that probiotics can be used to prevent and to treat this condition. What is still unclear is what dosage is suitable and how effective different probiotics are at alleviating this condition (Hempel *et al.* 2012).

1.2.4.3 Anti-carcinogenicity

Cancer has become one of the leading causes of death in the world, with more than 200 different forms recorded to date. Colon cancer particularly, is the second leading cause of cancer deaths worldwide. Various factors contribute to its development including both environmental and genetic aspects. Although no cure has been found for the disease it has been documented that probiotics can aid in the prevention of colon cancer through different mechanisms. Certain LAB are known to bind carcinogens such as those formed during the cooking of meat and aflaxatoxins produced by certain fungal species (Burns and Rowland 2000; Zhong, Zhang and Covasa 2014). Furthermore, many inhabitants of the gut possess enzymes that change pre-carcinogens into active carcinogens. However, probiotic inhabitants belonging to *Bifidobacteria* spp. and *Lactobacillus* spp. have low activity of these enzymes, can alter the xenobiotic metabolizing enzymes, reduce reactive oxygen species, prevent processes of oxidative stress, lower intestinal inflammation, bind or adsorb carcinogens and thus reduce the incidence of colon cancer. (Burns and Rowland 2000; Rolfe 2000; Khorshidian *et al.* 2016).

1.2.4.4 Probiotics and mental health

There is compelling new evidence that shows potential of probiotics in treating stress, anxiety, mood disorders and Major depressive disorder (MDD) (Wallace and Milev 2017; Misra and Mohanty 2019). MDD affects 20% of the population at some time in their life. Its symptoms include feelings of hopelessness, guilt, lack of interest, altered appetite and sleep patterns. Its

effects can extend to performance at school or work and also impair ones social relationships. At present, treatment for this condition involves altering neurotransmitter activity in the brain but with the new research in neuro-gastroenterology a new course of treatment is on the horizon. Researchers have found a complex signalling network between the central nervous system and the GIT known as the gut-brain axis, this research has shown a link between psychiatric disorders and the changes that occur within the gut microbiota. These recent developments in neuroscience potentially offer an alternative for treatment of disorders such as MDD (Wallace and Milev 2017). A study conducted by Huang, Wang and Hu in (2016) showed that different strains of probiotics were successful in decreasing symptoms of depression in affected individuals as well as preventing depressive symptoms in healthy individuals. Other studies had similar outcomes, petrochemical workers who had consumed probiotic yoghurt or probiotic supplementation for a period of six weeks showed improved mental health biomarkers (Mohammadi *et al.* 2016). These reports are opening a door for a new class of probiotics called "Psychobiotics" "which are a group of probiotics, capable of producing and delivering neuroactive substances that act across the brain gut axis (Misra and Mohanty 2019). There is still much work to be done in understanding the complex mechanisms involved in the gut-brain axis and how that relates to treating mental disorders such as MDD, however, probiotic offer a potential solution to this end.

1.2.5 Mechanisms of Action

1.2.5.1 Competitive exclusion of pathogens

To grow and thrive inside the GIT probiotics need to outcompete other microorganisms for critical resources such a food and adherence space. Probiotics are so good at doing so that they growth is favoured over that of pathogens. While competing for nutrients probiotics release fatty acids that lower the pH of the environment so that those pathogens incapable of growth at acidic levels are inhibited (Callaway *et al.* 2008; Mathipa and Thantsha 2017).

In addition to nutrients probiotics can also compete for adherence sites. In this case they are found present at sites where pathogens would usually adhere so that they are unable to colonize and initiate infection. Binding to the various attachment sites can be achieved through specific surface proteins, steric forces or electrostatic interactions. The various mechanisms of binding offers an advantage as they are able to do so in high quantities thereby outcompeting pathogens (Fukuda 2017; Mathipa and Thantsha 2017).

1.2.5.2 Production of antimicrobial compounds

The ability of probiotics to exclude pathogens depends on the production of inhibitory substances such as lactic and acetic acid. In addition to directly inhibiting pathogens by lowering the pH of the surroundings the production of antimicrobial substances affects pathogen metabolism and toxin production which can aid in disease prevention. Moreover, probiotics can produce other inhibitory substances known non-lactic acid molecules. These molecules can either be small ribosomally synthesised peptides referred to as bacteriocins or hydrogen peroxide which both aid in the inhibition of intestinal pathogens using different mechanisms (Volzing *et al.* 2013). The latter is known to have oxidising effects that destroy the molecular structures of cell protein, it is also thought to be responsible for the prevention of urogenital infections (Arena *et al.* 2016). While bacteriocins produced by many LAB have a wide or narrow host range with its activity aimed at low G+C gram positive species. Bacteria can also use bacteriocins for competition in natural ecosystem. Their production by probiotic species can be advantageous in food industry as they are known to be active against many foodborne pathogens and thus act as a natural preservative (Šušković *et al.* 2010; Mathipa and Thantsha 2017).

1.2.5.3 Improved Barrier Function

The prevention of systemic and local infection depends on the integrity of the intestinal barrier; pathogens should not be able to breech it. The critical role played by intestinal epithelial cells is known as the "barrier function". Once the barrier has been interrupted the host becomes susceptible to infection by pathogens, allergens, toxins which result in an immunological stress response. Therefore, intact barrier function maintains the integrity of gastrointestinal mucosa (Rao and Samak 2013). Tight junctions are a component of the barrier function which forms a barricade between the paracellular space and the luminal contents so that no macromolecules move through the epithelium. Probiotics aid in the protection of the barrier function by releasing metabolites that regulate tight junctions. For example, certain probiotics release the short chain fatty acid butyrate which regulates the expression of tight junction proteins and thus enhances barrier function. They can also restore the barrier after injury sustained from invasion of gut pathogens. They do so by either stimulating the secretion of water, chlorides and mucus

as well as using tight junction proteins to unite submucosa cells (Rao and Samak 2013; Mathipa and Thantsha 2017).

1.2.5.4 Enhancement of Host Immune Function

The cells involved in innate and adaptive immunity; dendritic cells, B cells, T cells, natural killer cells (NK), macrophages are stimulated to varying degrees by the presence of probiotics in the gut. Probiotics can also protect the host from antigens produced by pathogens through activating lymphocytes and antibody production. This is crucial as the antibody immunoglobulin A offers the first line of defence in intestinal lumen by preventing pathogens from adhering to the epithelial cells (Ng *et al* 2009, Viasu-Bolocan *et al* 2013). Another way that probiotics enhance immunity is through the production of intestinal mucins. The mucins are responsible for preventing pathogen translocation through disrupted adherence to epithelial cells. Additionally, secretion of immunoglobulin A is increased through enhanced expression of epithelial cell interleulikns (Hardy *et al* 2013).

1.2.6 Adverse effects of Probiotics

Although a great majority of the literature highlights the advantages of consuming probiotics. That is not to say that no adverse effects can result from their consumption. This is especially documented among immunocompromised patients. Floch (2013) reported a 17 year old immunocompromised patient with universal ulcerative colitis who was being treated using antibiotics, corticosteroids and was also given *L. rhamnosus* GG. This resulted in sepsis developing. Although incidence of infection by probiotics is rare it does occur. It can be further accelerated by the lack of regulation surrounding purchase of probiotics products especially since these products are readily available over the counter. Floch also identified major and minor risk factors that could lead to infection. The two major factors include being immunocompromised another is administering probiotics to preterm babies. While the minor risk factors are administering probiotics to individuals with damaged epithelial barrier, cardiac valve disease, taking broad spectrum antibiotics that probiotics are resistant to and introducing the probiotics via jejunostomy (Floch 2013). It should also be noted that no complications have ensued following consumption of probiotics by healthy individuals. And thus should only be

used by individuals without any pre-existing conditions (Boyle, Robins-Browne and Tang 2006).

Another concern is that probiotics are resistant to various antibiotics, meaning that they could potentially transfer resistance genes to pathogenic species in the gut. If this were the case antibiotic intervention to eliminate these pathogens would fail. However, many of the antibiotic genes are carried on the chromosome and not on transferable elements such as plasmids meaning that transfer to pathogenic species should not occur (Boyle, Robins-Browne and Tang 2006).

1.2.7 Precautions when using probiotics

Other than the risk of infection after consumption of probiotics, there are additional precautions that should be taken. Needless to say taking broad-spectrum antibiotics in conjunction with probiotics could be counteractive Blaser (2011) noted that antibiotic treatment not only alters the microbiota in the gut but they also kills the residential probiotics. The author suggests that taking narrow-spectrum antibiotics as an alternative. This would only affect the target organisms rather than killing majority of the gut inhabitants including the probiotic species (Blaser 2011).

Precautions must also be taken when probiotics are taken in conjunction with garlic, a plant used as a natural remedy. The recorded health benefits of garlic include anticancer, antimicrobial, antiarthritic and antithrombotic properties. Additionally, it has been used for treating acne, asthma as well as gastrointestinal issues (Booyens, Labuschagne and Thantsha 2014). Therefore, it is not surprising that many individuals have opted to add garlic to their diets, to optimise on its health benefit. However, recent studies have shown that garlic's antimicrobial activity damages probiotic cells. When different preparations of garlic were tested to determine their potency against *Bifidobacteria* the results from a disk diffusion assay showed different zones of inhibition ranging between 13.0 ± 1.7 to 36.7 ± 1.2mm. These preparations that included garlic paste, garlic spice, fresh garlic cloves and garlic powder showed fresh garlic cloves as the most potent against the *Bifidobacteria* strains (Booyens and Thantsha 2013). When looking at the mechanisms of garlic, another study showed that it not only destroys the cells morphological structure but it also ruptures internal organs (Booyens, Labuschagne and Thantsha 2014). Taken together, these studies show that garlic not only works in eliminating pathogenic bacteria but also works against probiotic cells. Therefore,

caution is necessary when garlic and probiotic are taken simultaneously as this could counteract the effectiveness of the probiotics.

1.3 Application of Probiotics

1.3.1 Food Industry

Food plays an integral part in human health and the type of food ingested not only has physiological effects on host but can have an impact on ones quality of life (Moura 2005). The public were first made aware of foods with health benefits in the 1960's and by the 1980's these foods had their own category called functional foods. Functional foods are defined as "foods that provide health benefits beyond basic nutrition and promote health through mechanisms not addressed by conventional nutrition models". These foods possess one or more components that impart a positive physiological effect on the body (Cruz, Faria and Dender 2007).

The past twenty years has seen a drastic increase in the interest of functional foods especially those containing probiotics, this due to an increase in consumer awareness. From 2000 to 2013 the global market for functional food grew from $33 billion to $176 billion (Granato *et al.* 2010; Hennessy 2013). As such, the industry seeks to meet this demand by producing a variety of edible goods that carry probiotics. These typically include dairy products; cheese, yoghurt, drinking yoghurt, ice-cream, freeze-dried milk powder (Nagpal *et al.* 2012). And because of the increase in vegetarianism and lactose intolerance non-dairy variants like; fruit juice, breakfast cereals and nutrition bars have also been developed (Granato *et al.* 2010). Still, the recommended probiotic intake is approximately 10^9 CFU/ml/g daily to acquire desired health benefits which means the food must have sufficient cells during its shelf life to meet this requirement (Ross *et al.* 2002). However, large scale production and processing of probiotics as well as the conditions under which they are stored pose a threat to their viability and presents major challenges for the probiotic industry (Stanton *et al.* 2005).

1.3.1.1 Dairy products

Dairy products are still the leading vehicles of choice for delivering probiotics to humans. Yoghurt, fermented sour milk, and cheese being among the most popular (Ranadheera *et al.* 2017). Furthermore, the constituents of dairy are thought to protect probiotic cells against the harsh conditions of the GIT. This is due to their high content of milk fats which buffer against

the deleterious effects of gastric acid and intestinal bile salts by reducing their direct exposure to them and thus increasing probiotic viability (Amund 2016; Ranadheera *et al.* 2017). In addition to milk fats, dairy products are also a source of the minerals calcium, inorganic phosphate and citrate. They also contain water soluble vitamins, C, B1 and B2 as well as the disaccharides alpha and beta lactose which act as probiotic substrates during fermentation (Flach *et al.* 2018). Furthermore, constituents of milk possess certain therapeutic properties. This was demonstrated in a study by de Vrese *et al.* (2011), who reported that milk containing probiotics or lactic acid could significantly reduce *Helicobacter pylori* activity.

1.3.1.1.1 Yoghurt

Of the many probiotic products available, yoghurt is still the most popular. It accounts for 78% of the worldwide probiotic sales in the developed world (Kandylis *et al.* 2016). This is because yoghurt has many known desirable attributes and is considered "healthy" by most. It is made from the symbiotic interaction of starter culture *Streptococcus thermophilus* and *Lactobacilli delbrueckii* subsp. *bulgaricus* (Lourens-hattingh and Viljoen 2001; Ashraf and Shah 2011). The presence of the starter cultures changes the nutritional content of the milk, their proteolytic activity results in higher amino acid content. Additionally, the starter cultures can produce vitamins, however, this process does not change the mineral content of the milk. So by the end of fermentation there is a decrease in lactose and an increase in the lactic acid, free amino acids and fatty acids (Lourens-hattingh and Viljoen 2001). The probiotics are usually added with the starter culture at the beginning of fermentation, after fermentation, or are used solely to carry out fermentation (Flach *et al.* 2018).

Adequate probiotic viability of up to 6 weeks can be achieved in yoghurt stored at refrigerated temperatures. Moreover, Lee *et al.* (2015) showed that in mice, GIT survival of probiotics was better when delivered in milk rather than a nutrient free buffer. The disadvantages of using yoghurt as a probiotic carrier is its low pH, and the oxygen often incorporated during production and packaging usually hinders the survival of certain *Bifidobacteria* strains (Flach *et al.* 2018).

1.3.1.2 Non Dairy Probiotic Foods

The popularity of dairy based products is mainly in developed countries. Even in these regions the rise in vegetarianism and the health risks involved with eating dairy has limited the use of

dairy based functional foods (Granato *et al.* 2010; Kandylis *et al.* 2016; Shori 2016a). In Asia and Africa, where dairy products are not popular among consumers, non-dairy based products are likely to be commercially more successful. Furthermore, in these countries non-dairy based probiotic beverages offer a cheaper alternative to dairy (Kandylis *et al.* 2016).

1.3.1.2.1 Fruits and Vegetables

A promising new alternative is that which involves probiotic beverages from fruit or vegetables. When consumers were asked to rank their top functional foods, fruit and vegetables were among the most highly rated (Dey 2018). They are packed with antioxidants, dietary fibre, vitamins and minerals. Their perceived health benefits is the reason many food manufacturers have commercialised fruits and vegetables as well as their derivatives (Dey 2018). Recently, to add more value to these products, nutraceuticals and probiotics are being introduced to fruit and vegetable juices (Granato *et al.* 2010; Shori 2016a; Dey 2018). Fruit juices have a taste profile that can be enjoyed by people from all age groups. They can be good substrates because of their high nutrient and sugar content, which facilitates probiotic growth and survival. Moreover, they spend a short amount of time in the harsh acidic conditions of the stomach which leads to high cell viability (Kumar, Vijayendra and Reddy 2015; Kandylis *et al.* 2016). Certain fruit and vegetables are high in prebiotic material which aid in growth and stimulation of the intestinal microflora. However, fruit and vegetable juices have a low pH and as such can lower probiotic survival. How well a probiotic survives during storage in fruit juices is largely strain dependent (Shori 2016a).

1.4 Threats to Probiotic Viability

The definition of probiotics stipulates that they be present in the end product in "adequate amounts". These exact numbers have not been determined however, the agreed upon number is between 10^8-10^9 cfu/ml, with at least 10^7cfu/ml microorganisms present at the end of a products shelf life. This is a general guideline as the number of probiotics will vary according to the strain and preparation process in which they undergo (FAO/WHO 2001; Corcoran *et al* 2008). However, there are many factors during the manufacture of probiotic products that present imminent threat to organism viability. These include the different conditions encountered during production such as temperature extremes, high salinity, and exposure to oxygen to name a few. In addition to production conditions the probiotics should also safely

navigate through the gastrointestinal tract where a host of dangers await them such as the digestive enzymes of the saliva, the acidic nature of the stomach and the bile secretions into the duodenum. Despite these physiological assaults which all have adverse effects on probiotic function and viability, the microorganisms must still reach the target organ in sufficient numbers or else they cease to confer the desired effects (Corcoran *et al.* 2008; du Toit *et al.* 2013). This presents a problem because many of the microorganisms used as commercial probiotics strains are sensitive to the stress conditions mentioned above (Gueimonde and Sánchez 2012).

1.4.1 Stress Conditions that Threaten Probiotic Survival

It is common practice to introduce probiotics to the host in dried forms as dietary supplements and in food carriers. It is also the norm to select probiotics of human origin for human hosts. Meaning that bacteria found in the stress free habitat of the small intestine are selected for use as probiotics (Ross *et al.* 2005; Corcoran *et al.* 2008). Before these probiotics are reintroduced to the GIT they must overcome two major types of stresses namely; technological stresses, those involved in production and storage of probiotic formulations. These stresses can range from but are not limited to; temperature extremes, acidification and osmotic stress. In addition to this, during storage they must survive in adequate amounts often for extended periods of time. The second type of stress, gastrointestinal, happens during passage through the GIT and includes; nutrient deficiency, bile, acid and oxidative stresses. The combination of these can lead to altered probiotic function and stability, so bacterial strains used must be able to overcome this (Culligan, Hill and Sleator 2009b; Amund 2016).

1.4.1.1 Technological stress

1.4.1.1.1 Temperature

Food industry applications use heat in many of their production processes, this is also true for the manufacture of functional foods. Probiotics grow optimally between the temperatures of 37^{o}C–43^{o}C, so when exposed to temperatures higher than this it should only be for short periods or they should be added downstream of cooking/pasteurization process (Tripathi and Giri 2014). Moreover, starter cultures that are directly added to milk are often in freeze dried or frozen forms. Thus, probiotics can experience different temperature extremes while in production (Ross *et al.* 2005; Haddaji *et al.* 2015). For any organism, including probiotics, to

remain viable at these temperature extremes the cellular components need to remain intact (Haddaji *et al.* 2015). Spray drying of probiotics usually occurs after they have been grown to large quantities and are dried for use in powdered formulations. This process can expose them to temperatures up to 200^{o}C. Moreover, Due to inability of probiotics to grow in milk they are often inoculated in high concentrations after freeze drying or as frozen cultures, where the former can reach temperatures of -197^{o}C and the latter -70^{o}C (Haddaji *et al.* 2015; Amund 2016). In the case of both, these bacteria are being exposed to temperature extremes. Although temperature sensitivity varies among strains, probiotic bacteria belonging to *Lactobacilli* and *Bifidobacteria* are generally susceptible to temperatures above 50^{o}C (Corcoran *et al.* 2008). Therefore, heat stress can cause considerable damage to these bacterial cells. Likewise cold exposure can also be detrimental.

While spray drying only exposes cells to temperature highs for a short amount of time, it is enough to cause considerable amount of heat damage. This kind of damage manifests itself in the cytoplasmic membrane where fatty acids, aggregation of proteins, intracellular proteins, RNA and ribosomes are adversely impacted. Furthermore, the spray dying process can also be a gateway to additional stress factors such as dehydration, oxidative and osmotic stress (Mills *et al.* 2011). Freeze-drying on the other hand is done in two steps, cells are frozen to below critical temperature for the formulation and using sublimation, are dried in a vacuum. During this procedure, the cell wall and its constituents are severely compromised and this usually leads to cell death. Furthermore, the fluidity of the cell membrane is also significantly reduced (Jalali *et al.* 2012). Therefore, probiotics produced for human consumption must overcome the temperature extremes encountered during production.

1.4.1.1.2 Oxygen

Probiotic bacteria used for human consumption often originate from the human intestine. These microorganisms are usually microaerophilic or strictly anaerobic, as is the case for *Lactobacillus* and *Bifidobacterium* spp., respectively. Unlike aerobic counterparts who have the ability to reduce oxygen to water, they do not possess effective oxygen scavenging mechanisms and exposure to oxygen results in oxygen toxicity; a phenomena where toxic oxygenic metabolites build up resulting in cell death (Talwalkar and Kailasapathy 2004b).

The sensitivity of probiotics to oxygen poses a problem, as many of these strains are incorporated into functional foods where preparation leads to inevitable oxygen exposure. This

occurs during the production of yoghurt where dissolved oxygen is required for starter culture *Streptococcus thermophilus* to ferment lactose. Furthermore, oxygen is incorporated at other stages in yoghurt production resulting in substantial oxygen concentrations in the final product, which consequently pose a threat to probiotic survival (Talwalkar and Kailasapathy 2004a).

Either than product manufacture, oxygen can also be introduced during storage. Many functional foods are kept in plastic packages that allow high levels of oxygen to diffuse through. The amount of this dissolved oxygen increases significantly during storage. This is especially concerning since numbers of the viable probiotics need to remain high throughout storage (Anand, Beniwal and Singh 2018). Other authors have noted that adding deoxidant and desiccant, which remove oxygen and water, respectively, improves probiotic survival (Chen, Chen and Shiu 2008). Moreover, the encapsulation of probiotics improves losses encountered during the storage period (Amund 2016).

1.4.1.1.3 Acidification

Although LAB like *Lactobacillus* are tolerant to acidic environments, it is not uncommon to see these bacteria sustain damage due to auto-acidification. In the process of sugar fermentation starter cultures produce a large amount of lactic acid as a by-product. This is usually an attractive quality to have as competing microorganisms are inhibited. However, too much lactic acid in the product can halt growth of LAB and can ultimately kill the cells (Papadimitriou *et al.* 2016).

Post acidification is another related phenomena that involves the increase in acid levels due to the continued production of acid by LAB in a product. This is especially problematic during storage when pH continues to decrease well after the fermentation. The amount of post-acidification experienced depends on the type of bacteria being used. Usually probiotic bacteria such as *L. acidophilus* and *Bifidobacterium* spp. promote low post-acidification while the commonly used starter culture *L. delbrueckii* subsp. *bulgaricus* are notorious for their acidification levels (Cavalcante *et al.* 2017). Conventional starter cultures *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are still preferred over probiotic bacteria because of their superior fermentation abilities. To attempt to combat the issue of post-acidification during storage slow acid producing strains of *L. delbrueckii* subsp. *bulgaricus* can now be used in fermentation process instead.

1.4.1.2 Gastrointestinal Stress Factor

Besides technological challenges encountered during processing and storage, probiotics must further endure host-associated stresses. Upon ingestion, they travel through the GIT where they face a decrease in pH in the stomach, bile salts and an increase in osmolarity in the intestines. All these diminish viability making them ineffective and unable to produce health benefits in the host. Therefore, it is imperative that strains that show promise for probiotic use are not technologically and physiologically fragile. Instead they should persist and survive not only technological hurdles but their travel in the GIT (Culligan, Hill and Sleator 2009b).

1.4.1.2.1 Stomach Acidity

One of the main factors that cause probiotic decline within the GIT is the acidity found inside the stomach. This acidity can vary from a pH of 5 to a low of 1.5 depending on the kind of food consumed. *Lactobacilli* spp. have intrinsic acid tolerance but decline when acidity drops below pH 3. However, *Lactobacillus rhamnosus* GG, can persist up to four hours at a pH 2.5 (Corcoran *et al.* 2005). Ability of other probiotics to survive the drop in pH is strain dependent, however, survival can be greatly increased if the probiotic is consumed with food containing a high buffering capacity such as dairy products (Corcoran *et al.* 2008).

1.4.1.2.2 Bile Salts

After encountering stomach acid another threat in the form of bile salts must be overcome in the small intestine. Bile salts, the main component of bile, function in solubilising and absorbing dietary fats. Their production involves conjugating amino acids, taurine and glycine with bile acids produced in the liver. Thereafter, they are stored in the gall bladder ready for secretion into the duodenum during digestion. The bile salt concentration within the intestine ranges from 2% to 0.05%, therefore, this environment can be lethal to microorganisms that are not adapted to these conditions (Ruiz, Margolles and Sánchez 2013b). A study by Hassanzadazar *et al.* in (2012) showed that of 28 strains of *Lactobacilli*, only five could survive bile exposure at 0.3% for four hours. The authors further noted that bile exposure led to the damage to the lipid bilayer and its associated proteins, which resulted in cell content leakage and ultimately cell death. Therefore, tolerance to bile salts is important when being selecting

strains for probiotic use. Authors like Havenaar, Brink and Huis In't Ved , go as far as stating that during probiotic selection, tolerance to the small intestine is more important than tolerance to stomach acidity because probiotics can be delivered to the small intestine using foods that buffer the stomach's acidic effect.

1.5 Ways to Enhance Probiotic Survival

Beneficial microorganisms are expected to pass through the gastrointestinal tract in a viable and active state but also sufficiently numerous in order to colonize the human gut (Kailasapathy and Chin 2000; Corcoran *et al.* 2005; Ruiz, Margolles and Sánchez 2013b). As such, measures must be in place to ensure that they survive manufacture, storage and gastrointestinal transit. Food additives, microencapsulation and increasing intrinsic stress tolerance are some of the ways in which probiotic survival can be improved (Kailasapathy and Chin 2000).

1.5.1 Food Additives

There are various additions to food that aid in the survival of probiotics. For instance, tomato juice added to skimmed milk improved the survival of *L. acidophilus* in addition to increasing viable counts, shortening generation time and enhancing sugar utilization (Babu, Mital and Garg 1992). Similar results were observed when milk was supplemented with casitone and fructose (Saxena, Mital and Garg 1994). This could be accredited to simple sugars and minerals like (magnesium and manganese) that are growth promoters for *L. acidophilus* (Ahmed, Mital and Garg 1990). *Bifidobacteria* which demonstrate poor growth in milk can be stimulated by adding vitamins, dextrin and maltose. *B. longum* in particular survives better in the presence of 0.01% baker's yeast (Medina and Jordano 1994).

1.5.2 Cell Immobilization

Another way in which probiotic survival can be boosted is by offering physical protection against threats found in the GIT. To this end, immobilization and encapsulation techniques are used. Cell immobilization and microencapsulation are often used interchangeably but to be more specific, cell immobilization is the process where material i.e. bacterial cells, are trapped throughout or within a matrix. This means that during this process, some of the material could be exposed to the surface, this is not the case for microencapsulation (Vidhyalakshimi,

Bhakyaraj and Subhasree 2009). However, cells that have been immobilized are offered more protection against harsh environment than free cells, they can also be separated from the product and yield greater productivity due to high cell concentrations present (Gbassi *et al.* 2012; Rathore *et al.* 2013).

Different techniques can be used to immobilize cells, adsorption is one of these; it takes advantage of the cells natural ability to adhere to solid support and form biofilms (Rathore *et al.* 2013). And for those cells that do not possess this trait, cross linking chemically using glutaraldehyde and salinization onto silica support can offer an alternative. These processes are simple to perform and inexpensive. However, cell leakage from support which affects the ability to obtain cell free effluent downstream, is a major limitation (Zhu 2007; Kosseva 2011).

Moreover, the refinement of the immobilization process gave birth to the more commonly used cell encapsulation technologies which address issues like cell leakage, contamination, low mechanical stability encountered by many immobilization techniques (Gbassi *et al.* 2012; Rathore *et al.* 2013).

1.5.3 Microencapsulation

Microencapsulation unlike immobilization is a matrix surrounded by a coat contained within a capsule wall (Vidhyalakshimi, Bhakyaraj and Subhasree 2009; Gbassi *et al.* 2012). Encapsulation of bacteria in this way can offer safe GIT passage and targeted release in the small and large intestine (Mandal, Puniya and Singh 2006). Furthermore, the use of encapsulation stimulates the production and excretion of secondary metabolites it also increases operating efficiency during fermentation (Nazzaro *et al.* 2009). The microparticles used for the encapsulation process must remain structurally intact within food matrix and in the GIT therefore water insolubility is imperative (Nazzaro *et al.* 2012). Caution should be taken to ensure that the polymer used is safe for both host and bacteria and therefore should not be antimicrobial or cytotoxic (Mandal, Puniya and Singh 2006). Which is why naturally occurring polysaccharides and proteins are often used (Cook *et al.* 2012). Additionally, these natural polymers offer a large range of functional properties as a result of their many variations in chemical structures, sugar composition, charge density, anomeric configuration, repeat sequences and degree of polymerization. Many of them being derived from higher plants (cellulose, starch and pectin) or seaweed (alginate, agar and carrageenan) (Murano 1998).

1.5.4 Stress Adaptation

Survival of probiotics can also be enhanced by manipulating its stress responses through pre-exposure to sub-lethal stress (Amund 2016). This process of pre-stress adaptation creates an adaptive response by inducing heat shock proteins that allow microorganisms to survive encounters with lethal stress levels (Yousef and Courtney 2003). Although this could also breed stress tolerant pathogens, it offers a relatively inexpensive solution to the problem of maintaining probiotic viability during product manufacture. To this end, sub-lethal conditions of acid and heat are often used to induce stress tolerance as these are the two factors that adversely affect probiotic survival. Other findings reveal that after pre-exposure to these, the probiotic survival increased upon encountering lethal doses of heat and acid (Gueimonde and Sánchez 2012). The mechanism of pre-stress treatment is not limited to heat and acid, others stress conditions can also be used to achieve stress tolerance. Furthermore, the exposure to just one of these conditions can lead to protection against other types of hostile conditions, a mechanism known as cross protection. This is especially useful for overcoming the different types of stresses encountered during industrial processes (Begley, Gahan and Hill 2002; Champagne, Gardner and Roy 2005).

A recent study done on *Lactobacillus kefiranofaciens* looked at the effects of acid, bile, hot and cold temperatures on this bacterium. It showed that exposure to one condition offered cross protection against other stresses, the extent of protection depended on the kind of stress experienced. The study further revealed that the proteomic profile of the treated vs untreated cells differed by 27 proteins. These proteins are thought to be involved in improving the stress response of the treated bacteria (Chen, Tang and Chiang 2017). In an earlier study by (Mathipa and Thantsha 2015) multiple stress adaptation was proven to be better at enhancing probiotic survival compared to adaptation to a single stress factor. This study will explore this further by looking at whether probiotic properties of multi-stress adapted strains are affected by the process of long-term freezing.

1.6 Conclusion

Probiotics products are popularly consumed due to their health benefits, however, their effectiveness hinges on their ability to remain viable in high numbers until they reach their target organ, the large intestine. Many of the microorganisms used are fastidious and are sensitive to environmental stress leading to losses in viability during manufacture and passage through the GIT. Inability to deliver on the promised health benefits will lead to economic losses for manufacturers of these products. Multi-stress adaptation is one way in which the survival of probiotics can be improved upon. Another way is to combine multi-stress adapted strains to food matrices that facilitate survival during storage and GIT transit. However, because many probiotic strains are often subjected to long term cold-storage, any acquired functional enhancements should be retained during storage period. In this study, previously multi-stress adapted strains that were stored at cold temperatures (-20^{o}C) were evaluated for any functional and morphological changes by comparing them to their freshly and non-adapted strains. And because food matrices play a role in the probiotic survival, the stability of these strains in yoghurt, carrot and cranberry juice and under simulated GIT conditions was also assessed.

1.7 References

Ahmed B, Mital BK, Garg SK. Effect of magnesium and manganese ions on the growth of *Lactobacillus acidophilus*. *J Food Sci Technol* 1990;**27**:228–9.

Amund OD. Exploring the relationship between exposure to technological and gastrointestinal stress and probiotic functional properties of *Lactobacilli* and *Bifidobacteria*. *Can J Microbiol* 2016;**62**:715–25.

Anand S, Beniwal A, Singh KS. Significance of probiotic encapsulation and deficiencies within. *Pharma Innov J* 2018;**7**:434–9.

De Angelis M, Gobbetti M. Environmental stress responses in *Lactobacillus*: A review. *Proteomics* 2004;**4**:106–22.

Arena MP, Silvain A, Normanno G *et al.* Use of *Lactobacillus plantarum* strains as a bio-control strategy against food-borne pathogenic microorganisms. *Front Microbiol* 2016;**7**:464.

Ashraf R, Shah NP. Selective and differential enumerations of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium* spp. in yoghurt - A review. *Int J Food Microbiol* 2011;**149**:194–208.

Babu V, Mital BK, Garg SK. Effect of tomato juice addition on the growth and activity of *Lactobacillus acidophilus*. *Int J Food Microbiol* 1992;**17**:67–70.

Bäumler AJ, Sperandio V. Interactions between the microbiota and pathogenic bacteria in the gut. *Nature* 2016;**535**:85–93.

Begley M, Gahan CGM, Hill C. Bile stress response in *Listeria monocytogenes* LO28: adaptation, cross-protection, and identification of genetic loci involved in bile resistance. *Appl Environ Microbiol* 2002;**68**:6005–12.

Bezkorovainy A. Probiotics: determinants of survival and growth in the gut. *Am J Clin Nutr* 2001;**73**:399s-405s.

Blaabjerg S, Artzi DM, Aabenhus R. Probiotics for the prevention of antibiotic-associated diarrhea in outpatients-A systematic review and meta-analysis. *Antibiotics* 2017;**6**, DOI: 10.3390/antibiotics6040021.

Blaser M. Stop the killing of beneficial bacteria. *Nature* 2011;**476**:393–4.

Blum S, Haller D, Pfeifer A *et al.* Probiotics and immune response. *Clin Rev Allergy Immunol* 2002;**22**:287–309.

Booyens J, Labuschagne MC, Thantsha MS. In vitro antibacterial mechanism of action of crude garlic (*Allium sativum*) clove extract on selected probiotic *Bifidobacterium* species as revealed by SEM, TEM, and SDS-PAGE analysis. *Probiotics Antimicrob Proteins* 2014;**6**:82–7.

Booyens J, Thantsha MS. Antibacterial effect of hydrosoluble extracts of garlic (*Allium sativum*) against *Bifidobacterium* spp. and *Lactobacillus acidophilus*. *African J Microbiol Res* 2013;**7**:669–77.

Bottacini F, Ventura M, Sinderen D *et al.* Diversity, ecology and intestinal function of bifidobacteria. *Microb Cell Fact* 2014;**13**:S4.

Boyle RJ, Robins-Browne RM, Tang ML. Probiotic use in clinical practice: what are the risks? *Am J Clin Nutr* 2006;**83**:1256–64.

Burns AJ, Rowland IR. Anti-carcinogenicity of probiotics and prebiotics. *Curr Issues Intest Microbiol* 2000;**1**:13–24.

Callaway TR, Edrington TS, Anderson RC *et al.* Probiotics, prebiotics and competitive exclusion for prophylaxis against bacterial disease. *Anim Heal Res Rev* 2008;**9**:217–25.

Cavalcante D, Santos DOS, Timoni L *et al.* Post-acidification and evaluation of anthocyanins stability and antioxidant activity in acai fermented milk and yogurts. *Rev Bras Frutic* 2017;**39**:1–13.

Champagne CP, Gardner NJ, Roy D. Challenges in the addition of probiotic cultures to foods. *Crit Rev Food Sci Nutr* 2005;**45**:61–84.

Chen KN, Chen MJ, Shiu JS. Development of probiotic candies with optimal viability by using response surface methodology and sequential quadratic programming. *Asian-Australasian J Anim Sci* 2008;**21**:896–902.

Chen M, Tang H, Chiang M. Effects of heat, cold, acid and bile salt adaptations on stress tolerance and protein expression of kefir-isolated probiotic *Lactobacillus kefiranofaciens*. *Food Microbiol* 2017;**66**:20–7.

Cook MT, Tzortzis G, Charalampopoulos D *et al.* Microencapsulation of probiotics for gastrointestinal delivery. *J Control Release* 2012;**162**:56–67.

Corcoran B, Stanton C, Fitzgerald G *et al.* Life under stress: The probiotic stress response and how it may be manipulated. *Curr Pharm Des* 2008;**14**:1382–99.

Corcoran BM, Stanton C, Fitzgerald GF *et al.* Survival of probiotic *Lactobacilli* is enhanced in acidic environments in the presence of metabolizable sugars. *Appl Environ Microbiol* 2005;**71**:3060–7.

Cruz AG, Faria DAF, Dender AGF Van. Packaging system and probiotic dairy foods. *Food Res Int* 2007;**40**:951–6.

Culligan EP, Hill C, Sleator RD. Probiotics and gastrointestinal disease: successes, problems and future prospects. *Gut Pathog* 2009;**1**:19.

Dey G. Non-dairy probiotic foods: innovations and market trends. *Innovations in Technologies for Fermented Food and Beverage Industries*. Cham: Springer International Publishing, 2018, 159–73.

Flach J, van der Waal MB, van den Nieuwboer M *et al.* The underexposed role of food matrices in probiotic products: Reviewing the relationship between carrier matrices and product parameters. *Crit Rev Food Sci Nutr* 2018;**58**:2570–84.

Flint HJ, Scott KP, Louis P *et al.* The role of the gut microbiota in nutrition and health. *Nat Rev Gastroenterol Hepatol* 2012;**9**:577–89.

Floch MH. Probiotic safety and risk factors. *J Clin Gastroenterol* 2013;**47**:375–6.

Fooks LJ, Fuller R, Gibson GR. Prebiotics, probiotics and human gut microbiology. *Int Dairy J* 1999;**9**:53–61.

Fukuda K. Is it feasible to control pathogen infection by competitive binding of probiotics to the host? *Virulence* 2017;**8**:1502–5.

Fuller R. Probiotics in man and animals. *J Appl Bacteriol* 1989;**66**:365–78.

Fuller R. History and development of probiotics. *Probiotics*. Dordrecht: Springer Netherlands, 1992, 1–8.

Gbassi GK, Vandamme T, Gbassi GK *et al.* Probiotic encapsulation technology: From

microencapsulation to release into the gut. *Pharmaceutics* 2012;**4**:149–63.

Gill HS. Probiotics to enhance anti-infective defences in the gastrointestinal tract. *Best Pract Res Clin Gastroenterol* 2003;**17**:755–73.

Granato D, Branco GF, Nazzaro F *et al.* Functional foods and nondairy probiotic food development: trends, concepts, and products. *Compr Rev Food Sci Food Saf* 2010;**9**:292–302.

Gueimonde M, Sánchez B. Enhancing probiotic stability in industrial processes. *Microb Ecol Heal Dis* 2012;**23**, DOI: 10.3402/mehd.v23i0.18562.

Haddaji N, Krifi B, Lagha R *et al.* Effect of high temperature on viability of *Lactobacillus casei* and analysis of secreted and GroEL proteins profiles. *African J Bacteriol Res* 2015;**7**:29–34.

Halasz A. *Food Quality And Standards - Volume III*. Lasztity R (ed.). Oxford: Eolss Publishers Co. Ltd, 2009.

Hao W-L, Lee Y-K. Microflora of the gastrointestinal tract: A Review. *Public Health Microbiology*. New Jersey: Humana Press, 2004, 491–502.

Hassanzadazar H, Ehsani A, Mardani K *et al.* Investigation of antibacterial, acid and bile tolerance properties of *lactobacilli* isolated from Koozeh chees. *Vet Res forum an Int Q J* 2012;**3**:181–5.

Havenaar R, Brink NG, Huis In't Ved JHJ. Probiotics; A general review. In: Fuller R (ed.). *Lactic Acid Bacteria Volume 1*. London: Elsevier, 1992, 210–40.

Hempel SJ, Newberry SJ, Maher AR *et al.* Probiotics for the prevention and treatment of antibiotic-associated diarrhea. *JAMA* 2012;**307**:1959.

Hennessy M. Whats drivong growh in functional foods and beverages? *What's Driv Funct food beverages? A Converg Nutr Conv Tast* 2013.

Hsiao EY, McBride SW, Hsien S *et al.* Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* 2013;**155**:1451–63.

Huang R, Wang K, Hu J. Effect of probiotics on depression: A Systematic review and meta-analysis of randomized controlled trials. *Nutrients* 2016;**8**:483.

Jalali M, Abedi D, Varshosaz J *et al.* Stability evaluation of freeze-dried *Lactobacillus paracasei* subsp. tolerance and *Lactobacillus delbrueckii* subsp. *bulgaricus* in oral capsules. *Res Pharm Sci* 2012;**7**:31–6.

Kailasapathy K, Chin J. Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and Bifidobacterium spp. *Immunol Cell Biol* 2000;**78**:80–8.

Kandylis P, Pissaridi K, Bekatorou A *et al.* Dairy and non-dairy probiotic beverages. *Curr Opin Food Sci* 2016;**7**:58–63.

Kechagia M, Basoulis D, Konstantopoulou S *et al.* Health benefits of probiotics: a review. *ISRN Nutr* 2013;**2013**:481651.

Khorshidian N, Yousefi Asli M, Hosseini H *et al.* Potential anticarcinogenic effects of lactic acid bacteria and probiotics in detoxification of process-induced food toxicants. *Iran J Cancer Prev* 2016;**In Press**, DOI: 10.17795/ijcp-7920.

Kolida S, Saulnier DM, Gibson GR. Gastrointestinal microflora: probiotics. *Adv Appl Microbiol* 2006;**59**:187–219.

Kosseva MR. Immobilization of microbial cells in food fermentation. *Food Bioprocess Technol* 2011;**4**:1089–118.

Kumar VB, Vijayendra SVN, Reddy OVS. Trends in dairy and non-dairy probiotic products - a review. *J Food Sci Technol* 2015;**52**:6112–24.

Lahtinen SJ, Davis E, Ouwehand AC. *Lactobacillus* species causing obesity in humans: where is the evidence? *Benef Microbes* 2012;**3**:171–4.

Lee B, Yin X, Griffey SM *et al.* Attenuation of colitis by *Lactobacillus casei* BL23 is dependent on the dairy delivery matrix. *Appl Environ Microbiol* 2015;**81**:6425–35.

Levri KM, Ketvertis K, Deramo M *et al.* Do probiotics reduce adult lactose intolerance? A systematic review. *J Fam Pract* 2005;**54**:613–20.

Li Q, Lui X, Dong M *et al.* Aggregation and adhesion abilities of 18 Lactic acid bacteria strains isolated from traditional fermented food. *Int J Agric Policy Res* 2015;**3**:84–92.

Lilly DM, Stillwell RH. Probiotics: Growth-promoting factors produced by microorganisms. *Science (80-)* 1965;**147**:747–8.

Ljungh A, Wadström T. *Lactobacillus Molecular Biology : From Genomics to Probiotics*. Ljung A, Wadstrom T (eds.). Caister Academic, 2009.

Lourens-hattingh A, Viljoen BC. Yogurt as probiotic carrier food. 2001;**11**:1–17.

Ma L, Lv Z, Su J *et al.* Consistent condom use increases the colonization of *Lactobacillus crispatus* in the vagina. Drews SJ (ed.). *PLoS One* 2013;**8**:e70716.

Mandal S, Puniya AK, Singh K. Effect of alginate concentrations on survival of microencapsulated *Lactobacillus casei* NCDC-298. *Int Dairy J* 2006;**16**:1190–5.

Mathipa M, Thantsha M. Cocktails of probiotics pre-adapted to multiple stress factors are more robust under simulated gastrointestinal conditions than their parental counterparts and exhibit enhanced antagonistic capabilities against *Escherichia coli* and *Staphylococcus aureus*. *Gut Pathog* 2015;**7**:5.

Mathipa MG, Thantsha MS. Probiotic engineering: towards development of robust probiotic strains with enhanced functional properties and for targeted control of enteric pathogens. *Gut Pathog* 2017;**9**:28.

McFarland L V. Meta-Analysis of Probiotics for the Prevention of Antibiotic Associated Diarrhea and the Treatment of Clostridium difficile Disease. *Am J Gastroenterol* 2006;**101**:812–22.

Medina LM, Jordano R. Survival of constitutive microflora in commercially fermented milk containing *Bifidobacteria* during refrigerated storage. *J Food Prot* 1994;**57**:731–3.

Mills S, Stanton C, Fitzgerald GF *et al.* Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again. *Microb Cell Fact* 2011;**10**:S19.

Misra S, Mohanty D. Psychobiotics: A new approach for treating mental illness? *Crit Rev Food Sci Nutr* 2019;**59**:1230–6.

Mohammadi AA, Jazayeri S, Khosravi-Darani K *et al.* The effects of probiotics on mental health and hypothalamic–pituitary–adrenal axis: A randomized, double-blind, placebo-controlled trial in petrochemical workers. *Nutr Neurosci* 2016;**19**:387–95.

Moura MRL. Alimentos funcionias: seus beneficious e a ligislacao. 2005.

Murano E. Use of natural polysaccharides in the microencapsulation techniques. *J Appl Ichthyol* 1998;**14**:245–9.

Nagpal R, Kumar A, Kumar M *et al.* Probiotics, their health benefits and applications for developing healthier foods: a review. *FEMS Microbiol Lett* 2012;**334**:1–15.

Nazzaro F, Fratianni F, Coppola R *et al.* Fermentative ability of alginate-prebiotic encapsulated *Lactobacillus acidophilus* and survival under simulated gastrointestinal conditions. *J Funct Foods* 2009;**1**:319–23.

Nazzaro F, Orlando P, Fratianni F *et al.* Microencapsulation in food science and biotechnology. *Curr Opin Biotechnol* 2012;**23**:182–6.

Nissen L, Chingwaru W, Sgorbati B *et al.* Gut health promoting activity of new putative probiotic/protective *Lactobacillus* spp. strains: A functional study in the small intestinal cell model. *Int J Food Microbiol* 2009;**135**:288–94.

Ouwehand AC, Salminen SJ. The health effects of cultured milk products with viable and non-viable bacteria. *Int Dairy J* 1998;**8**:749–58.

Ouwehand AC, Tolkko S, Salminen S. The effect of digestive enzymes on the adhesion of probiotic bacteria in vitro. *J Food Sci* 2001;**66**:856–9.

Papadimitriou K, Alegría Á, Bron PA *et al.* Stress physiology of lactic acid bacteria. *Microbiol Mol Biol Rev* 2016;**80**:837–90.

Parker RB. Probiotics. The other half of the antibiotics story. *Anim Nutr Heal* 1974;**29**:4–8.

Picard C, Fioramonti J, Francois A *et al.* Review article: bifidobacteria as probiotic agents - physiological effects and clinical benefits. *Aliment Pharmacol Ther* 2005;**22**:495–512.

Poupard JA, Husain I, Norris RF. Biology of the *Bifidobacteria*. *Bacteriol Rev* 1973;**37**:136–65.

Ranadheera CS, Vidanarachchi JK, Rocha RS *et al.* Probiotic delivery through fermentation : dairy vs non-dairy beverages. *Fermentation* 2017;**3**:1–17.

Rao KR, Samak G. Protection and restitution of gut barrier by probiotics: Nutritional and clinical implications. *Curr Nutr Food Sci* 2013;**9**:99–107.

Rathore S, Desai PM, Liew CV *et al.* Microencapsulation of microbial cells. *J Food Eng* 2013;**116**:369–81.

Rolfe RD. The role of probiotic cultures in the control of gastrointestinal health. *J Nutr*

2000;**130**:396S-402S.

Ross, Fitzgerald P;, Collins G; *et al.* Cheese delivering biocultures-probiotic cheese. *Aust J Dairy Technol* 2002;**57**:71.

Ross RP, Desmond C, Fitzgerald GF *et al.* Overcoming the technological hurdles in the development of probiotic foods. *J Appl Microbiol* 2005;**98**:1410–7.

Ruiz L, Margolles A, Sánchez B. Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium*. *Front Microbiol* 2013;**4**:396.

Saarela M, Mogensen G, Fondén R *et al.* Probiotic bacteria: safety, functional and technological properties. *J Biotechnol* 2000;**84**:197–215.

Sanz Y. Ecological and functional implications of the acid-adaptation ability of *Bifidobacterium*: A way of selecting improved probiotic strains. *Int Dairy J* 2007;**17**:1284–9.

Savage DC. Microbial ecology of the gastrointestinal tract. *Annu Rev Microbiol* 1977;**31**:107–33.

Saxena SN, Mital BK, Garg SK. Effect of casitone and fructose on the growth of *Lactobacillus acidophilus* and its survival during storage. *Int J Food Microbiol* 1994;**21**:271–6.

Shori AB. Influence of food matrix on the viability of probiotic bacteria: A review based on dairy and non-dairy beverages. *Food Biosci* 2016;**13**:1–8.

Stanton C, Ross RP, Fitzgerald GF *et al.* Fermented functional foods based on probiotics and their biogenic metabolites. *Curr Opin Biotechnol* 2005;**16**:198–203.

Šušković J, Kos B, Beganović J *et al.* Antimicrobial activity - The most important property of probiotic and starter lactic acid bacteria. *Food Technol Biotechnol* 2010;**48**:296–307.

Talwalkar A, Kailasapathy K. A review of oxygen toxicity in probiotic yogurts : Influence on the survival of probiotic bacteria and protective techniques. *Compr Rev Food Sci Food Saf* 2004a;**3**:117–24.

Talwalkar A, Kailasapathy K. The role of oxygen in the viability of probiotic bacteria with reference to *L. acidophilus* and *Bifidobacterium* spp. *Curr Issues Intest Microbiol* 2004b;**5**:1–8.

Tharmaraj N, Shah NP. Selective enumeration of *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, Bifidobacteria, *Lactobacillus casei*, *Lactobacillus rhamnosus*, and Propionibacteria. *J Dairy Sci* 2003;**86**:2288–96.

du Toit E, Vesterlund S, Gueimonde M *et al.* Assessment of the effect of stress-tolerance acquisition on some basic characteristics of specific probiotics. *Int J Food Microbiol* 2013;**165**:51–6.

Tojo R, Suárez A, Clemente MG *et al.* Intestinal microbiota in health and disease: Role of *bifidobacteria* in gut homeostasis. *World J Gastroenterol* 2014;**20**:15163.

Tripathi MK, Giri SK. Probiotic functional foods: Survival of probiotics during processing and storage. *J Funct Foods* 2014;**9**:225–41.

Urbańska M, Szajewska H. The efficacy of *Lactobacillus reuteri* DSM 17938 in infants and children: a review of the current evidence. *Eur J Pediatr* 2014;**173**:1327–37.

Vidhyalakshimi R, Bhakyaraj R, Subhasree R. Encapsulation "The Future of Probiotics"-A Review. *Adv Biol Res (Rennes)* 2009;**3**:96–100.

Volzing K, Borrero J, Sadowsky MJ *et al.* Antimicrobial peptides targeting gram-negative pathogens, produced and delivered by lactic acid bacteria. *ACS Synth Biol* 2013;**2**:643–50.

de Vrese M, Kristen H, Rautenberg P *et al.* Probiotic *lactobacilli* and *bifidobacteria* in a fermented milk product with added fruit preparation reduce antibiotic associated diarrhea and *Helicobacter pylori* activity. *J Dairy Res* 2011;**78**:396–403.

Wallace CJK, Milev R. The effects of probiotics on depressive symptoms in humans: a systematic review. *Ann Gen Psychiatry* 2017;**16**:14.

Yousef AE, Courtney PD. *Microbial Stress Adaptation and Food Safety*. Yousef AE, Juneja VK (eds.). Boca Ranton: CRC Press LLC, 2003.

Zhong L, Zhang X, Covasa M. Emerging roles of lactic acid bacteria in protection against colorectal cancer. *World J Gastroenterol* 2014;**20**:7878–86.

Zhu Y. Immobilized cell fermentation for production of chemicals and fuels. In: Yang ST (ed.). *Bioprocessing for Value-Added Products from Renewable Resources*. Elsevier B.V, 2007, 373–96.

Zotta T, Parente E, Ricciardi A. Aerobic metabolism in the genus *Lactobacillus* : impact on stress response and potential applications in the food industry. *J Appl Microbiol* 2017;

Chapter 2

Morphological and functional properties of *Lactobacilli* and *Bifidobacetrium* strains as influenced by acid-bile-temperature stress adaptation process

2.1 Abstract

It is well known that probiotics are microorganisms with health benefits. They lose their viability and hence their functionality as a result of exposure to unfavourable conditions during production and when passing through the gastrointestinal tract. Multi-stress adaptation is one of the ways that this can be averted allowing probiotics to reach target site viable and in sufficient numbers. The current study investigated whether long term storage of multi- stress adapted (acid- bile- temperature) probiotics had an effect on their functional performance and morphological properties. Cells were exposed to pH 2, 2.5 and 3 for 3 hours. For bile tolerance the cells were exposed to 0.3, 0.5 and 2% bile salts for 3 hours. The bile salt hydrolase activity and antimicrobial susceptibility profiles of the adapted and non-adapted cells were determined. Morphological changes of the cells were of observed using scanning electron microscopy and the auto-aggregation abilities and cell surface hydrophobicity of the cells were measured. The results showed that adapting the cells sequentially to different stress factors increased their survival. Exposure of strains to pH 2, 2.5 & 3 showed no notable difference between non-adapted, old-adapted and freshly adapted cells ($p>0.05$). The survival in bile salts was better for the non-adapted cells; however, this could not be attributed to bile salt hydrolase activity as all non-adapted cultures lacked this activity. All probiotic cultures were capable of inhibiting *Staphylococcus aureus* and *Escherichia coli* but significant inhibition of *S. aureus* was only achieved for the old adapted cells of *Lactobacillus plantarum* ($p<0.005$). The strains also shared the same antibiotic susceptibility profile with deviations occurring in *L. plantarum* and *Bifidobacterium longum* Bb46. Scanning electron microscopy revealed that stress adaptation changed the morphology of many of the probiotic strains. Multi-stress adaptation led to significant differences between the cell surface hydrophobicity of adapted and non-adapted cells ($p<0.0001$). Non-adapted cells had better aggregation abilities than adapted equivalents. The results indicate storage following adaptation process can affect certain functions of probiotic bacteria, however these are limited to certain strains. Therefore, caution should be taken when freezing probiotics for storage as this method can impair their function and can thereby limit the benefits experienced by consumers.

2.2 Introduction

Probiotics have been defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on host" (FAO/WHO 2001) and often belong to the genera *Lactobacillus* and *Bifidobacterium*, although others do exist (Vizoso Pinto *et al.* 2006). As stated in the definition, probiotics should maintain viability and functional abilities from the time of production throughout gastrointestinal passage. It has been stated that probiotics must be at a concentration of 10^6-10^7 CFU/ml when they reach the ileum, for beneficial effects to be exerted (Sanz 2007). However, the strains usually encounter stresses at various places of production, during storage and in the gastrointestinal tract (GIT) (Amund 2016).

Many technological processes involved in making probiotic products pose a threat to their viability. Acidification of fermented products is one of the important factors limiting probiotic viability during production (Sánchez *et al.* 2007). Moreover, because these probiotics are administered orally they must make their way through the gastric juices of the stomach, through to the small intestine where bile salts are excreted. All while maintaining sufficient cell numbers (Corcoran *et al.* 2008; Mills *et al.* 2011; Amund 2016). It is critical that strains selected as probiotics not only survive these conditions in sufficient numbers but are able to colonize the host (Lv *et al.* 2017). However, many of the strains used do not survive technological and gastrointestinal challenges that they face (Corcoran *et al.* 2008).

Different strategies are employed to boost probiotic survival within the host. Among the many strategies used to improve viability of probiotics, stress adaptation has proved to be successful. During this process the bacteria are exposed to moderate or sub-lethal stresses which often leads to the development or an increase in the tolerance to the same or different stress (Yousef and Courtney 2003). Previous research has shown that the use of stress adaptation is effective for increasing survival of these microorganisms. After exposure to heat shock and moderate exposure to acid, *Lactobacilli* were able to survive subsequent exposure to the same stress (Saarela *et al.* 2004). A similar pattern was observed for *Bifidobacteria* after being pre-exposed to acid (Park *et al*. 1995). Mathipa and Thantsha (2015) showed how multi-stress adaptation not only improved functional abilities of probiotic strains but were superior to those strains that had only been adapted to one stress. Using the process of multi-stress adaptation will help produce strains that remain resilient to the different lethal environmental conditions they face. When using multi-stress adapted probiotics their properties must be reassessed to determine whether they have retained their functional properties (Sánchez *et al.* 2013). This should also

be done for strains that were adapted and then stored for long periods. The current study aims to determine the effects long-term cold storage has on the functionality and structural properties of multi-stress adapted probiotics.

2.3 Materials and Methods

2.3.1 Bacterial Cultures

Bifidobacterium bifidum LMG 11041, *Bifibacterium longum* LMG 13197, *Bifidobacterium longum* Bb46, *Lactobacillus acidophilus* La14 150B *and Lactobacillus plantarum* glycerol stock from Probiotics Research Group, University of Pretoria were used as test probiotic cultures. Non-adapted and previously adapted cultures (acid-bile-temperature) of *Lactobacilli* and *Bifidobacteria* that were stored at -20^{o}C for 24 months, hereafter referred to as old-adapted, were sub-cultured twice in de Man Rogosa (MRS) broth (Merck, South Africa) and *Bifidobacterium* spp. in MRS broth supplemented with 0.05% v/v L-cysteine hydrochloride monohydrate (MRS-cys-HCL). They were incubated at 37^{o}C for 72 hours in anaerobic jars containing Anaerocult A gaspacks with anaerostrips (Merck, South Africa). All anaerobic incubations were performed in this same way. Before each experiment probiotic cultures were standardised to an optical density of 0.2 at 600_{nm} which approximately equates to 10^{8} CFU/ml. Two foodborne pathogens, *Escherichia coli* and *Staphylococcus aureus* were used for antimicrobial testing. They were revived by growing in Luria Bertani (LB) broth incubated in an orbital shaking incubator (LM- 530R) (100 rpm) at 37^{o}C, overnight. They were then be plated onto Mannitol salt agar (Merck, South Africa) and MacConkey agar (Merck, South Africa) for *S. aureus* and *E. coli*, respectively, and incubated at 37^{o}C for 48 hours. These pathogens were then sub-cultured twice for use in experiments.

2.3.2 Stress Adaptation of Probiotics

2.3.2.1 Acid Adaptation

All the stress adaptation of probiotics was done according to (Mathipa and Thantsha 2015). Overnight cultures were harvested by centrifugation at 604 x g for 15 minutes and the pellets were re-suspended in 1ml ¼ strength Ringer's solution (Merck, South Africa). Then 1ml of the cultures was added to 9ml of MRS or MRS-cys broth at pH 2 (adjusted using 1M HCL) for two hours. The acid adaptation was done at 37^{o}C. After two hours, 100 µl from incubated suspensions was transferred to 900µl of MRS or MRS-cys-HCL Broth. Serial dilutions up to 10^{-7} using ¼ strength Ringer's solution were performed. The diluted cultures were then recovered by plating onto MRS and MRS-cys-HCL agar plated for *Lactobacilli* and

Bifidobacteria, respectively. The plates were incubated anaerobically for 72 hours at 37ºC. The surviving cells were used for bile adaptation.

2.3.2.2 Bile Adaptation

Previously acid adapted cultures were grown overnight and aseptically transferred to Falcon tubes containing 2% bile solution (w/v). The cultures were then incubated in shaking incubator (100 rpm) for 60 minutes at 37ºC. Following incubation, 1ml of each strain was added to 9ml of MRS or MRS-cys-HCL broth. Following which the cell suspensions were serially diluted up 10^{-7} using ¼ strength Ringer's solution and 100µl of each dilution was plated onto MRS or MRS-cys–HCL plates in triplicates. The plates were incubated anaerobically at 37ºC for 72 hours. Those cells that survived bile exposure were used for temperature adaptation.

2.3.2.3 Temperature Adaptation

Acid- bile- adapted cells that had been grown overnight in shaking incubator (100 rpm) were used. One millilitre from overnight cultures were transferred into 9ml of MRS and MRS-cys-HCL broth and placed in a water bath set at 55ºC (AccuBlock digital dry bath) for 120 minutes. The surviving cultures were then serially diluted up to 10^{-7} using ¼ strength Ringer's solution and 100µl of each dilution were plated in triplicated onto MRS or MRS-cys-HCL agar plates. The plates were then incubated at 37ºC under anaerobic conditions. After the temperature adaptation, the stress adapted cultures were recovered by growing them overnight in MRS or MRS-cys-HCL broth at 37ºC. The resulting freshly adapted acid- bile- temperature strains were stored in 20% glycerol stock (1:1) at -20ºC.

2.3.3 Acid Tolerance

Acid tolerance of the non-adapted, old-adapted and freshly adapted cells was done using the method referred to in (Mathipa and Thantsha 2015). Cultures of *Lactobacilli* and *Bifidobacteria* grown overnight at 37ºC in MRS and MRS- cys- HCL were used. Ten millilitres of fresh MRS and MRS-cys-HCL adjusted to pH (2, 2.5 and 3) with 1M HCL was used to sub-culture cells, followed by an incubation step at 37ºC. One hundred millilitres aliquots were drawn hourly for 3 hours and placed into 1ml MRS-cys-HCL broth. Resulting suspensions were serially diluted using ¼ strength Ringer's solution and 100µl from each dilution was plated onto MRS or MRS-cys- HCL plates in triplicates. Anaerobic incubation of plates was then done using anaerobic jars with Anaerocult gaspacks and Anaerotest strips at 37ºC for 72 hours. Plates with colonies between 30-300 were counted following incubation.

2.3.4 Bile Tolerance

Probiotic tolerance to bile salts was done according to (Mathipa and Thantsha 2015) with minor modifications. Briefly, non-adapted, old-adapted and freshly adapted probiotic cells were grown overnight in MRS or MRS-cys-HCL broth at 37ºC. These cells were harvested by centrifugation and washed twice in ¼ strength Ringer's solution before 100µl was added to MRS or MRS-cys-HCL broth adjusted to 0.3, 0.5 and 2% (w/v) bile salts. The cells suspensions were incubated at 37ºC and 100µl aliquots were taken each hour for 3h. The aliquots were used to perform serial dilutions up to 10^{-7} using ¼ strength Ringer's solution and 100µl from each dilution was plated onto MRS or MRS-cys-HCL agar plates in triplicates. Plates were incubated anaerobically using anaerobic jars with Anaerocult gaspacks and Anaerotest strips at 37ºC for 72 hours. Plates with colonies between 30-300 were counted following incubation.

2.3.5 Bile Salt Hydrolase Activity

Bile salt hydrolase (BSH) activity was performed using method by (Sedlackova *et al.* 2015), with minor modifications. Overnight cultures were grown and used to assess BSH activity. Soft agar was prepared as follows: MRS broth (50g/L), bile salts (0.3% v/w; Sigma, South Africa), bacteriological agar (75g/L; Merck, South Africa) and $CaCl_2$ (0.375g/L; Sigma, South Africa). Sterile pipette tips were used to puncture holes into agar. Two hundred microliters of probiotics were placed into the holes, after leaving plates in the laminar flow for ten minutes they were incubated under anaerobic conditions at 37ºC for 72 hours. Plates that contain no bile salts were used as a negative control. The presence of translucent halos around holes indicated bile salt hydrolase activity. The experiment was done a total of three times in triplicates.

2.3.6 Antibiotic Susceptibility

Mastring antibiotic discs (Davies diagnostics, SA) were used to determine sensitivity to antibiotics. Chloramphenicol 25µg, erythromycin 5µg, Fusidic acid 10µg, penicillin G 1 unit, streptomycin 10µg and tetracycline 25µg were the specific antibiotics used. One hundred microliters of overnight probiotic cultures were spread onto MRS and MRS- cys- HCL plates. The antibiotic disc were placed in the centre of the plates. Plates were incubated anaerobically using anaerobic jars with Anaerocult gaspacks and Anaerotest strips at 37ºC for 72 hours, in

triplicates. The resulting diameters of inhibition zones were measured to determine sensitivity of probiotic cells to antibiotics. Antibiotic susceptibility was done three times in triplicates.

2.3.7 Antimicrobial Activity Assay

The antimicrobial tests were done using the method from (Mohankumar and Murugalatha 2011) with modifications. Centrifugation was used to harvest overnight cultures of probiotics (14691 x g for 20 minutes at 4^{o}C) and obtain cell free supernatants. *E. coli* and *S. aureus* were the pathogens used for the test. One hundred microliter of pathogen overnight cultures adjusted to OD_{600} 0.2 were inoculated onto separate Mueller Hinton Agar. Using sterile pipette, holes were punctured onto agar plates and l00µl of cell free supernatants, harvested from probiotic cultures were added to the wells. The plates were incubated overnight at 37^{o}C. Zones of inhibition around the wells indicated antimicrobial activity. The experiment was done a total of three times in triplicates.

2.3.8 Scanning Electron Microscopy (SEM)

The preparation of the samples for microscopy was done according to (Booyens, Labuschagne and Thantsha 2014) with modification. Cultures that had been previously grown in MRS or MRS-cys-HCL broth overnight were harvested using centrifugation at 604 x g for 2 min. The cells were then fixed using 2.5% glutaraldehyde in 0.075 mol^{-1} phosphate buffer (pH 7.4) for 1 hour. They were subsequently washed three times in 0.15 mol^{-1} phosphate buffer solution (PBS) before being dehydrated in a series of graded alcohol concentrations (25%, 50%, 75%, and 100% ethanol). Finally, the cells were critically dried for 24 hours before being coated with carbon. The resulting cells were viewed using a JSM-840 and JSM-5800 LV scanning electron microscope.

2.3.9 Auto-aggregation

The auto-aggregation was done according to (Li *et al.* 2015) with slight alterations. For aggregation assay the overnight probiotic cultures were harvested using centrifugation (1152 x g, 10min, 4^{o}C) followed by washing in 0.9% saline solution twice, before being re-suspended in 10 ml of the same buffer. The suspensions were adjusted to an absorbance reading of 0.3nm (± 0.05) at 600_{nm}. Each bacterial suspension was then incubated at room temperature and spectrophotometric readings (600_{nm}) using 1ml of suspension was taken at time interval 0, 3, 6

hours. Auto-aggregation experiment was done three times using triplicate readings. The following equation was used to measure auto-aggregation percentage:

Auto-aggregation (%) = 1- (A_t/A_0) × 100

A_t represents absorbance at different time intervals and A_0 represents the initial absorbance reading.

2.3.10 Cell Surface Hydrophobicity

The cell surface hydrophobicity was determined as described by (Abdulla, Abed and Saeed 2014). This test was based on probiotics ability to separate into hydrocarbons from (PBS). The probiotic strains were grown in MRS or MRS-cys-HCL broth for 24h at 37^{o}C, followed by centrifugation at 1677 x g for 10 minutes and two wash steps in PBS (pH 7). The solution containing probiotic cells was measured using spectrophotometry (540_{nm}) and adjusted to an optical density (OD) of 1. A millilitre of probiotic solution was added to 1ml of chloroform and mixed vigorously by vortexing for 30s. Following phase separation (30 minutes) the OD_{540} of the aqueous phase was taken. Cell surface hydrophobicity was done a total of three times using triplicate readings. The following equation was used to determine hydrophobicity:

(A_{540} initial – A_{540} aqueous phase)/A_{540} initial] × 100 = % hydrophobicity.

A_t represents absorbance at different time intervals and A_0 represents the initial absorbance reading.

2.3.11 Statistical Analysis

After means and standard deviation were determined software from GraphPad Prism 7.01 was used to analyse the results. ANOVA (Analysis of Variance) was used and P values < 0.05 were considered statistically significant and P values > 0.05 were taken as statistically non-significant.

2.4 Results

2.4.1 Acid- bile and temperature adaptation

Figure 2.1 shows the number of surviving *Bifidobacteria* and *Lactobacilli* cells after successive exposure to acid, bile and temperature. The number of surviving cells after acid exposure ranged between 7.67- 7.88 log CFU/ml, with *Lactobacilli* surviving better than *Bifidobacteria*. The tolerance to acid was as follows, from highest survival to lowest; *L. acidophilus* La14 150B, *L. plantarum*, *B. longum* LMG 13197, *B. longum* Bb46, *B. bifidum* LMG 11041. The reductions of cells after exposure to acid were, 0.085, 0.21, 0.36, 0.31 and 0.30 log CFU/ml, respectively. Following exposure to acid the cells were subjected to bile adaptation and the range in survival was between 6.51 – 7.58 log CFU/ml and the cell reductions were as follows: 0.42, 0.51, 1.39, 1.41, 1.49 log CFU/ml for *L. acidophilus* La14 150B, *B. longum* LMG 13197, *B. bifidum* LMG 11041, *L. plantarum* 7.1 E, *B. longum* Bb46, respectively. The decrease in viability after bile exposure from the initial number (10^8 log CFU/ml) was greater than the decrease observed after acid exposure. Finally, acid- bile adapted cells were treated with a temperature of 55^{o}C for 120 minutes. Following temperature adaptation the number of viable cells ranged from 7.49-8.85 log CFU/ml, an increase from the cells that survived bile adaptation. The increase in viability from bile adaptation was as follows: 1.27, 1.18, 1.00, 0.88 and 0.09 log CFU/ml for *L. acidophilus* La14 150B, *L. plantarum*, *B. longum* Bb46, *B. longum* LMG 13197 and *B. bifidum* LMG 11041, respectively. However, only the increase experienced by *L. acidophilus* La14 150B was statistically significant ($p< 0.05$).

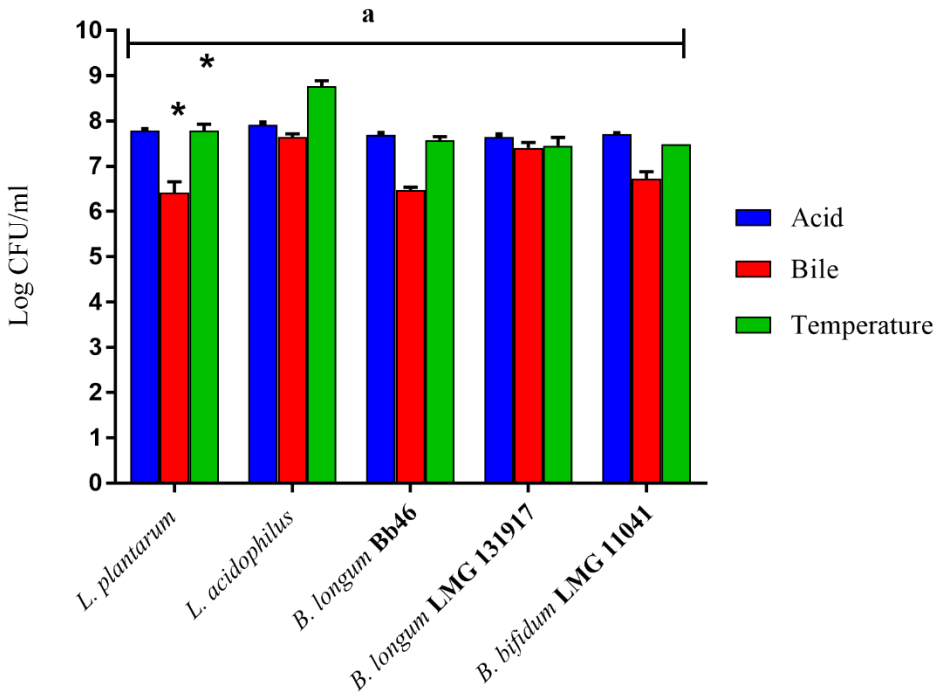

Figure 2. 1 Survival of probiotics after exposure to sub-lethal stresses acid (pH 2), bile (2%) and temperature (55ºC). The log CFU/ml of probiotic cultures were determined upon completion of each stress adaptation step. Each bar represents the mean of three replicates, error bars are standard deviation. *, represents statistically significant difference, $p<0.05$, between stress conditions and, a, shows statistical insignificance.

2.4.2 Acid Tolerance

Figure 2.2 depicts survival of non-adapted, old-adapted and freshly adapted of probiotic cells in MRS/ MRS-cys broth adjusted pH 2, 2.5 and 3 over a duration of 180 minutes. The results showed that the survival range was between 6.44- 8.42 log CFU/ml. At pH 2 (Figure 2.2A) the non-adapted cells of *B. longum* Bb46 exhibited the poorest survival throughout all three treatments, while *L. acidophilus* La14 150and *L. plantarum* were the best surviving for old-adapted and *B. bifidum* LMG 11041 and *B. longum* LMG 13197 were the best surviving for freshly adapted, respectively. At pH 2.5 (Figure 2.2B), *L. acidophilus* La14 150B is the best surviving strain across all three cultures, no other recognizable trend is observed at this pH. The order of best surviving to least at pH 3 (Figure 2.2C) was *B. bifidum* LMG 11041, *B. longum* LMG 13197, *L. plantarum*, *L. acidophilus* La14 150B, *B. longum* Bb46, respectively. Overall the survival shows no significant difference in acid resistance between the three cultures (non-adapted, freshly adapted and old-adapted) of the probiotic strains ($p>0.05$).

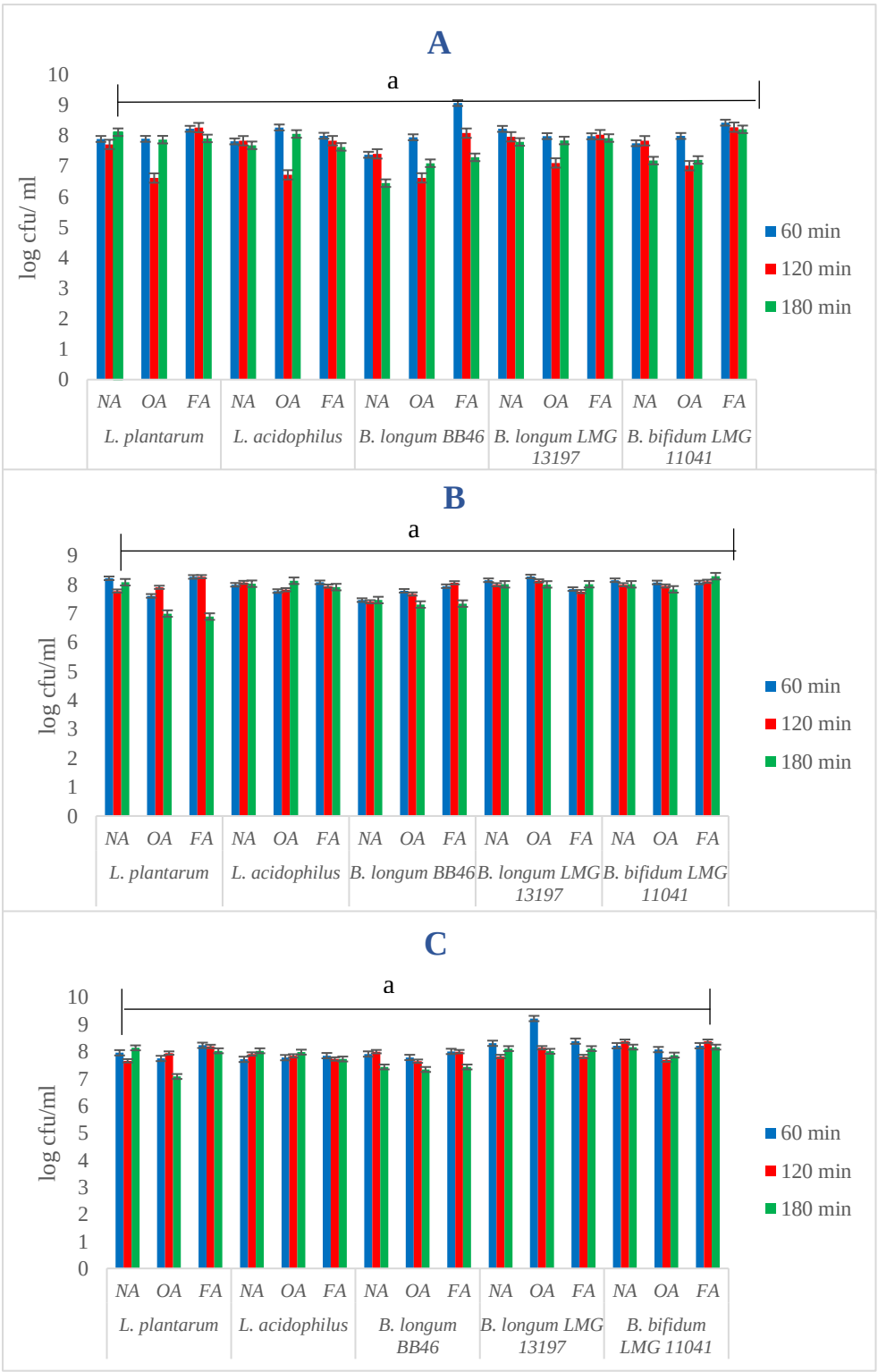

Figure 2. 2 Survival of non-adapted (NA), old-adapted (OA) and freshly adapted (FA) probiotic cells in pH 2 (A), 2.5 (B) and 3 (C) over a duration of 180 minutes. Each bar representing the mean of three replicates, error bars are standard deviation. a, shows that no significant difference existed between the three cultures $p > 0.05$.

2.4.3 Bile Tolerance

Figure 2.3 shows the survival of non-, old- and freshly adapted probiotics in 0.3, 0.5 and 2% bile salts concentrations overtime. Exposure to bile salts decreased the number of viable cells after 180 minutes. The survival of the cells in different bile concentrations ranged from 4.55-7.86 log CFU/ml. In 0.3% bile salts the non-adapted cells, except *L. acidophilus* La14 150B had better survival than both adapted cells, however, freshly adapted cells were less than old-adapted counterparts. Though, the differences were non-significant ($p>0.05$) (Figure 2.3A).

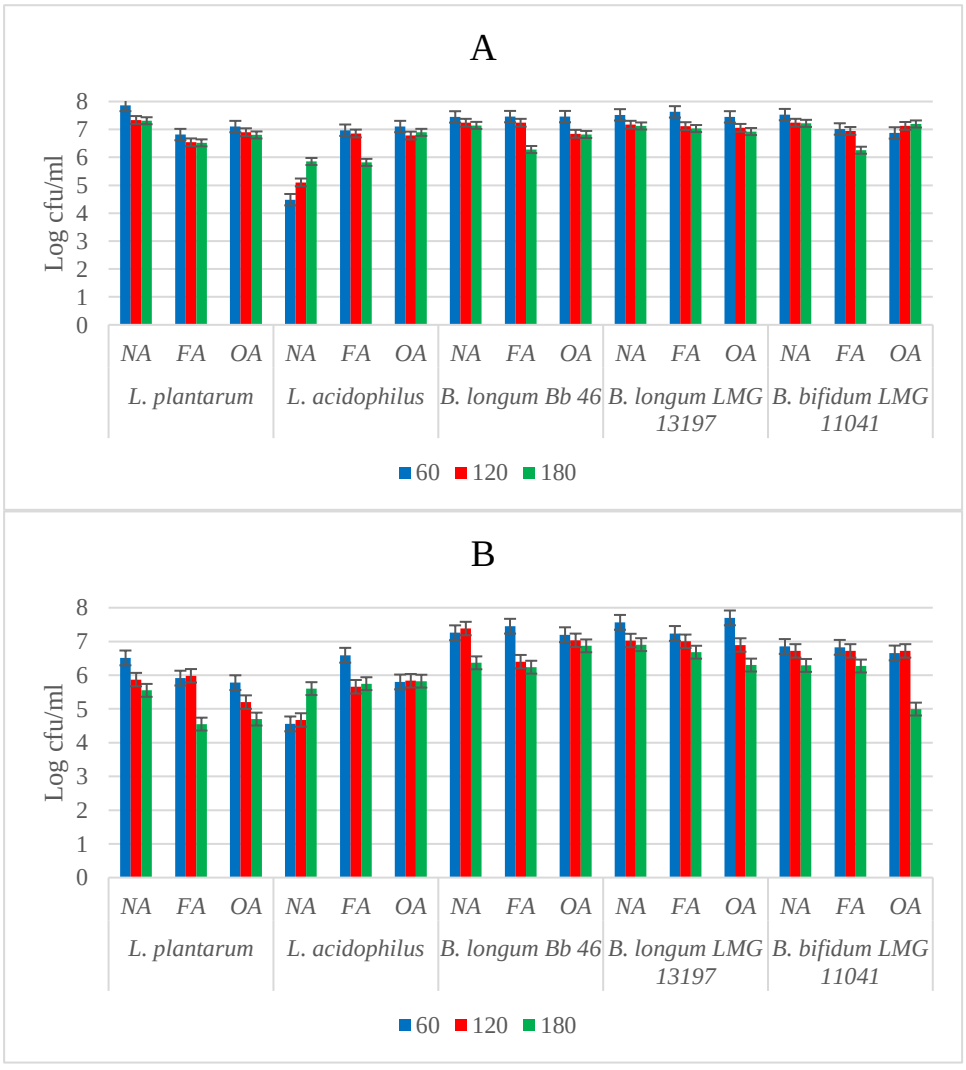

Figure 2. 3 Survival of probiotic strains in different concentrations (A: 0.3%, B: 0.5%) of bile salts over time. Each bar represents the mean of three replicates, error bars are standard deviation.

The probiotic cells experienced a bigger reduction in viable cells when exposed to 0.5% bile salts than in 0.3% bile concentration. The non-adapted cells of all strains except *L. acidophilus* La14 150B were the best surviving at this concentration, there was no significant difference between the losses in viability of both adapted cultures. (Figure 2.3B). The exposure of cells to 2% bile resulted in a complete loss of viability for all cells (Data not shown).

2.4.4 Bile Salt Hydrolase Activity

Only the non-adapted cells of *L. acidophilus* La 14 150B showed BSH activity while the rest tested negative (Table 2.1). However, all old-adapted were negative while all freshly adapted tested positive for BSH activity.

Table 2. 1 Bile salt hydrolase activity of probiotic strains. The presence of opaque halos around punctured holes indicated bile salt hydrolase activity

Probiotic cells	**Non- adapted**	**Old- Adapted**	**Freshly- Adapted**
L. plantarum	-	-	+
***L. acidophilus* La 14 150B**	+	-	+
***B. longum* Bb46**	-	-	+
***B. longum* LMG 13197**	-	-	+
***B. bifidum* LMG 11041**	-	-	+

+: Halo present, - : Halo absent

2.4.5 Antimicrobial Activity

Figure 2.4 shows the antagonistic effect of probiotic cell free supernatants against *E. coli* and *S. aureus*. All the supernatants were capable of inhibiting both pathogens, with *S. aureus* being highly inhibited. For *E. coli*, the range of inhibition was between 12mm -14.5mm (Figure 2.4A) while for *S. aureus* it was between 15.2mm-18.6mm (Figure 2.4B).

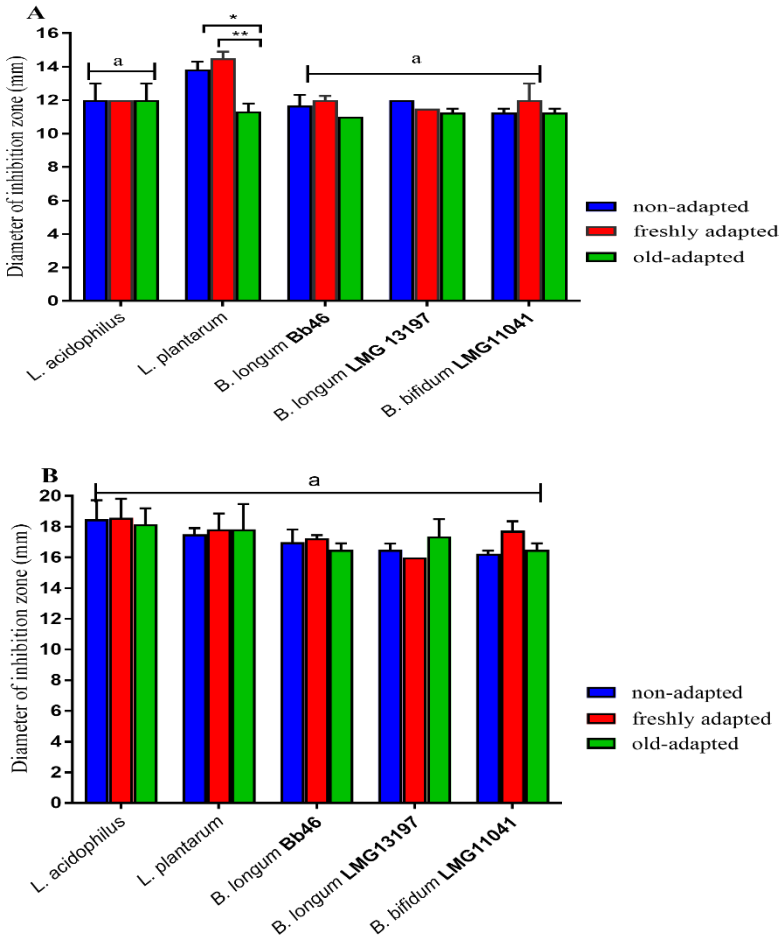

Figure 2. 4 Antimicrobial activity of cell free supernatants of probiotics against pathogens *E. coli* (A) and *S. aureus* (B) depicted as zones of inhibition in millimetres. Each bar represents mean of triplicates from three separate trials. The error bars showing standard deviation. *, represents statistically significant difference, $p < 0.010$ and **, $p < 0.001$ between probiotic adaptations and, a, shows statistical insignificance $p > 0.05$.

There were no significant differences between inhibition of *E. coli* by the three cultures of the different probiotic strains ($p>0.05$) except for *L. plantarum*, where non-adapted cells achieved significantly higher inhibition than old-adapted cells ($p<0.010$) and inhibition by freshly

adapted cells was significantly more than for old-adapted counterparts, $p<0.001$. There were no statistically significant differences between inhibitions of *S. aureus* the three cultures of the different probiotic strains (Figure 2.4B).

2.4.6 Antibiotic Susceptibility

Resistance and sensitivity of the probiotics cultures to different antibiotics was assessed and the study revealed that all the cultures except the old-adapted *L. plantarum* were resistant to Penicillin G. All cultures demonstrated sensitivity towards chloramphenicol, erythromycin, fusidic acid, oxacillin, novobiocin, streptomycin, tetracycline with the exception of non-adapted *B. longum* Bb46, which was resistant to oxacillin. Therefore the process of long-term storage allowed old-adapted *L. plantarum* to become susceptible to penicillin G. On the other hand both fresh adaptation and long term storage resulted in *B. longum* Bb46 developing sensitivity to oxacillin. Zones of inhibition ranged from 0.8±0.14-3.1±0.08 mm for all tested probiotics. Larger inhibition zones were observed for both adapted cultures compared to non-adapted counterparts, indicating more susceptibility. However, the differences between the two groups were non-significant ($p<0.05$) (Table 2.2).

Table 2. 2 The zones of inhibition of the, non-adapted, old-adapted and freshly adapted strains resulting from different antibiotics. NA: Non-adapted, OA: Old-adapted and FA: Freshly adapted.

Probiotic strains		**Antibiotics**							
		S	T	C	E	FC	OX	NO	PG
		Zone of inhibition mean diameter (cm)							
L. acidophilus	NA	0.97±0.00	1.36±0.38	2.55±0.04	3.00±0.08	1.17±0.12	1.53±0.05	2.03±0.09	0.00
L. acidophilus	OA	0.96±0.047	1.20±0.08	2.30±0.08	2.90±0.08	1.20±0.21	1.50±0.08	2.21±0.080	0.00
L. acidophilus	FA	1.06±0.04	2.13±0.04	2.51±0.27	3.10±0.08	1.21±0.21	2.00±0.08	2.10±0.08	0.00
L. plantarum	NA	1.13±0.05	2.33±0.09	2.20±0.08	1.20±0.08	1.56±0.05	1.60±0.00	1.96±0.34	0.00
L. plantarum	OA	1.03±0.17	2.17±0.17	2.28±0.08	1.13±0.04	1.76±0.18	1.83±0.12	1.42±0.88	1.21±0.16
L. plantarum	FA	1.03±0.09	2.26±0.16	2.67±0.04	1.33±0.09	1.53±0.25	2.20±0.08	1.91±0.33	0.00
B. longum Bb46	NA	1.00±0.14	1.20±0.14	2.74±0.18	1.96±0.68	1.90±0.21	0.00	1.83±0.24	0.00
B. longum Bb46	OA	1.00±0.06	1.23±0.17	2.32±0.34	1.50±0.52	1.57±0.33	2.00±0.05	1.96±0.08	0.00
B. longum Bb46	FA	1.18±0.14	1.73±0.14	2.17±0.18	1.87±0.67	1.43±0.21	1.83±0.00	2.00±0.25	0.00
B. bifidum LMG 11041	NA	0.80±0.14	1.08±0.13	2.21±0.19	1.43±0.21	1.56±0.04	1.71±0.08	1.58±0.11	0.00
B. bifidum LMG 11041	OA	0.00±0.00	1.25±0.11	2.48±0.08	1.41±0.06	1.65±0.04	2.73±0.05	1.60±0.08	0.00
B. bifidum LMG 11041	FA	1.00±0.14	1.77±0.33	2.20±0.08	2.13±0.40	1.16±0.16	1.63±0.04	1.76±0.18	0.00
B. longum LMG 13197	NA	1.00±0.08	1.13±0.09	2.07±0.13	2.37±0.05	1.17±0.17	2.00±0.36	1.96±0.20	0.00
B. longum LMG 13197	OA	1.00±0.08	1.28±0.06	2.23±0.26	1.43±0.09	1.23±0.19	1.53±0.05	1.63±0.13	0.00
B. longum LMG 13197	FA	0.99±0.15	1.23±0.09	2.23±0.04	1.53±0.09	1.37±0.17	2.13±0.12	1.77±0.18	0.00

Each value is a mean of 3 triplicates from 3 separate experiments, ± standard deviation (SD) C, Chloramphenicol; E, Erythromycin; FC, Fusidic acid; OX, Oxacillin; NO, Novobiocin; PG, Penicillin G; S, Streptomycin; T, Tetracycline; 0.00, no zone of inhibition

2.4.7 Scanning Electron Microscopy

The scanning electron micrographs of *Bifidobacteria* and *Lactobacilli* show the changes in morphology of probiotic cells (Figure 2.5 and Figure 2.6). The images of *Bifidobacteria* in Figure 2.5 show that both non-adapted cells of *B. longum* Bb46 (Figure 2.5d) and *B. longum* LMG 13197 (Figure 2.5g) were uniform in appearance, presenting as rods or coccoid, respectively. Non-adapted cells of *B. bifidum* LMG 11041 (Figure 2.5a), were pleomorphic. Fresh adaptation changed the morphology to rods, pleomorphic and coccoids for *B. bifidum* LMG 11041, *B. longum* Bb46 and *B. longum* LMG 13197 to rods, respectively (Figure 2.5b, e and h) The process of freezing multi stress adapted probiotics overtime changed the morphology of *B. bifidum* LMG 11041, from rods (Figure 2.5b) to coccoids (Figure 2.5c) and diminished the number of rods present in *B. longum*. Bb46 and *B. longum* LMG 13197, (Figure 2.5f and i).

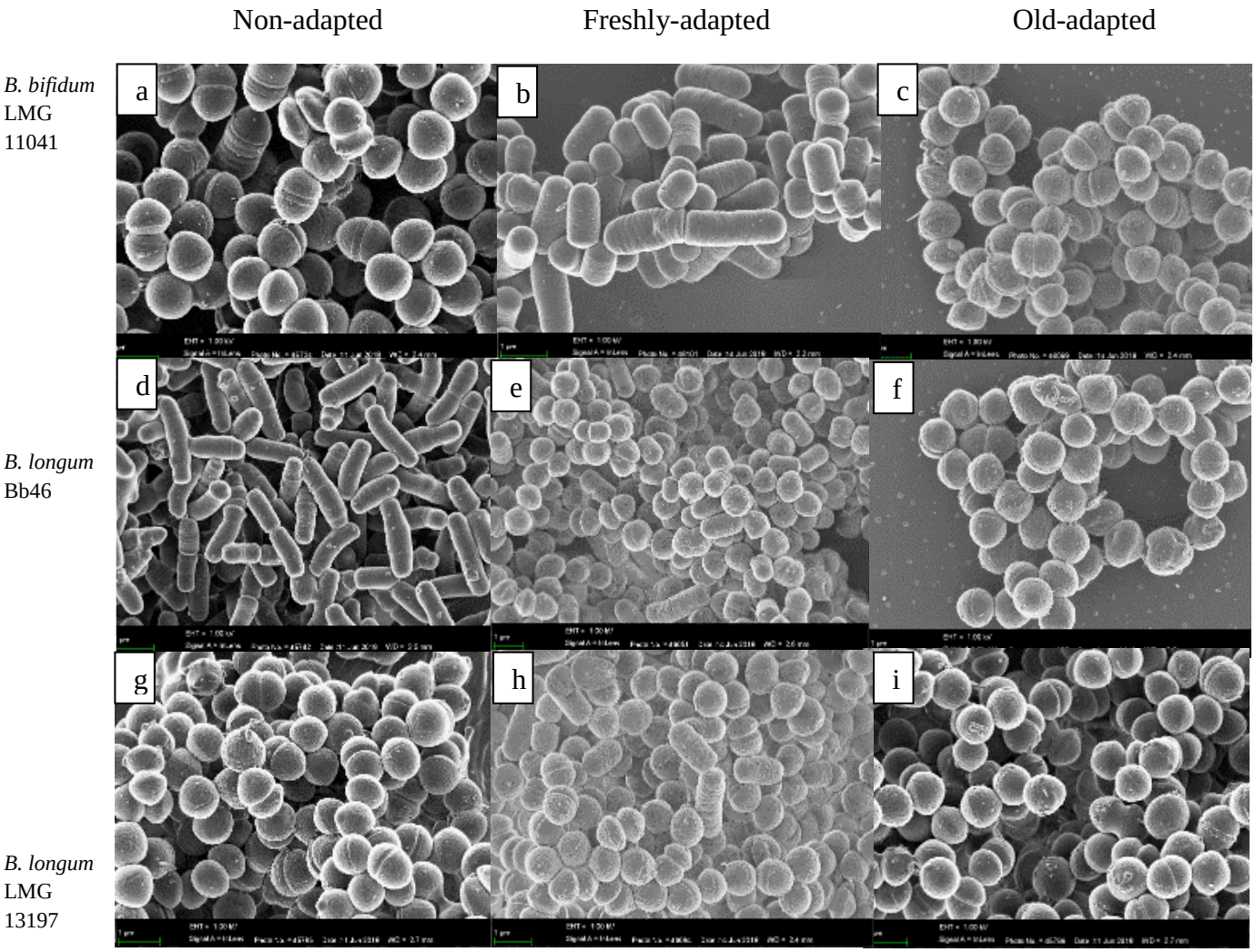

Figure 2. 5 Scanning electromicrographs of *B. bifidum* LMG 11041, *B. longum* Bb46, *B. longum* LMG 13197 as non-adapted (a, d & g), freshly-adapted (b, e & h), and (c, f & i) old-adapted cultures, respectively.

Figure 2.6 depicts the scanning electron images of *Lactobacilli*, both non-adapted cultures were rod shaped (Figure 2.6a & d). Subjection of the cells to stress adaptation process produced shorter rods and some coccoids in *L. acidophilus* but left the morphology of *L. plantarum* unchanged (Figure 2.6 b & e). The subsequent freezing of adapted cells resulted in cells changing to coccoids for both *Lactobacilli* strains (Figure 2.6c & f).

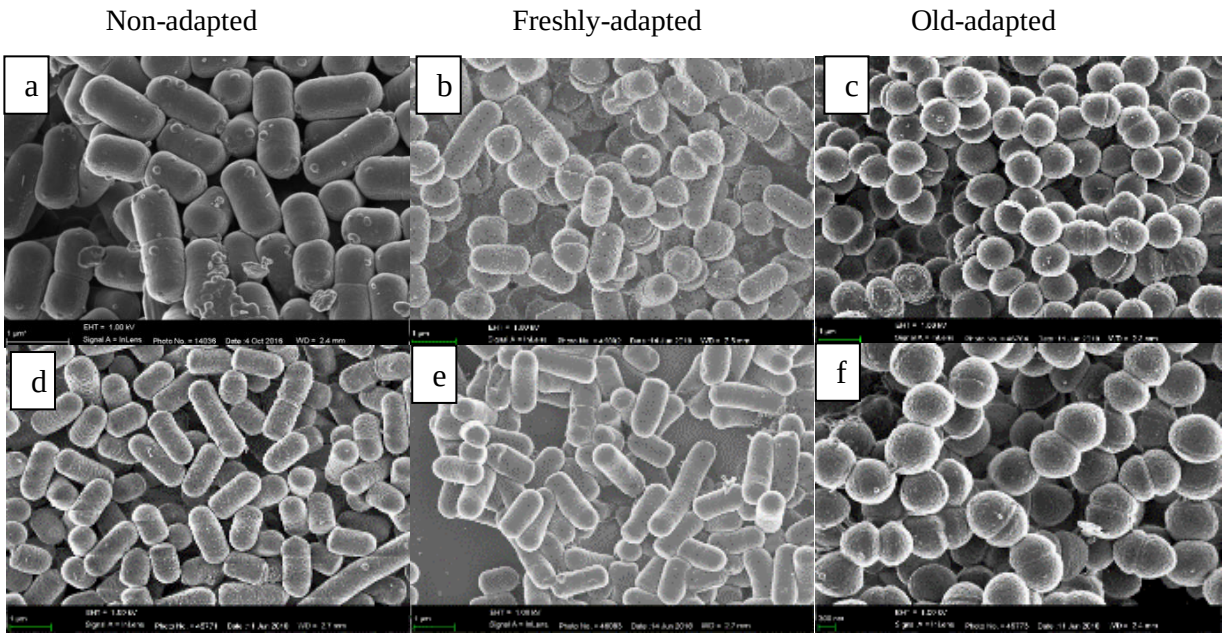

Figure 2. 6 Scanning electromicrograph of *L. acidophilus* La14 150B and *L. plantarum*, as non-adapted (a & d), freshly-adapted (b & e), and old-adapted (c & f) cultures, respectively.

2.4.8 Auto-aggregation

All probiotic cells were able to auto-aggregate, with the percentages ranging between 5.66±4.50-24.00±3.50 after six hours. Table 2.3 shows those percentages as a function of time and shows the order of aggregation from largest to least as *B. longum* Bb46, *B. bifidum* LMG 11041, *B. longum* LMG 13197, *L. acidophilus* and *L. plantarum*. Percentages of aggregation of most strains reduced between the 3rd and 6th hour with the exception of both freshly adapted cultures of *Lactobacilli* and the old adapted *B. longum* Bb46. Overall, non-adapted cells possessed better aggregation abilities than stress-adapted cells. When comparing the adapted cells, freshly adapted cells demonstrated superior aggregation abilities than old adapted ones. However, the statistical analysis revealed that all differences encountered were not significant ($p > 0.05$).

Table 2. 3 Auto-aggregation percentages of probiotic strains over a six hour period as determined by spectrophotometry.

Probiotic Strains	**Auto-aggregation (%)**		
	0 h	**3h**	**6h**
L. plantarum NA	0.00	15.80 ± 10.06	10.33 ± 2.88
L. plantarum FA	0.00	8.73 ± 4.03	9.04 ± 1.41
L. plantarum OA	0.00	9.33 ± 3.09	7.06 ± 7.73
L. acidophilus NA	0.00	27.00 ± 5.88	12.73 ± 7.20
L. acidophilus FA	0.00	6.87 ± 2.63	13.67 ± 1.09
L .acidophilus OA	0.00	19.17 ± 6.38	9.03 ± 2.37
B. longum Bb46 NA	0.00	19.70 ± 6.93	17.57 ± 6.93
B. longum Bb46 FA	0.00	14.56 ± 2.60	12.57 ± 4.30
B. longum Bb46 OA	0.00	6.00 ± 4.90	10.83 ± 0.62
B. bifidum LMG 11041 NA	0.00	24.00 ± 3.50	18.87 ± 5.50
B. bifidum LMG 11041 FA	0.00	15.00 ± 10.57	11.90 ± 5.48
B. bifidum LMG 11041 OA	0.00	19.53 ± 5.81	5.66 ± 4.50
B. longum LMG 13197 NA	0.00	13.56 ± 9.63	14.93 ± 0.99
B. longum LMG 13197 FA	0.00	18.76 ± 2.98	14.80 ± 1.31
B. longum LMG 13197 OA	0.00	18.86 ± 4.49	6.20 ± 2.31

Each value is a mean of 3 replicates from 3 independent trials ± standard deviation. NA: Non- adapted, OA: Old-adapted and FA: Freshly adapted.

2.4.9 Cell Surface Hydrophobicity

The cell surface hydrophobicity (CSH) was high among all bacterial strains and across the three cultures (non-, old- and freshly adapted) (Figure 2.7**)**. The hydrophobicity percentages ranged from 54.9%-99.2%. For all strains, the process of multi-stress adaptation altered CSH. For *L. plantarum*, *B. longum* LMG 13197 and *B. bifidum* LMG 11041 stress adaptation (fresh and old) led to significant increases in hydrophobicity when compared to their non-adapted counterparts ($p<0.0001$). On the other hand, hydrophobicity of *L. acidophilus* La14 150B was significantly decreased by fresh adaptation and cold storage following pre-stress treatment, compared to the non-adapted parental strain ($p<0.0001$). The hydrophobicity of *B. longum* Bb46 was significantly diminished by adaptation and freezing ($p<0.0001$) compared to its non- and freshly adapted counterparts. The results also reveal significant differences between the freshly and old-adapted cells for all strains ($p<0.005$) except *B. bifidum* LMG 11041 where the differences were non- significant ($p= 0.058$).

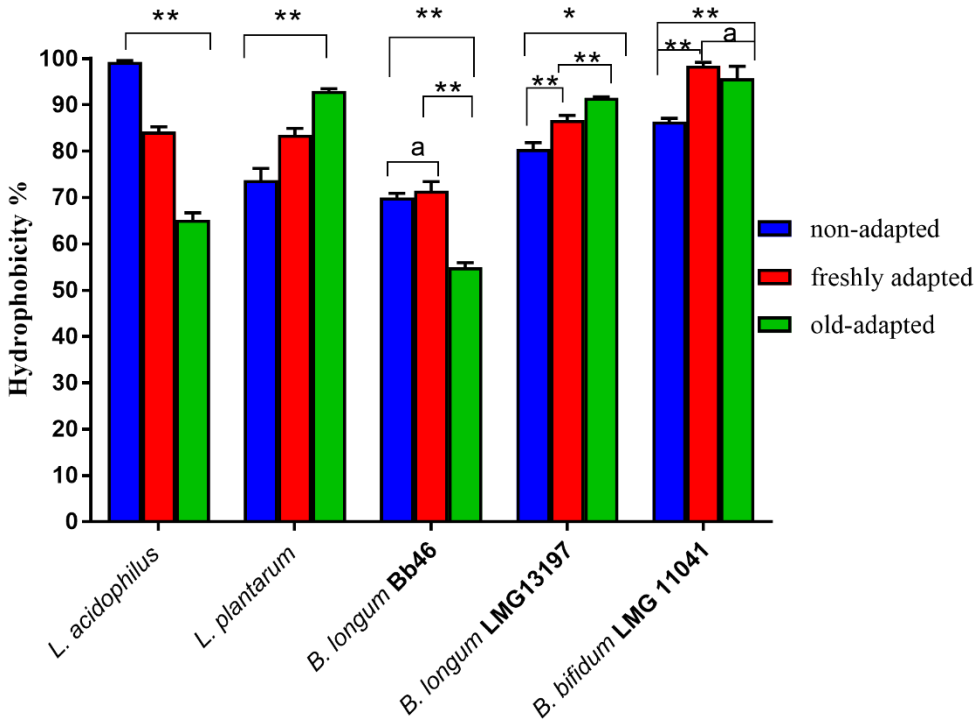

Figure 2. 7 Cell surface hydrophobicity of non-, old- and freshly- adapted *Lactobacilli* and *Bifidobacteria* species. Each bar represents the mean of three replicates, error bars represent standard deviation. The error bars showing standard deviation. *, ** represents statistically significant differences between probiotic adaptations with p values: $p < 0.005$ and $p <0.0001$ respectively, while a, shows statistical insignificance ($p > 0.05$).

2.5 Discussion

In recent times there has been a rise in the awareness of probiotics and the benefits they possess. This in turn has resulted in the demand of their commercialized products, as such, the functional foods industry has seen rapid growth. However, their use has not come without challenges. Firstly, the bacteria used for probiotic application are sensitive to environmental conditions, have specific nutrition requirements and their viability is threatened by the processes involved in their manufacture and storage (Gueimonde and Sánchez 2012; Sánchez *et al.* 2012). Although, the number of viable cells during and at the end of shelf life can vary, the general consensus is that they should be high enough as stipulated by the definition of a probiotic, so to administer the desired benefits. This, however, has not been the case for many probiotic products as many have been found to contain lower bacterial numbers than stated on their label (Saarela *et al.* 2004). The reasons being, probiotic cells are posed with many threats during the manufacturing of products right up until storage. These dangers can be anything from the extreme temperatures they encounter during production to the low pH found within the stomach (Ross *et al.* 2005; Amund 2016). It is imperative that strains are capable of withstanding these harsh assaults in order to maintain viability, functionality and stability. Several authors have studied ways which can be used to attain this (Gueimonde and Sánchez 2012; Nguyen *et al.* 2016). Cross protection and stress adaptation is one of the mechanisms that can produce stress resistant strains (Saarela *et al.* 2004). Moreover, the use of multi-stress adapted strains has proved better than strains adapted to a single stress factor (Sumeri *et al.* 2010; Mathipa and Thantsha 2015).

In this study probiotic strains of *Lactobacilli* and *Bifidobacteria* were successively treated with acid, bile and temperature (Figure 2.1). The cells initially decreased after exposure to acid, however, they increased following exposure to bile. When Mathipa and Thantsha (2015) performed the same stress adaptation to probiotic cells they found that they decreased to a lesser extent with exposure to each stress factor. This study, however, showed that after bile exposure the cells were not only capable of surviving but also increasing, *L. acidophilus* in particular showed a significant increase after following bile exposure ($p<0.05$). This indicated that adaptation to acid and bile offered cross protection to temperature as well. A study by (Saarela *et al.* 2004) showed that in certain *Lactobacilli* and *Bifidobacteria* strains, heat stress could offer cross protection against acid and bile stress, the inverse was also true for the current. The freshly adapted cells were then used for comparison studies with old-adapted and

non-adapted strains to check whether or not storage affected functional and structural properties.

One of the important properties probiotics must possess is resistance to acid. This is because the pH in the stomach can reach levels as low as 1.5 (Mills *et al.* 2011). A good probiotic should be able to survive pH 3 (Sahadeva *et al.* 2011). In the current study *Bifidobacteria* were more resistant to acid than *Lactobacilli* (Figure 2.2) which is not the norm as *Lactobacilli* are known to be intrinsically acid tolerant, however, behaviour of probiotics are largely strain dependent (Corcoran *et al.* 2005). Still, all the probiotic cultures (non-, old- and freshly adapted) exhibited considerable acid resistance at all pHs, with survival ranging from 6.44-8.42 log CFU/ml. However, there were no significant differences found between the survival of the three different cultures (non-, old- and freshly adapted) at different acid concentrations (Figure 2.2). This was in agreement with a study by (Waddington *et al.* 2010) which found that pre-exposure to sub-lethal acid stress did little to improve survival of *Bifidobacteria* during subsequent exposure to low pH.

The survival rate in bile salts was different to that encountered in acid, in bile, the number of surviving cells decreased with the increase of bile concentration and ultimately resulted in total loss of viable cells at 2% bile salt (Figure 2.3). Sahadeva *et al.* (2011) indicated that stress adapted *Lactobacilli* and *Bifidobacteria* spp. were capable of surviving 2% bile concentrations at levels as high as 8.51 log units. However, in this study none of the cells could survive this concentration. Bile acts as a biological detergent which damages cell membranes. In high enough concentrations it dissolves membrane lipids resulting in leakage and cell death (Urdaneta and Casadesús 2017). Unlike the finding by (Sahadeva *et al.* 2011) the process of stress-adaptation also impeded many of the strains ability tolerate bile salts as non-adapted cells survived in higher numbers (Figure 2.3). The activity of the inducible *bsh* gene through the de-conjugation of bile salts is thought to be one of the ways that cells deal with bile stress (Ruiz, Margolles and Sánchez 2013b). So, it was expected that cells exhibiting BSH activity were capable of surviving better in bile concentrations. On the contrary, the findings of this study suggest that, though non-adapted cells for all strains but *L. acidophilus* La14 150B showed better bile tolerance than the two adapted cultures (Figure 2.3) BSH activity could not have been the reason for this as non-adapted strains lacked the ability to de-conjugate bile salts (Table 2.1). Interestingly, *L. acidophilus* La14 150B, the only non-adapted strain with BSH activity, showed the lowest survival in both bile concentrations compared to its adapted counterparts (Figure 2.3). Moreover, the freshly-adapted cells which also tested positive for

BSH activity (Table 2.3) had the lowest survival in 0.3% bile concentration. Moser and Savage (2001) showed no link between BSH activity and the lethal effects of conjugated bile salts. Other work by (Lv *et al.* 2017) demonstrated how under bile stress *Lactobacilli salivarus* LI01 used a strengthened bile efflux and general stress response systems rather than BSH activity to combat the stress. These systems were most likely the reason that the non-adapted cells were capable of demonstrating superior bile resistance over adapted cells at both bile concentrations, despite lacking BSH activity. Although, BSH activity did not result in increased bile salt resistance, this trait is still desirable because bile salt hydrolysis can reduce serum cholesterol levels, therefore, it is beneficial that this activity was induced in freshly-adapted cells (Sridevi, Vishwe and Prabhune 2009).

In addition to acid and bile tolerance probiotic bacteria should be able to prevent or inhibit pathogens. One of the ways they do this is through the production of antimicrobial substances such as bacteriocins, organic acids or inhibitors of virulence gene expression, which target gut pathogens (Corr, Hill and Gahan 2009). In our study the antimicrobial activity against *S. aurues* was notably larger than against *E. coli* ($p < 0.05$) and non-adapted and fresh adapted culture of *L. plantarum* was able to inhibit *E. coli* significantly better than old-adapted cells ($p<0.001$) (Figure 2.4A). A study where *Lactobacilli* were tested for antimicrobial activity against several enteropathogens (Tebyanian *et al.* 2017), found that the gram positive pathogens were more susceptible than their gram negative counterparts. Much like the current findings where *S. aureus* was more sensitive than *E. coli*. Furthermore, a similar experiment by Mathipa and Thantsha (2015) found that freshly adapted probiotic combinations were more potent against *E. coli* and *S. aureus* than their non-adapted counterparts. This is contrary to our findings where differences encountered by the two were statistically insignificant ($p<0.05$) except for *L. plantarum* as previously mentioned. Meaning, long term storage lowered the antimicrobial abilities of *L. plantarum.*

Resistance to antibiotics that control gastrointestinal infection is another important property of probiotics. This allows the GIT microflora to persist during this type of treatment (Gueimonde *et al.* 2013). Many of the probiotic species carry intrinsic antibiotic resistance. This kind of resistance does not pose a threat, however, when resistant genes are mobile (capable of transfer), they can move from beneficial bacteria to pathogens within the gut especially since these bacteria find themselves in close proximity of each other (Liu *et al.* 2009; Georgieva *et al.* 2015). When this kind of resistance arises the probiotics are no longer deemed safe for human consumption. Therefore, no change in resistance should occur between the adapted and

non- adapted probiotic cells. This study showed all cells were sensitive to oxacillin, except non-adapted *B. longum* Bb46, erythromycin, novobiocin, fusidic acid and broad spectrum antibiotics chloramphenicol, streptomycin, tetracycline and resistant to Penicillin G with the exception of old- adapted *L. plantarum* (Table 2.2). Our findings were in correlation with reports by researchers elsewhere, who reported that strains of *Lactobacilli* and *Bifidobacteria* were susceptible to the antibiotics chloramphenicol, tetracycline, novobiocin, erythromycin, ampicillin and sensitive to fusidic acid and streptomycin (Zhou *et al.* 2005; Ammor *et al.* 2008; Georgieva *et al.* 2015). What is more important to ascertain is why the deviation in susceptibility occurred in *B. longum* Bb46 and *L. plantarum* because if it resulted from gene transfer then these bacteria would not be safe for probiotic use but if the change happened as a result of a genetic mutation then it is unlikely that resistance would be transferrable.

Bacterial morphology plays an important role in cell surface attachment, hence any variations in probiotic cells shape can influence their ability to attach to epithelial cells (Young 2007). The differences in cell morphology of non-adapted and the adapted cells, as well as the differences between freshly-adapted and old-adapted cells suggest that adaptation and long term storage modified the morphology of these bacteria (Figure 2.5 and Figure 2.6). Typically, *Bifidobacteria* morphology ranges from straight rods, curved rods to rods that branch and form the characteristic Y-shaped bifid. It is also not uncommon to see the swelling in the rod shapes, all these variations can occur within the same strain (Husain, Poupard and Norris 1972). Similar work done by these authors showed *Bifidobacteria* cells that resembled coccoid granules (Poupard, Husain and Norris 1973). The non-adapted *Bifidobacteria* cells in Figure 2.5 demonstrates these morphological variations that can occur within this species. When these cells were subjected to sub-lethal doses of acid, bile and temperature stress their morphology was altered (Figure 2.5 b, e & h). The subsequent freezing of the adapted cells resulted in further morphological changes (Figure 2.5 c, f & i). Bacterial cells are known to alter their morphology in response to changing environments (Young 2006, 2007). The presence of oxidative stress increased the coccoid forms of *Campylobacter jejuni* (Oh, McMullen and Jeon 2015). Similarly, treating *Bifidobacteria* with garlic resulted in shorter rods or becoming all together cocci (Booyens, Labuschagne and Thantsha 2014). This is similar to what occurred to the cells of *B. longum* Bb46, which became shorter following fresh adaptation (Figure 2.5 e) and eventually were just cocci after storage (Figure 2.5 f). The freshly-adapted cells of *B. bifidum* LMG 11041, *B. longum* LMG 13197 and *Lactobacilli* followed the same trend after long-term cold storage. The shrinking of rod-shaped bacteria to coccoids during periods of

stress is believed to be a protective mechanism that limits cell maintenance requirements (Chaiyanan *et al.* 2007). These results corresponds to those reported by Chen and colleagues in 2009 where *Vibrio cholerae* incubated at 5^{o}C over several days changed its morphology from rod-shaped to coccoid by the end of storage. In the current study, it was shown that the cold stress encountered during long-term storage by the old-adapted cells increased the population of coccoidal cells in both *Bifidobacteria* and *Lactobacilli* (Figure 2.5 c, f & i and Figure 2.6 c & f).

Before any of the other benefits are derived from probiotics, the cells must first attach and colonize the epithelial cells of the intestines, making this another criterion when selecting probiotic strains (Collado, Meriluoto and Salminen 2007). The process of attachment involves multiple steps with different interactions that allow for bacterial adhesion to the intestinal mucosa (Darilmaz, Beyatli and Yuksekdag 2012). Auto-aggregation, known to aid in adherence as well as form a protective barrier which prevents infection by enteropathogens, is one such interactions (Kos *et al.* 2003; Collado, Meriluoto and Salminen 2007; Tareb *et al.* 2013). Furthermore, hydrophobicity as measured by microbial adhesion to hydrocarbons, has also been linked to adhesion abilities in certain *Lactobacilli* strains (Darilmaz, Beyatli and Yuksekdag 2012). Certain authors have demonstrated that environmental stresses can affect these cell surface properties and thus the cells ability to attach to intestinal cells (Gong *et al.* 2012; Shakirova *et al.* 2013; Haddaji *et al.* 2015). When these properties were evaluated in pre-stress adapted strains Ferrando *et al.* (2016) found no significant difference between auto-aggregation abilities of heat pre-treated *L. plantarum* and its parental strain. The current study concurs with theirs in that, the differences in auto-aggregation percentages occurring between adapted and non-adapted cells were non-significant (Table 2.3). However, unlike this study their auto-aggregation percentages were higher, which is not uncommon as auto-aggregation percentages can vary largely among strains of probiotics (Del Re *et al.* 2000; Ramos *et al.* 2013). Findings by (Haddaji *et al.* 2015) showed that two strains of *Lactobacilli casei* under osmotic stress showed significantly lower hydrophobicity than non-stressed strains. Gomez Zavaglia *et al.* (2002) also reported significant decreases in auto-aggregation and hydrophobicity of *Bifidobacteria* following pro-longed exposure to bile salts. Findings from the current study also show that significant changes in hydrophobicity occurred following stress adaptation (Figure 2.7). It was only in *L. acidophilus* La14 150B where adaptation resulted in diminished CSH, for *L. plantarum*, *B. bifidum* LMG 11041 and *B. longum* LMG 13197 the changes led to an increase in hydrophobicity unlike the findings from the other

authors (Figure 2.7). Moreover, the difference found between the freshly adapted and old-adapted cells indicates that long-term storage also played a role in changing cell surfaces properties of probiotics. Since morphology, auto-aggregation and CSH are properties linked to adherence it is likely that adherence to epithelial surfaces will also be affected by the long-term storage. If that were the case only changes that increase adherence would be deemed acceptable.

2.6 Conclusions

There are certain criteria that bacterial species must meet in order to be called probiotic. These include but are not limited to the following: should possess acid and bile tolerance, *bsh* and antimicrobial activity, as well as adhere to intestinal cells. In addition, they must retain high numbers throughout storage and through GIT transit. Multi-stress adaptation is one of the ways in which robust strains are created, however, this treatment should not interfere with the functional traits of strains. The focus of this study was to compare previously multi-stress adapted probiotics which underwent long-term cold storage with its non-adapted and freshly counterparts. This work was an expansion of the study done by (Mathipa and Thantsha 2015) who showed the robustness of multi-stress adapted (acid-bile-temp) strains of *Lactobacilli* and *Bifidobacteria*.

This study showed that the acid tolerance of all cultures (non-, freshly, and old- adapted) was high throughout the 3h exposure period. The bile tolerance of non-adapted cells, except *L. acidophilus* La14 150B was better than both adapted cells but, tolerance was not linked to BSH activity. The supernatants of the probiotics were capable of inhibiting both *E. coli* and *S. aureus*, however, the latter was inhibited to a greater extent. Furthermore, the inhibition of *E.coli* by non- and freshly adapted *L. plantarum* was significantly higher compared to old-adapted cells. Strains had a similar antibiotic sensitivity profile, apart from *L. plantarum* and *B. longum* Bb46. SEM images reveal that both fresh adaptation and long term storage resulted in morphological changes of the strains. While auto-aggregation abilities of the strains were lowered by the adaptation process, and further diminished by long term storage. The CSH of the freshly and old-adapted strains was significantly different from each other.

The results show how storage of pre-stress treated probiotics can alter certain properties. Many of these properties were limited to specific strains. Therefore, it is important that storage

conditions for those strains are determined individually to prevent counteracting the process of stress adaptation.

2.7 References

Abdulla AA, Abed TA, Saeed AM. Adhesion, autoaggregation and hydrophobicity of six *Lactobacilli* strains. *Br Microbiol Res J* 2014;**4**:381–91.

Ammor MS, Flórez AB, van Hoek AHAM *et al.* Molecular characterization of intrinsic and acquired antibiotic resistance in lactic acid bacteria and *Bifidobacteria*. *J Mol Microbiol Biotechnol* 2008;**14**:6–15.

Amund OD. Exploring the relationship between exposure to technological and gastrointestinal stress and probiotic functional properties of *Lactobacilli* and *Bifidobacteria*. *Can J Microbiol* 2016;**62**:715–25.

Booyens J, Labuschagne MC, Thantsha MS. In vitro antibacterial mechanism of action of crude garlic (*Allium sativum*) clove extract on selected probiotic *Bifidobacterium* species as revealed by SEM, TEM, and SDS-PAGE analysis. *Probiotics Antimicrob Proteins* 2014;**6**:82–7.

Chaiyanan S, Chaiyanan S, Grim C *et al.* Ultrastructure of coccoid viable but non-culturable *Vibrio cholerae*. *Environ Microbiol* 2007;**9**:393–402.

Collado MC, Meriluoto J, Salminen S. Measurement of aggregation properties between probiotics and pathogens: In vitro evaluation of different methods. *J Microbiol Methods* 2007;**71**:71–4.

Corcoran B, Stanton C, Fitzgerald G *et al.* Life under stress: The probiotic stress response and how it may be manipulated. *Curr Pharm Des* 2008;**14**:1382–99.

Corcoran BM, Stanton C, Fitzgerald GF *et al.* Survival of probiotic *Lactobacilli* is enhanced in acidic environments in the presence of metabolizable sugars. *Appl Environ Microbiol* 2005;**71**:3060–7.

Corr SC, Hill C, Gahan CGM. Understanding the mechanisms by which probiotics inhibit gastrointestinal pathogens. *Adv Food and Nutr Res*. Vol 56. 2009, 1–15.

Darilmaz DO, Beyatli Y, Yuksekdag ZN. Aggregation and hydrophobicity properties of 6 dairy *Propionibacteria* strains isolated from homemade Turkish cheeses. *J Food Sci* 2012;**77**:20–4.

FAO/WHO. Evaluation of health an nutitional properties of powdered milk and lactic acid

bacteria. 2001.

Ferrando V, Quiberoni A, Reinheimer J *et al.* Functional properties of *Lactobacillus plantarum* strains: A study in vitro of heat stress influence. *Food Microbiol* 2016;**54**:154–61.

Georgieva R, Yocheva L, Tserovska L *et al.* Antimicrobial activity and antibiotic susceptibility of *Lactobacillus* and *Bifidobacterium* spp. intended for use as starter and probiotic cultures. *Biotechnol Biotechnol Equip* 2015;**29**:84–91.

Gomez Zavaglia A, Kociubinski G, Perez P *et al.* Effect of bile on the lipid composition and surface properties of *Bifidobacteria*. *J Appl Microbiol* 2002;**93**:794–9.

Gong X, Yu H, Chen J *et al.* Cell surface properties of *Lactobacillus salivarius* under osmotic stress. *Eur Food Res Technol* 2012;**234**:671–8.

Gueimonde M, Sánchez B. Enhancing probiotic stability in industrial processes. *Microb Ecol Heal Dis* 2012;**23**, DOI: 10.3402/mehd.v23i0.18562.

Gueimonde M, Sánchez B, G. de los Reyes-Gavilán C *et al.* Antibiotic resistance in probiotic bacteria. *Front Microbiol* 2013;**4**:202.

Haddaji N, Krifi B, Lagha R *et al.* Effect of high temperature on viability of *Lactobacillus casei* and analysis of secreted and GroEL proteins profiles. *African J Bacteriol Res* 2015;**7**:29–34.

Husain I, Poupard JA, Norris RF. Influence of nutrition on the morphology of a strain of *Bifidobacterium bifidum*. *J Bacteriol* 1972;**111**:841–4.

Kos B, Šušković J, Vuković S *et al.* Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *J Appl Microbiol* 2003;**94**:981–7.

Li Q, Lui X, Dong M *et al.* Aggregation and adhesion abilities of 18 Lactic acid bacteria strains isolated from traditional fermented food. *Int J Agric Policy Res* 2015;**3**:84–92.

Liu C, Zhang Z-Y, Dong K *et al.* Antibiotic resistance of probiotic strains of lactic acid bacteria isolated from marketed foods and drugs. *Biomed Environ Sci* 2009;**22**:401–12.

Lv L-X, Yan R, Shi H-Y *et al.* Integrated transcriptomic and proteomic analysis of the bile stress response in probiotic *Lactobacillus salivarius* LI01. *J Proteomics* 2017;**150**:216–29.

Mathipa M, Thantsha M. Cocktails of probiotics pre-adapted to multiple stress factors are more robust under simulated gastrointestinal conditions than their parental counterparts and exhibit enhanced antagonistic capabilities against *Escherichia coli* and *Staphylococcus aureu*s. *Gut Pathog* 2015;**7**:5.

Mills S, Stanton C, Fitzgerald GF *et al.* Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again. *Microb Cell Fact* 2011;**10**:S19.

Mohankumar A, Murugalatha N. Characterization and antibacterial activity of bacteriocin producing *Lactobacillus* isolated from raw cattle milk sample. *Int J Biol* 2011;**3**:p128.

Moser SA, Savage DC. Bile salt hydrolase activity and resistance to toxicity of conjugated bile salts are unrelated properties in *Lactobacilli*. *Appl Environ Microbiol* 2001;**67**:3476–80.

Nguyen H-T, Truong D-H, Kouhoundé S *et al.* Biochemical engineering approaches for increasing viability and functionality of probiotic bacteria. *Int J Mol Sci* 2016;**17**:867.

Oh E, McMullen L, Jeon B. Impact of oxidative stress defense on bacterial survival and morphological change in *Campylobacter jejuni* under aerobic conditions. *Front Microbiol* 2015;**6**:295.

Poupard JA, Husain I, Norris RF. Biology of the *Bifidobacteria*. *Bacteriol Rev* 1973;**37**:136–65.

Ramos CL, Thorsen L, Schwan RF *et al.* Strain-specific probiotics properties of *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus brevis* isolates from Brazilian food products. *Food Microbiol* 2013;**36**:22–9.

Del Re B, Sgorbati B, Miglioli M *et al.* Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. *Lett Appl Microbiol* 2000;**31**:438–42.

Ross RP, Desmond C, Fitzgerald GF *et al.* Overcoming the technological hurdles in the development of probiotic foods. *J Appl Microbiol* 2005;**98**:1410–7.

Ruiz L, Margolles A, Sánchez B. Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium*. *Front Microbiol* 2013;**4**:396.

Saarela M, Rantala M, Hallamaa K *et al.* Stationary-phase acid and heat treatments for improvement of the viability of probiotic *lactobacilli* and *bifidobacteria*. *J Appl*

Microbiol 2004;**96**:1205–14.

Sahadeva RPK, Leong SF, Chua KH *et al.* Survival of commercial probiotic strains to pH and bile. *Int Food Res J* 2011;**18**:1515–22.

Sánchez B, Champomier-Vergès M-C, Collado M del C *et al.* Low-pH adaptation and the acid tolerance response of *Bifidobacterium longum* biotype longum. *Appl Environ Microbiol* 2007;**73**:6450–9.

Sánchez B, Ruiz L, Gueimonde M *et al.* Toward improving technological and functional properties of probiotics in foods. *Trends Food Sci Technol* 2012;**26**:56–63.

Sánchez B, Ruiz L, Gueimonde M *et al.* Adaptation of *Bifidobacteria* to the gastrointestinal tract and functional consequences. *Pharmacol Res* 2013;**69**:127–36.

Sanz Y. Ecological and functional implications of the acid-adaptation ability of *Bifidobacterium*: A way of selecting improved probiotic strains. *Int Dairy J* 2007;**17**:1284–9.

Sedlackova P, Horackova S, Shi T *et al.* Two different methods for screening of bile salt hydrolase activity in *Lactobacillus* Strains. *Czech J Food Sci* 2015;**33**:13–8.

Shakirova L, Grube M, Gavare M *et al. Lactobacillus acidophilus* La5 and *Bifidobacterium lactis* Bb12 cell surface hydrophobicity and survival of the cells under adverse environmental conditions. *J Ind Microbiol Biotechnol* 2013;**40**:85–93.

Sridevi N, Vishwe P, Prabhune A. Hypocholesteremic effect of bile salt hydrolase from *Lactobacillus buchneri* ATCC 4005. *Food Res Int* 2009;**42**:516–20.

Sumeri I, Arike L, Stekolštšikova J *et al.* Effect of stress pretreatment on survival of probiotic bacteria in gastrointestinal tract simulator. *Appl Microbiol Biotechnol* 2010;**86**:1925–31.

Tareb R, Bernardeau M, Gueguen M *et al.* In vitro characterization of aggregation and adhesion properties of viable and heat-killed forms of two probiotic *Lactobacillus* strains and interaction with foodborne zoonotic bacteria, especially *Campylobacter jejuni*. *J Med Microbiol* 2013;**62**:637–49.

Tebyanian H, Bakhtiari A, Karami A *et al.* Antimicrobial activity of some *Lactobacillus* species against intestinal pathogenic bacteria. *Int Lett Nat Sci* 2017;**65**:10–5.

Urdaneta V, Casadesús J. Interactions between bacteria and bile salts in the gastrointestinal

and hepatobiliary tracts. *Front Med* 2017;**4**:163.

Vizoso Pinto MG, Holzapfel WH, Franz C *et al. Lactobacillus* spp. with in vitro probiotic properties from human faeces and traditional fermented products. *Int J Food Microbiol* 2006;**109**:205–14.

Waddington L, Cyr T, Hefford M *et al.* Understanding the acid tolerance response of *bifidobacteria*. *J Appl Microbiol* 2010;**108**:1408–20.

Young KD. The selective value of bacterial shape. *Microbiol Mol Biol Rev* 2006;**70**:660–703.

Young KD. Bacterial morphology: why have different shapes? *Curr Opin Microbiol* 2007;**10**:596–600.

Yousef AE, Courtney PD. *Microbial Stress Adaptation and Food Safety*. Yousef AE, Juneja VK (eds.). Boca Ranton: CRC Press LLC, 2003.

Zhou JS, Pillidge CJ, Gopal PK *et al.* Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. *Int J Food Microbiol* 2005;**98**:211–7.

Chapter 3

Survival of multi-stress adapted *Lactobacillus plantarum* in different food matrices and in simulated gastrointestinal fluids

3.1 Abstract

Probiotic functional foods make up the bulk of the functional food market with dairy leading the sales. However, allergens in dairy products have created a need for alternatives, of which fruit and vegetable juices are a feasible option. Foods containing probiotics should allow them to survive in significant numbers during shelf life; their ability to protect these cultures during gastrointestinal (GIT) transit would be an added advantage. This study investigated the survival of multi- stress adapted *Lactobacillus plantarum* in yoghurt, carrot and cranberry juice, as well as in simulated GIT conditions. *L. plantarum* cells (non-adapted, freshly adapted and old-adapted) were inoculated into each of the abovementioned products, followed by storage at 4^{o}C for six weeks. Then the viable counts of *L. plantarum* in the products, the pH and ^{0}Brix content of the products were measured on a weekly basis. The GIT survival of the *L. plantarum* within the food products was also determined. A significant decreases ($p<0.05$) of the *L. plantarum* cells during storage in all foods was observed. Survival of the multi-stress adapted (old and fresh) *L. plantarum* cells in the foods surpassed that of the non- adapted cells. The reduction in pH was higher for both juices than for yoghurt. The ^{0}Brix of cranberry juice was higher than that of carrot juice. In the GIT non-adapted cells were the most negatively affected while the freshly adapted cells survived better than their old-adapted counterparts. Adaptation of *L. plantarum* to multiple stress factors enhanced its stability during storage, however, without notable differences between the freshly and old- adapted cells. Various food products affected survival of *L. plantarum* under simulated GIT conditions differently. The carrot juice allowed better survival of *L. plantarum* followed by cranberry juice and then yoghurt. Therefore, care should be taken when choosing probiotic food vehicle. As they affect probiotic viability differently during storage as well as offer different levels of protection during passage through the GIT.

3.2 Introduction

Functional foods are defined as foods with ingredients that assist in specific functions within the body. For example, dairy products like yoghurt are packed with bioactive components while fruit and vegetable juices contain dietary fibre that aids in digestion (Buttriss 2000; Lourens-hattingh and Viljoen 2001; Hasler 2002; Kumar, Vijayendra and Reddy 2015). An important class of functional foods are those which incorporate probiotics, prebiotics or the combination of both, synbiotics, which are largely dairy based (Cruz, Faria and Dender 2007). As the first food to which probiotics were added, yoghurt still enjoys commercial success as the leading probiotic food product, accounting for 78% of probiotic sales worldwide (Granato *et al.* 2010; Homayoni Rad *et al.* 2016). However, dairy products do not suit the dietary habits of vegans or vegetarians. Moreover, those with cardiovascular disease and obesity cannot consume them due to their high cholesterol content (Granato *et al.* 2010).

This has resulted in the use of alternate delivery vehicles; fruit and vegetable juice being one of them. These plant based matrices are good substitutes because of their high nutrient content and lack starter cultures that compete for them. Moreover, they contain antioxidants and sugars that help maintain anaerobic conditions and stimulate probiotic growth, respectively (Sheehan, Ross and Fitzgerald 2007; Ding and Shah 2008). The total soluble sugar content of juices, also known as the ^{0}Brix content, plays an important role in the taste and palatability of fruit juices. So, addition of probiotics to juices should not alter this quality in ways that would reduce consumer acceptability (Pimental *et al.* 2015). Whichever matrix is used, probiotics should remain viable in sufficient numbers throughout shelf life as well as during passage through the gastrointestinal tract (GIT) (Sultana *et al.* 2000).

For probiotics to offer therapeutic effects, a high daily dosage of probiotic microorganisms is needed in the product. It is recommended that probiotics stay at levels of 10^{7}CFU/ml or grams at the time of consumption to meet these requirements (Champagne *et al.* 2011). However, viability and stability is threatened by the environmental stressors encountered during production, storage and transition through the GIT (Min *et al.* 2018). Understanding the relationship between viability and a food matrix can help in developing effective probiotic products that can withstand stressful conditions and remain viable in sufficient numbers (Shori 2016b). This is especially important since commercial success of these products depends on their viability and ability to retain functionality (Kumar, Vijayendra and Reddy 2015).

In addition to playing a role in shelf life of probiotics, food matrices are also thought to enable GIT survival and clinical efficacy. For instance, bacteria have an affinity for fat found in milk, which could offer them protection inside the GIT. Moreover, the matrix can have its own benefit on the host, for example, consuming dairy products helps the body to meet its daily calcium requirement. Therefore, incorporating probiotics into foods that have their own benefit allows the consumer to enjoy dual benefits, from food and probiotics, respectively. So, factors like the type of matrix used must be taken into consideration when formulating probiotic food products, not doing this can impede innovative efforts (Sanders and Marco 2010; Sánchez, Sánchez and Salas-Salvadó 2016).

Another aspect that can affect the quality of a probiotic product is the strain used. Not all strains offer the same health benefits or exhibit the same properties and should be studied individually as survival in food and subsequent exposure to GIT can vary among them (Corcoran *et al.* 2005; Corr, Hill and Gahan 2009).

Lactobacilli are known to be intrinsically acid tolerant and would be suitable for use in foods like yoghurt and fruit juices which are known for their acidity (Corcoran *et al.* 2008). In the previous chapter, *L. plantarum* adapted to multiple stresses, namely, temperature, acid and bile, demonstrated robust functional abilities such as acid tolerance, BSH activity and high antimicrobial activity compared to the other probiotic strains tested, which would make it a good candidate for incorporation into the chosen food matrices. It is further envisaged that this stress adaptation could allow *L. plantarum* to survive the conditions of the GIT in sufficient numbers. Therefore, the current study aims to determine the survival of multi-stress adapted *L. plantarum* in selected food matrices, the resulting effect on the physicochemical properties and simulated gastrointestinal survival following incorporation into food.

3.3 Materials and Methods

Stress Adaptation of *L. plantarum*

Adaptation of *L. plantarum* to multiple stresses was conducted as described in Chapter 2, section 2.3.2.

Briefly, acid adaptation was done by adding an overnight culture of *L. plantarum* to MRS both adjusted to pH 2 for 2h. Acid adapted cells were then recovered by growing them overnight at 37ºC. These cells were then bile adapted by incubating them in a 2% bile solution (w/v) for 1h at 37ºC. Acid-bile adapted cells were recovered overnight and adapted to temperature by placing them in a water bath at 55ºC for 2h.

3.3.1 Preparation of Yoghurt

Yoghurt was prepared in the laboratory according to Amakiri and Thantsha (2016). Briefly, 8 g of powdered milk (NIDO, Nestle, South Africa) was added to 250ml of skimmed milk (Long Life, Pick n Pay Dairy Promise, South Africa) in three separate flasks. The flasks were swirled to homogenise the mixture and then placed in a water bath at 72ºC for 3min to pasteurise the milk. The pasteurised milk was cooled to 42ºC and then inoculated with a spoon of plain yoghurt containing starter cultures, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus*. The freshly inoculated milk was then incubated at 37ºC until the pH dropped to 4.53. To ensure that the starter cultures were no longer alive in the yoghurt, following incubation the yoghurt was pasteurised for 30 minutes at 72ºC. A tenfold serial dilution was performed for the yoghurt using ¼ strength Ringer's solution and 100µl of each dilution were plated onto M-17 and MRS for *S. thermophilus* and *L. delbruecki* subsp *bulgaricus*, respectively. Both sets of plates were incubated for 72h at 37ºC in anaerobic jars containing Anaerocult A gaspacks and Anaerocult C test strip. The colonies counted were reported as CFU/ml.

3.3.2 Selection of fruit and vegetable juice

Commercial cranberry and carrot juices were obtained from retail stores in Pretoria. Cranberry was used as a fruit based matrix and carrot juice served as a vegetable based matrix. The same brand of juice was used in all three independent trials. The juices were bought before their best before dates, and stored at 4ºC before their use in experiments.

3.3.3 Preparation of *L. plantarum*

L. plantarum strains were grown according to (Ding and Shah 2008). Briefly, 1.5 ml of the non-, old- and freshly adapted cultures of *L. plantarum* were separately grown in 50ml of MRS broth for 18h to have them reach early logarithm phase. The cells were then concentrated by centrifugation at 10707 x g for 30min at 4^{o}C. The cells were washed twice with sterile phosphate buffered saline (PBS), pH 7 by centrifuging at 3000× g for 5 minutes between each wash. The viable counts of cells were counted using the pour plate method using MRS agar.

3.3.4 Assessment of *L. plantarum* viability in yoghurt and juices during storage

For determining viability in food products, 30ml of yoghurt or juice was aseptically added into separate sterile glass bottles. The food matrix was then inoculated with *L. plantarum* to a final concentration of 10^{11} CFU/ml. Each sample was inoculated in triplicate. The inoculated food samples were stored at 4^{o}C for six weeks. A bottle containing either juice or yoghurt was used each week to perform viable plate counts, pH measurement and for juice, to measure the ^{0}Brix value. The first assessments were done in week 0, a day after storage in 4^{o}C.

3.3.5 Enumeration of *L. plantarum* over six weeks of storage

The samples were analysed weekly over the storage period. On each day of sampling, 1 ml subsample from yoghurt or juice was drawn and serially diluted into 9ml ¼ strength Ringer's solution up to 10^{-9} dilution. One hundred microliters of each dilution was spread plated onto MRS agar plates. The plates were then incubated at 37^{o}C for 72h under anaerobic conditions using Anaerocult A gaspaks and Anaerocult C test strips.

3.3.6 pH and ^{o}Brix measurements

^{o}Brix readings were taken for carrot and cranberry juice, while pH measurements were done for all the food matrices. The first readings were taken on week 0, a day after cold storage. A 1ml subsample was removed from either yoghurt or juices, respectively. The pH of the subsample was measured using a Crison Basic 20 (Denver instruments, USA) pH meter. The Brix reading was measured by a pocket refractometer (Atago, Japan)

3.3.7 Effect of food on the survival of *L. plantarum* in simulated GIT conditions.

3.3.7.1 Preparation of probiotic

Lactobacillus plantarum was grown for 18 hours in MRS broth at 37ºC. The cells were harvested by centrifugation (3000×g for 10min) and then washed twice in PBS (pH 7). They were the re-suspended in carrot juice, cranberry juice, and yoghurt, to a final concentration of approximately 10^{10} cfu/ml.

3.3.7.2 Survival of *L. plantarum* in different foods under simulated gastrointestinal conditions

Simulated gastrointestinal fluids were prepared using method by (Amakiri and Thantsha 2016). To make simulated gastric fluid (SGF), pepsin (P7000, 1: 10000, ICN) (3 g/L) was suspended into NaCl (0.5 w/v) and the pH was adjusted to 2 using 12M HCL. The solution was then filter sterilized using a 0.45µm membrane (Millipore) followed by a 0.22µm filter membrane. Simulated intestinal fluid (SIF) was prepared by dissolving 6.8g of monobasic potassium phosphate (Sigma, St Louis, MO, USA) into 250 ml of distilled water, after which 77ml of 0.2M NaOH was added, followed by 500ml of distilled water. The solution was then vortexed for 30min and then 10g of pancreatin (P-1500, Sigma, St Louis, MO, USA) was added and mixed. The pH of the solution was adjusted to 6.8 using 0.2M NaOH and 0.2M HCL before being made up to a final volume of 1000ml. The solution was sterilized by passing it through a 0.45 µm filter membrane (Millipore) followed by 0.22µm membrane.

One millilitre of yoghurt, carrot or cranberry juice containing *L. plantarum* was added to 9ml of SGF (pH 7) and vortexed for 30s. These were then incubated with shaking (50 rpm) at 37ºC for 2h. Every 30min during incubation a 1ml subsample was taken from the tubes containing *L. plantarum* and sequentially diluted in ¼ strength Ringer's solution and plated on MRS agar to determine the number of surviving bacteria. After 2 hours the remaining bacteria were pelleted 7000× g for 5min before being re-suspended in 5ml of SIF (pH6.8) and incubated as done previously. Bacterial enumeration was performed every 2h for 6h by withdrawing a 1ml subsample from SIF and plating serial dilutions up 10^{-7} onto MRS agar plates. The plates were incubated anaerobically using anaerobic jars containing Anaerocult A and Anaerocult C test strips at 37ºC for 72h. Each experimented in triplicate in three independent trials.

3.3.8 Statistical Analysis

Data captured from three independent trials was used to calculate means and standard deviations, the differences in the means were calculated using least significant difference, (LSD). Software from GraphPad prism 7.01 was used to analyse results. Two way ANOVA was used to perform analyses. Any p value less than 0.05 were taken as statistically significant.

3.4 Results

3.4.1 Survival of *L. plantarum* stored in different food matrices

Figure 3.1 depicts the survival of non-adapted and multi stress (old & fresh) adapted *L. plantarum* in yoghurt, carrot and cranberry juice at 4ºC over a period of six weeks. During storage there were significant reductions in viability of all the cells in yoghurt (Figure 3.1A). The viable numbers of non-adapted cells decreased by 3.02 log ($p<0.0001$) while those of the freshly and old-adapted cells decreased by 1.7 log ($p=0.0037$) and 1.93 log ($p=0.0003$), respectively.

Similarly, *L. plantarum* cells were observed to decrease in viability in carrot and cranberry juice by the end of storage (Figure 3.1B and C). The non- adapted cells in carrot juice decreased by 2.06 log CFU/ml ($p<0.0001$). The freshly adapted cells experienced a 1.31 log CFU/ml decrease ($p=0.0037$) while the old adapted cells decreased by 1.28 log CFU/ml ($p=0.0003$), after six weeks of storage. The same trend was recorded in cranberry juice (Figure 3.1C). In cranberry juice counts of non-adapted cells experienced a 3.21 log CFU/ml decrease, while the decrease in the freshly and old-adapted cells was 2.55 and 2.59 log CFU/ml, respectively. The decline of all cells in cranberry juice during storage time were significant $p<0.0001$.

From the results of all three food matrices, the non- adapted cells showed the largest decrease in viability during storage while the adapted (fresh and old) cells survived better, these differences were significant ($p<0.05$). When comparing the freshly and old- adapted cells, there was no significant differences in the number of viable cells by the end of storage period ($p>0.05$). The survival of cells within the different matrices was highest in carrot juice, followed by yoghurt and least in cranberry juice.

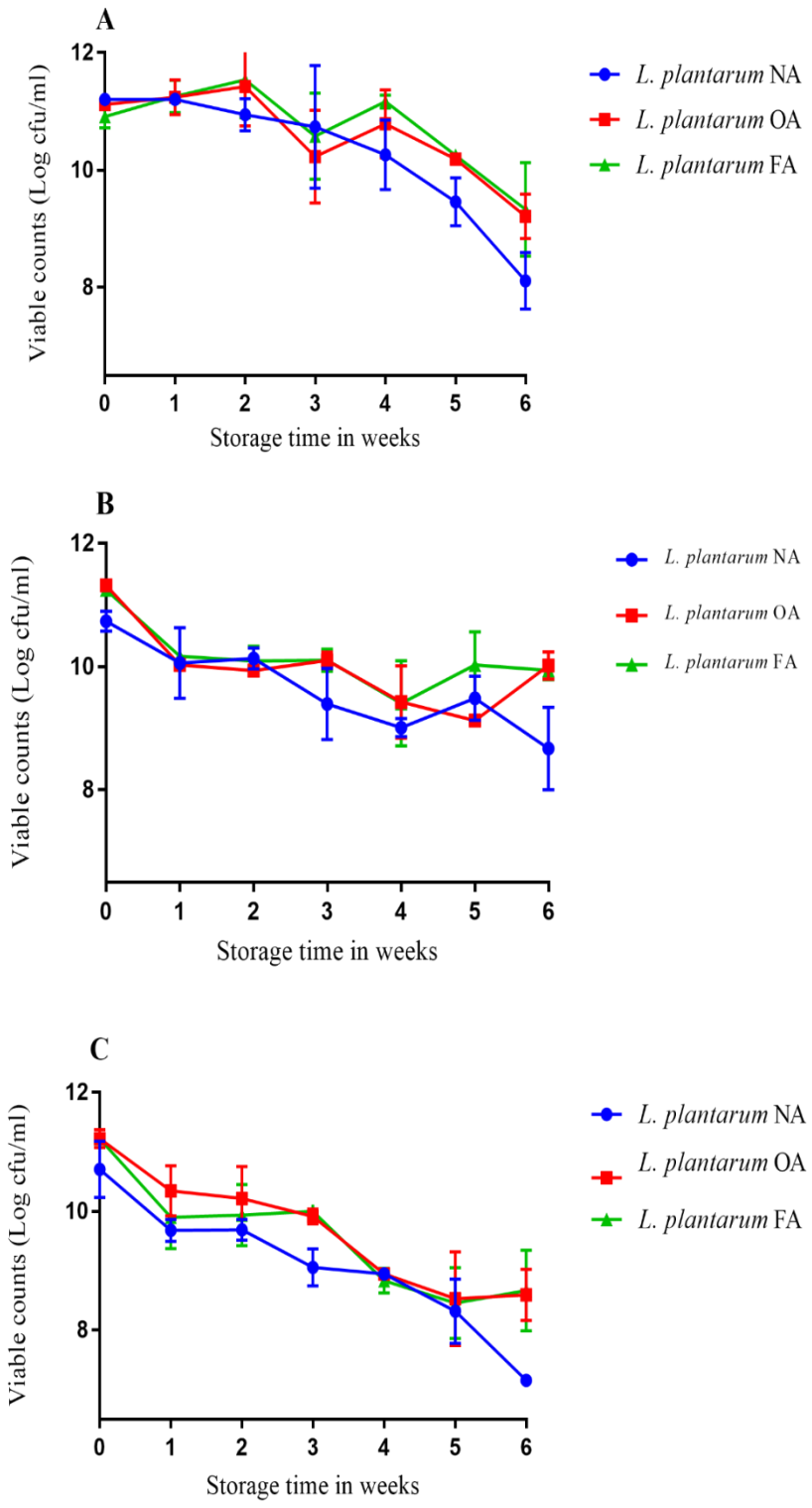

Figure 3. 1 Survival of non-adapted and multi- stress adapted *L. plantarum* in food matrices (Yoghurt (A), carrot (B) and cranberry juice (C)) over six weeks. NA= non- adapted, OA= old adapted, FA= freshly adapted. Error bars are standard deviation of means n=3.

3.4.2 Physico-chemical properties of foods containing *L. plantarum*

pH analysis

The pH values of the food samples containing *L. plantarum* over 6 weeks of storage are depicted in figure 3.2. For yoghurt, there was a decrease in pH, with the sample containing freshly adapted cells showing the largest decrease. There was a non-significant decrease ($p=0.351$) in the pH of the yoghurt containing non- adapted *L. plantarum* during the six weeks (Figure 3.2A). The pH decreased from 3.95-3.90 units (0.05 decrease). Similar results were observed for the yoghurts containing old- ($p=0.988$) and freshly ($p=0.456$) adapted cells, which showed decreases of (0.03 units) and (0.11 units), respectively. At the end of incubation, there was a significant difference between the pH of the yoghurt containing the non-adapted and freshly adapted cells ($p= 0.0389$), however, there was no significant difference ($p=0.506$) between the pH of the yoghurts containing freshly and old-adapted cells.

For carrot juice, there was a decrease in pH during storage, except for the juice with non-adapted *L. plantarum* (Figure 3.2B). However, when comparing the adapted cells, the decrease in the juice with old-adapted cells was slightly higher than the juice with freshly adapted cells. During storage, the pH of the juice containing non- adapted cells increased by 0.03 units from initial value while the pH of the juice with the old-adapted cells decreased by 0.36 unit. The freshly adapted cells caused a 0.34 unit decrease in the carrot juice. The pH changes that occurred from the start to the end of storage were all non-significant, ($p>0.05$) for non-adapted and adapted cultures (old- and fresh), respectively. There were also no significant differences at the end of storage between, non-adapted and adapted (old- and fresh) and between the old adapted and the freshly adapted, respectively ($p>0.05$).

The pH of cranberry juice containing non-adapted *L. plantarum* cells increased during the storage time. However, there was a decrease in the pH of the same juices containing old- and freshly adapted cells over the time of storage, these changes were all non-significant $p>0.05$. (Figure 3.2C). The pH of the cranberry juice containing non-adapted *L. plantarum* increased by 0.19 units while that of the juice with old-adapted cells decreased by 0.11 units during storage. The pH of juice with the freshly adapted cells also increased by 0.16 units during this storage period. At the end of storage there were no significant differences in pH between juices containing non-, old- and freshly adapted cells ($p>0.05$).

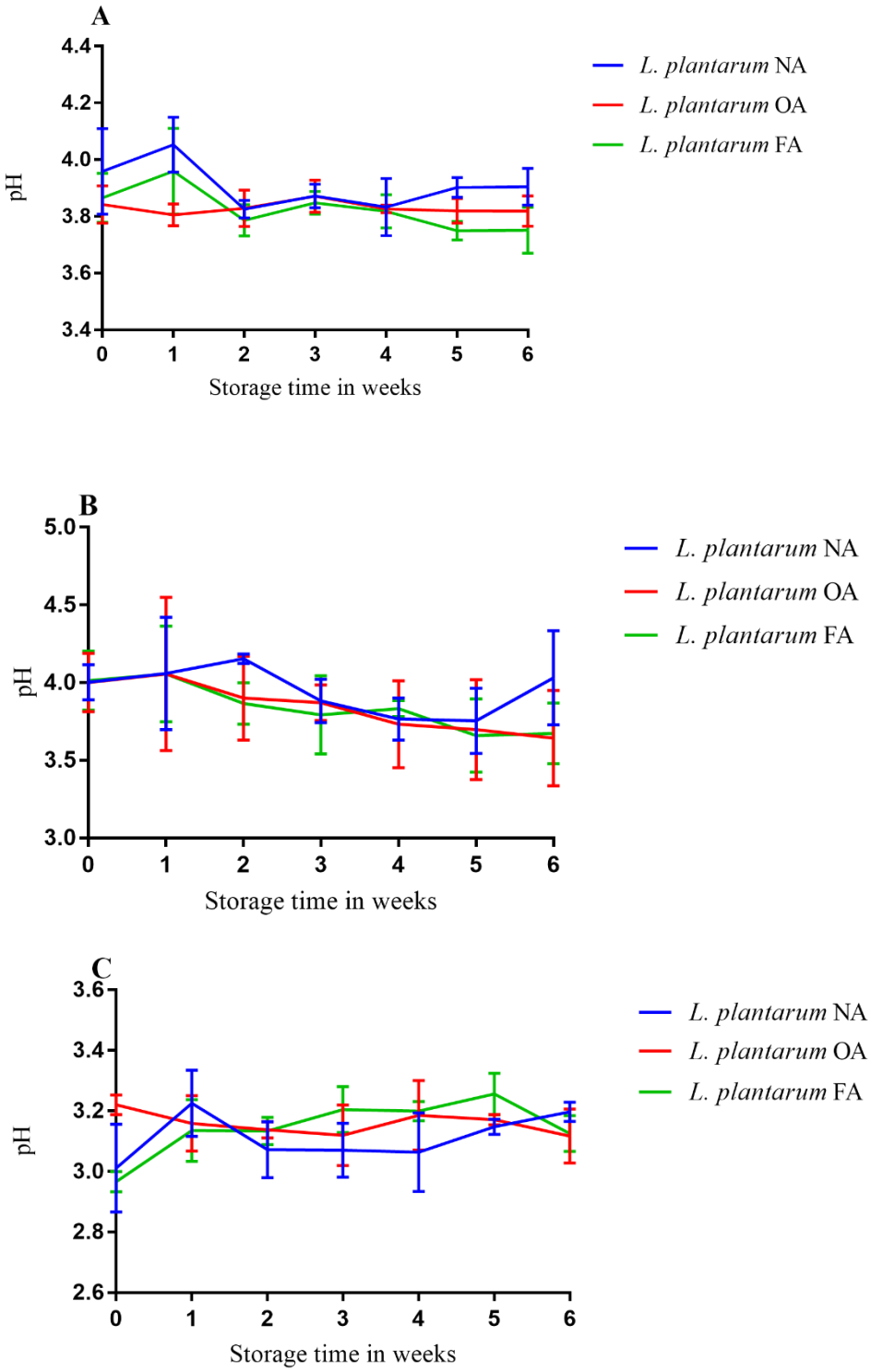

Figure 3. 2 pH changes in yoghurt (A), carrot (B) and cranberry juice (C) containing *L. plantarum* (non-, old- and freshly adapted) stored at 4ºC for six weeks. NA= non- adapted, OA= old adapted, FA= freshly adapted. Error bars are standard deviation of means n=3.

3.4.3 Changes in the ^{0}Brix content of juices containing *L. plantarum*

The sugar soluble contents of carrot (A) and cranberry juice (B) inoculated with *L. plantarum* was monitored throughout a storage period of six weeks and the results from each week were recorded (Figure 3.3). At the end of storage, the ^{0}Brix concentration of carrot juice decreased by 0.77%, 0.73% and 0.69% for samples containing non-, old- and freshly adapted cultures, respectively (Figure 3.3A). A significant reduction in the ^{0}Brix of carrot juice containing all three *L. plantarum* cultures took place between the 4th and the 5th week, otherwise no other significant changes occurred during storage. At the end of storage period there were no significant differences between juice containing non- adapted and old- adapted ($p=0.963$), non-adapted and freshly adapted ($p= 0.999$) and between the two adapted cultures ($p=0.942$). Figure 3.3B shows changes in ^{0}Brix content of cranberry juice containing the three cultures of *L. plantarum.* There was a decrease in the ^{0}Brix content of cranberry juice containing each culture, the non-adapted cells caused a 0.41% decrease while the old- adapted and freshly adapted cells resulted in a 0.19% and 0.92% decrease, respectively. None of these changes were significant $p=$ (0.873, 0.337 and 0.118, for old- and freshly- adapted cells, respectively). When comparing non-adapted and adapted (old- and fresh) cells and the two adapted cells against each other at the end of storage, the differences between them were non-significant ($p>0.05$).

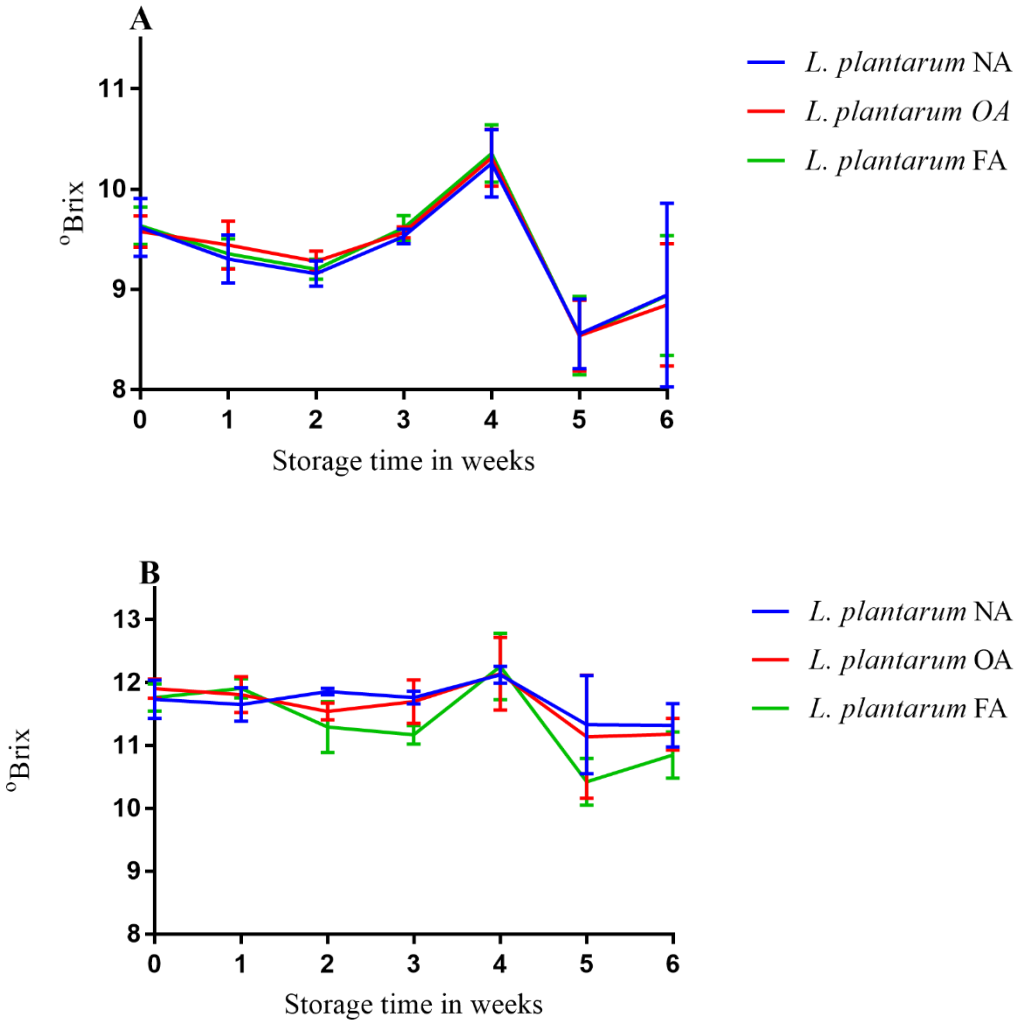

Figure 3. 3 Shows the changes in ^{0}Brix content of carrot (A) and cranberry juice (B) containing non- and multi-stress adapted *L. plantarum* stored at 4^{o}C for 6 weeks. NA= non-adapted, OA= old-adapted, FA= freshly adapted. Error bars are standard deviation of means n=3.

3.4.4 Gastrointestinal survival of non- old- and freshly adapted *L. plantarum* within different foods

Survival of *L. plantarum* in yoghurt (A), carrot (B) and cranberry juice (C) after exposure to simulated gastric (pH 2 for 2h) and intestinal fluids (pH 6.8 for 6h) is shown in figure 3.4. The greatest reduction in viable cells occurred during SGF exposure, while exposure to SIF initially resulted in the continued reduction of viable cells followed by increases in viability. In all food matrices, non-adapted cells showed the largest loss in viable cell counts, followed by old adapted cells, with the exception of carrot juice where old adapted cells had the least number of surviving cells. Freshly adapted cells had the highest numbers of viable cells after 480min exposure period. During exposure of yoghurt to SGF, all *L. plantarum* cells experienced a non-significant decline $p>0.05$ (Figure 3.4A). There was a decrease from 10.59-9.85 log CFU/ml (0.74 log decrease), 10.37-9.39 log CFU/ml (0.98 log decrease) and 10.74-10.25 log CFU/ml (0.53 log decrease), for non-, old- and freshly- adapted cells, respectively. Upon subsequent exposure to SIF, the cells continued to decrease until the 240^{th} minute after this point they began increasing, however, there was still an overall loss in viability at the end of exposure period from 10.59-8.73 log CFU/ml (1.86 log decrease), 10.37-9.34 log CFU/ml (1.03 log decrease and 10.74- 8.73 log CFU/ml (0.63 log decrease) for non- old- and freshly adapted cells.

There was a significant decrease ($p=0.003$) when carrot juice with non- adapted cells was exposed to SGF, the old-adapted also showed a significant decreased from 9.74-8.73 log CFU/ml (1.01 log decrease), ($p=0.103$). The decline in freshly adapted cells was non-significant $p=0.074$, from 9.59-9.51 log CFU/ml (0.08 log decrease) (Figure 3.4B). Subsequent SIF exposure resulted in initial decrease followed by an increase, however, at the end of the incubation there was an overall loss in viable cells for non- and old- adapted cells from 9.75 - 9.29 log CFU/ml (0.47 log decrease) and 9.74-9.05 log CFU/ml (0.23 log decrease), respectively. For the freshly adapted cells, there was an increase in viability at the end of the exposure period from 9.59-10.36 log CFU/ml (0.62 log increase).

Exposing *L. plantarum* in cranberry juice to SGF led to significant decreases from 10.23-8.71 log CFU/ml (1.52 log decrease) ($p=0.0012$) and 10.31-8.75 log CFU/ml (1.54 log decrease) $p=0.0015$ for non- and old- adapted cells, respectively (Figure 3.4C). There was no significant decrease ($p=0.143$) for the freshly adapted cells which showed a 0.95 log decrease from 10.46-9.51 log CFU/ml. The non-adapted *L. plantarum* in cranberry juice exposed to SIF showed an

increase in viability. However, when looking at the entire incubation period, there was an overall decrease from 10.29-9.59 log CFU/ml a 0.7 log decrease. The adapted cells also increased during SIF exposure but there was still a net loss in viable cells at the end of exposure period from 10.31-9.8 log CFU/ml (0.33 log decrease) and 10.46-10.22 log CFU/ml (0.24 log decrease) for old- and freshly adapted cells, respectively.

Yoghurt offered the best protection in SGF, all the losses encountered by the cells in this matrix were non-significant ($p>0.05$). However, the cells experienced the largest overall losses in yoghurt, followed by cranberry juice and carrot juice was able to maintain the highest number of viable cells under simulated GIT.

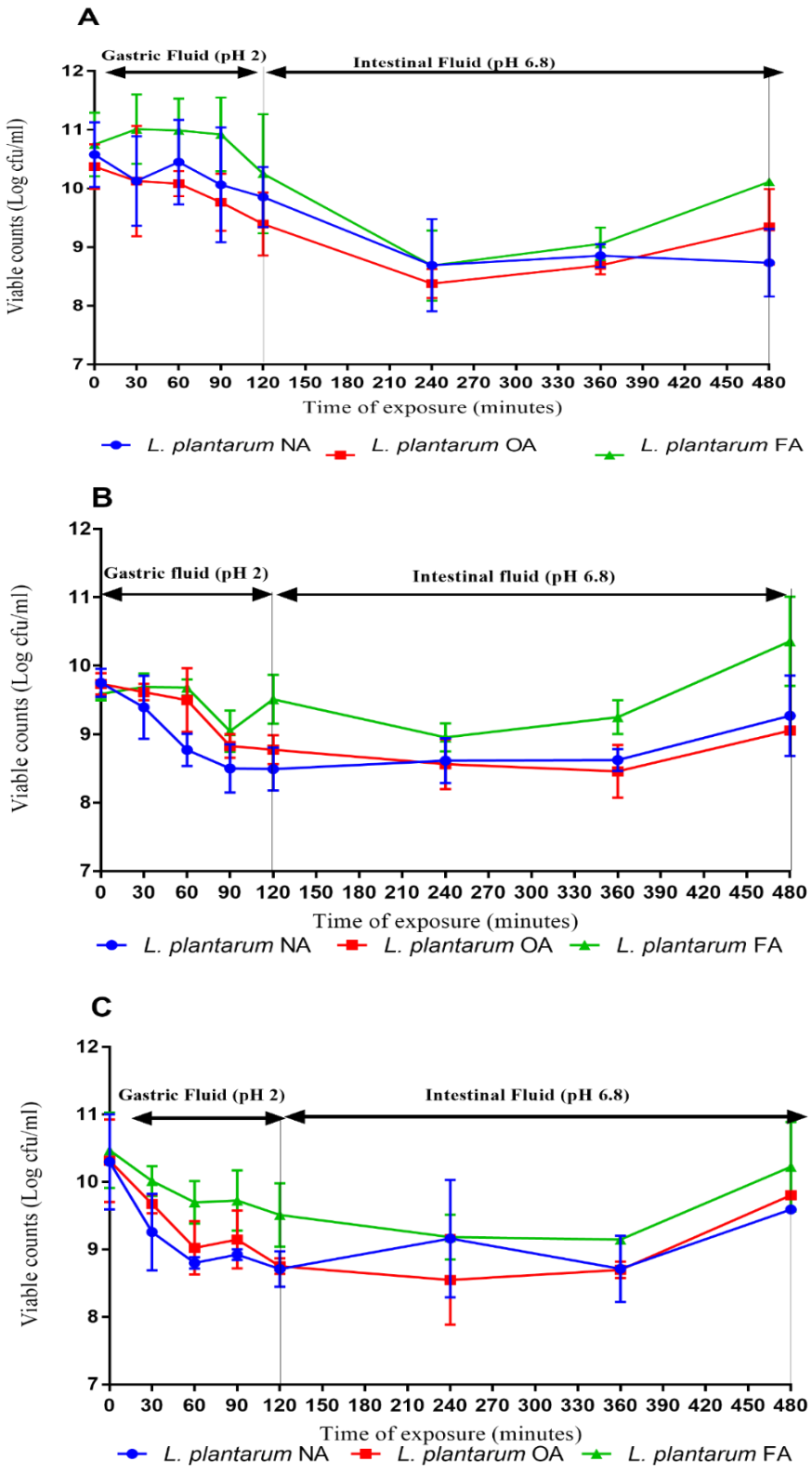

Figure 3. 4 Survival of *L. plantarum* in yoghurt (A), carrot (B) and cranberry (C) during exposure to simulated gastrointestinal conditions for 8 hours. NA= non- adapted, OA= old adapted, FA= freshly adapted. Error bars represent standard deviation of mean n=3.

3.5 Discussion

Consumers have become increasingly interested in foods that provide both quality nutrition and health benefits, specifically those capable of disease prevention and/or health maintenance. This has made the development of such products a priority in the food industry (Daneshi *et al.* 2013). Probiotic food products make up a significant portion of functional foods sales, of which dairy make up the bulk. However, dairy products have limitations, such as containing dairy allergens, high cholesterol and they exclude those practicing veganism, which has necessitated non-dairy alternatives (Granato *et al.* 2010; Min *et al.* 2018). Fruit and vegetable juices are promising in this regard because of their refreshing taste profile, nutrient content and can be enjoyed by a wider range of people (Panghal *et al.* 2018). Carrots are a popularly consumed vegetable that are high in vitamins, minerals and beta- carotene, an essential micronutrient which acts as a provitamin A as well as a powerful antioxidant (Daneshi *et al.* 2013; Vasudha and Mishra 2013). Cranberry juice is also a popular choice among consumers not just for its taste but it's proven benefits of treating urinary tract infections (Kontiokari *et al.* 2001). It is important that these food matrices allow probiotics to survive at levels that will offer therapeutic benefits during shelf life, which are between 10^6-10^7 CFU/ml (Ozer and Kirmaci 2010). The current study looked at survival of multi- stress adapted (old and fresh) *L. plantarum* in yoghurt, carrot and cranberry juice, which survived storage for six weeks at 4^{o}C (Figure 3.1). Additionally, viability and physicochemical changes (pH and ^{o}Brix content) were also assessed during the same period (Figure 3.2 and Figure 3.3).

The results showed a significant decline ($p<0.05$) of *L. plantarum* viability (non-,old-, and freshly adapted), in all three food matrices from the start to the end of storage time (Figure 3.1). However, all *L. plantarum* cells still survived at levels recommended for therapeutic benefits. As expected, the non- adapted culture showed the highest decrease in viability, while the old- and freshly adapted cells showed similar levels of survival. The decrease in viable count could be largely attributed to the acidic nature of all three food matrices, which remained below 4 during storage period (Figure 3.2). Acid stress causes an accumulation of protons within the cell which disrupts the proton motive force, which is essential for membrane transportation processes (Corcoran *et al.* 2008; Amund 2016). This kind of stress also damages the bacterial DNA and the proteins inside the cell (Corcoran *et al.* 2008). The ability of both the old- and freshly adapted cells of *L. plantarum* to maintain their high numbers despite significant viability losses is due to their pre-exposure to sub-lethal stresses. The ability of stress pre-adaptation treatment to enhance stress-tolerance of *Lactobacilli* and *Bifidobacteria* has been

reported by researchers elsewhere (Corcoran *et al.* 2008; Mills *et al.* 2011; Gueimonde and Sánchez 2012). Certain *Lactobacilli* species possess a natural ability to tolerate acidity, by maintaining a neutral cytoplasmic pH (De Dea Lindner *et al.* 2007). Although non- adapted cells were the least surviving compared to the other cultures (Figure 3.1), they remained above the recommended minimum levels due to the intrinsic acid tolerance *L. plantarum* possesses (G-Alegria *et al.* 2004). These results support the findings from chapter 2 where non- adapted *L. plantarum* demonstrated high levels of tolerance of up to pH 2 (Figure 2.2).

The properties of a food matrix also play a role on how well a probiotic survives in the product. In yoghurt, some properties known to affect probiotic survival include acidity (post acidification), specific strain used, dissolved oxygen, growth promoting factors and storage temperature (Lourens-hattingh and Viljoen 2001). The findings of this study showed that the changes in acidity of yoghurt were minor (Figure 3.2A). This was attributed to the starter culture *L. delbrueckii* subsp *bulgaricus*, responsible for post–acidification at cold temperatures, being killed off before *L. plantarum* was added (Lourens-hattingh and Viljoen 2001). It was already determined in chapter 2 that *L. plantarum* was acid tolerant and hence could survive in the low pH environment of the yoghurt. Fruit and vegetable juices as probiotic carriers have been less extensively studied than their dairy counterparts and using them presents unique challenges. According to Flach *et al.* (2018) juices are not conducive for probiotic survival due to their acidity, oxygen content, natural growth inhibitors and additives. Contrary to Flach *et al.* (2018), the results from the current study showed that despite both juices having a low pH, the survival of strains was still within the recommended limits for a probiotic product. Previous studies have reported on the ability of carrot juice to accommodate the survival and growth of *Lactobacilli*. Tamminem, Salminen and Ouwehand (2013) studied the survival of *L. rhamnosus* GG, *L. paracasei* Lpc-37, and *L. plantarum* Lp-115 in carrot juice, they found that the probiotics survived in high numbers for 30 days. However, after 12 weeks of storage the survival of *L. plantarum* dropped below 10^8 CFU/ml. The findings of the current study concurs with theirs, carrot juice had the highest number of surviving cells after six weeks of storage (Figure 3.1B). The two adapted cultures were above 10 log CFU/ml at the end of six weeks, while the non-adapted cells remained above 9 log CFUml after storage. When comparing the three food products, carrot juice had the highest pH (approximately 4) which could be the reason for the enhanced cell survival. Cranberry juice on the other hand is well known for incurring massive losses of probiotic viability during storage. Sheehan, Ross and Fitzgerald 2007) reported that when three strains of *Lactobacilli* were stored in cranberry, orange and

pineapple juice at 4ºC for 6 weeks, the biggest losses were among the cells in cranberry juice. A different study by Nualkaekul and Charalampopoulos (2011) reported that *L. plantarum* cells plummeted shortly after storage in cranberry juice. Our results were in agreement with these previous studies. However, the extent of viability loss was not the same as what the other authors had reported, in which cell viability dropped below 4 log CFU/ml. This is because, despite its low pH, cranberry juice also contains benzoic acid, at the same levels used to preserve perishable foods (Shori 2016b).

The sugars contained in juice are useful in promoting bacterial growth (Rivera-Espinoza and Gallardo-Navarro 2010; Kumar, Vijayendra and Reddy 2015; Min *et al.* 2018). In the current study, the total sugar soluble content of the two juices were monitored throughout the six weeks (Figure 3.3). There was no strong correlation found between increased probiotic survival during storage and decrease in ^{0}Brix content except between the fourth and fifth weeks, where the ^{0}Brix content of carrot juice declined significantly ($p<0.05$) (Figure 3.3A). Likewise, during storage of carrot juice (Figure 3.1B) there was a corresponding increase in non- and freshly adapted *L. plantarum* cells between the same times. During this period the bacteria utilized the sugars present in the carrot juice to promote their growth. A similar pattern was observed in the cranberry juice in which the freshly-adapted cells had the lowest ^{0}Brix concentration from the 1st week to the end of storage (Figure 3.3B). These cells were also the best surviving during storage (Figure 3.1C). This observation suggests that freshly-adapted cells were better at metabolizing the sugars present in the environment to aid their growth compared to the non- and old-adapted counterparts, moreover, since cranberry juice had a higher ^{0}Brix content there were more sugars available for these cells to use. These findings are similar to those by (Ding and Shah 2008) who showed a correlation between the growth of *Lactobacilli* and a decrease in ^{0}Brix concentration during storage in orange and apple juice. No other correlations were present between sugar utilization and increase of *L. plantarum* viability during storage.

Probiotics in functional foods are ingested following storage and must survive the GI conditions before reaching their target site, where they must still exist in large quantities (Amund 2016). So when assessing probiotic survival within the GIT, the two components (gastric and intestinal conditions) should not be separated as they could affect viability of probiotic in a synergistic manner (Vizoso Pinto *et al.* 2006). Moreover, there is a lack of studies on the role that food matrices play in GI survival of probiotics (Mortazavian, Mohammadi and Sohrabvandi 2012). Taking that into consideration, the current study investigated the survival of non- and multi stress adapted (old and fresh) *L. plantarum* in simulated GI conditions for 8

hours following incorporation into yoghurt, carrot and cranberry juice. Pre-adaptation significantly improved the survival of *L. plantarum* (Figure 3.4), the freshly-adapted cells were the best surviving in all three matrices. These results are similar to those reported by Mathipa and Thantsha (2015) where the survival of multi stress adapted *L. plantarum* was significantly higher than their non- adapted counterparts after exposure to simulated GIT. The survival of old-adapted cells was lower than freshly adapted cell in the foods, suggesting that long-term storage had an impact on gastrointestinal survival.

Food and food ingredients can enhance the survival of probiotics in the GIT (Pitino *et al.* 2012). This study showed that the type of food matrix used affected the survival of *L. plantarum* differently during simulated digestion. The cells were able to survive well in all three foods, maintaining viable counts greater than 8 logs throughout incubation period (Figure 3.4). Work by Ranadheera, Prasanna and Vidanarachchi (2014) suggested that using low pH fruit juices as carriers could make probiotics more resilient to subsequent acidic conditions such as those found in the gastric juice of the stomach. The current study verifies this, as both carrot and cranberry juice had the best surviving cells after GI exposure compared to yoghurt. This was contrary to the results reported by Champagne and Gardner (2008) where incorporating probiotics into a dairy rather than a juice matrix resulted in better GIT survival due to juices having a lower buffering capacity compared to dairy products. However, unlike dairy products, fruit and vegetable juice are high in carbohydrates and dietary fibre which act as natural prebiotics allowing survival in the adverse conditions of the stomach and intestines (Patel 2017). Furthermore, this study showed carrot juice as the matrix with the highest viable cells counts, in this matrix the freshly adapted cells grew and ultimately exceeded the initial viable count. Although survival in yoghurt was the lowest, probiotic levels were at acceptable levels, meaning that the dairy proteins and fats also offer sufficient probiotic protection (Amund 2016). Furthermore, the gastric survival of the cells in yoghurt was better than that of the juices suggesting that its components are better at shielding against acidic conditions of the stomach.

3.6 Conclusions

The study looked at the survival of multi-stress adapted *L. plantarum* in yoghurt, carrot and cranberry juice during storage and in simulated GIT conditions. During storage, the two adapted cultures survived better than the non-adapted cells, in all foods. All the losses in cell viability that occurred during storage were still with the acceptable minimum for probiotics. The low pH of the yoghurt and the juices could be the reason for the reduction in cell viability. A significant loss in ^{0}Brix content between the 4^{th} and 5^{th} week resulted in a corresponding increase in cell viability during storage. Although the survival of old- and freshly adapted cells was the same during storage, the freshly adapted cells were better at enduring GIT conditions compared to the old-adapted ones. Carrot juice was the matrix that had the highest number of surviving cells at the end of exposure period, however, yoghurt retained high cell numbers during exposure to gastric conditions.

Taken together, this study shows how multi- stress adaptation can be used to enhance survival of probiotics in food products during storage and passage through the GIT. However, when using this method to improve survival, care should be taken as pro-longed cold storage can affect acquired stress tolerance resulting in diminished survival during GIT transit. Furthermore, when developing probiotic products the type of food matrix should be taken into consideration as this also has a profound influence on survival of probiotic cultures during storage and subsequent exposure to GI stresses.

3.7 References

Amakiri AC, Thantsha MS. Survival of *Bifidobacterium longum* LMG 13197 microencapsulated in Vegetal or Vegetal - inulin matrix in simulated gastrointestinal fluids and yoghurt. *Springerplus* 2016;**5**, DOI: 10.1186/s40064-016-3010-y.

Amund OD. Exploring the relationship between exposure to technological and gastrointestinal stress and probiotic functional properties of *Lactobacilli* and *Bifidobacteria*. *Can J Microbiol* 2016;**62**:715–25.

Buttriss J. Is Britain ready for FOSHU? *Nutr Bull* 2000;**25**:159–61.

Champagne CP, Gardner N. Effect of storage in a fruit drink on subsequent survival of probiotic *Lactobacilli* to gastro-intestinal stresses. *Food Res Int* 2008;**41**:539–43.

Champagne CP, Ross RP, Saarela M *et al.* Recommendations for the viability assessment of probiotics as concentrated cultures and in food matrices. *Int J Food Microbiol* 2011;**149**:185–93.

Corcoran B, Stanton C, Fitzgerald G *et al.* Life under stress: The probiotic stress response and how it may be manipulated. *Curr Pharm Des* 2008;**14**:1382–99.

Corcoran BM, Stanton C, Fitzgerald GF *et al.* Survival of probiotic *Lactobacilli* is enhanced in acidic environments in the presence of metabolizable sugars. *Appl Environ Microbiol* 2005;**71**:3060–7.

Corr SC, Hill C, Gahan CGM. Understanding the mechanisms by which probiotics inhibit gastrointestinal pathogens. *Adv Food and Nutr Res*. Vol 56. 2009, 1–15.

Cruz AG, Faria DAF, Dender AGF Van. Packaging system and probiotic dairy foods. *Food Res Int* 2007;**40**:951–6.

Daneshi M, Ehsani MR, Razavi SH *et al.* Effect of refrigerated storage on the probiotic survival and sensory properties of milk/carrot juice mix drink. *Electron J Biotechnol* 2013;**16**:5–5.

De Dea Lindner J, Canchaya C, Zhang Z *et al.* Exploiting *Bifidobacterium* genomes: the molecular basis of stress response. *Int J Food Microbiol* 2007;**120**:13–24.

Ding WK, Shah NP. Survival of free and microencapsulated probiotic bacteria in orange and

apple juices. *Int Food Res J* 2008;**15**:219–32.

Flach J, van der Waal MB, van den Nieuwboer M *et al.* The underexposed role of food matrices in probiotic products: Reviewing the relationship between carrier matrices and product parameters. *Crit Rev Food Sci Nutr* 2018;**58**:2570–84.

G-Alegria E, Lopez I, Ruiz I *et al.* High tolerance of wild *Lactobacillus plantarum* and *Oenococcus oeni* strains to lyophilisation and stress environmental conditions of acid pH and ethanol. *FEMS Microbiol Lett* 2004;**230**:53–61.

Granato D, Branco GF, Nazzaro F *et al.* Functional foods and nondairy probiotic food development: trends, concepts, and products. *Compr Rev Food Sci Food Saf* 2010;**9**:292–302.

Gueimonde M, Sánchez B. Enhancing probiotic stability in industrial processes. *Microb Ecol Heal Dis* 2012;**23**, DOI: 10.3402/mehd.v23i0.18562.

Hasler CM. Functional foods: benefits, concerns and challenges—A position paper from the american council on science and health. *J Nutr* 2002;**132**:3772–81.

Homayoni Rad A, Vaghef Mehrabany E, Alipoor B *et al.* The comparison of food and supplement as probiotic delivery vehicles. *Crit Rev Food Sci Nutr* 2016;**56**:896–909.

Kontiokari T, Sundqvist K, Nuutinen M *et al.* Randomised trial of cranberry-lingonberry juice and *Lactobacillus* GG drink for the prevention of urinary tract infections in women. *Bmj* 2001;**322**:1571.

Kumar VB, Vijayendra SVN, Reddy OVS. Trends in dairy and non-dairy probiotic products - a review. *J Food Sci Technol* 2015;**52**:6112–24.

Lourens-hattingh A, Viljoen BC. Yogurt as probiotic carrier food. 2001;**11**:1–17.

Mathipa M, Thantsha M. Cocktails of probiotics pre-adapted to multiple stress factors are more robust under simulated gastrointestinal conditions than their parental counterparts and exhibit enhanced antagonistic capabilities against *Escherichia coli* and *Staphylococcus aureus. Gut Pathog* 2015;**7**:5.

Mills S, Stanton C, Fitzgerald GF *et al.* Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again. *Microb Cell Fact* 2011;**10**:S19.

Min M, Bunt CR, Mason SL *et al.* Non-dairy probiotic food products: An emerging group of

functional foods. *Crit Rev Food Sci Nutr* 2018:1–16.

Mortazavian AM, Mohammadi R, Sohrabvandi S. Delivery of probiotic microorganisms into gastrointestinal tract by food products. *New Adv Basic and Clin Gastroentereol*. 2012, 121–46.

Nualkaekul S, Charalampopoulos D. Survival of *Lactobacillus plantarum* in model solutions and fruit juices. *Int J Food Microbiol* 2011;**146**:111–7.

Ozer BH, Kirmaci HA. Functional milks and dairy beverages. *Int J Dairy Technol* 2010;**63**:1–15.

Panghal A, Janghu S, Virkar K *et al.* Potential non-dairy probiotic products – A healthy approach. *Food Biosci* 2018;**21**:80–9.

Patel AR. Probiotic fruit and vegetable juices- recent advances and future perspective. *Int Food Res J* 2017;**24**:1850–7.

Pimentel TC, Madrona GS, Garcia S *et al.* Probiotic viability, physicochemical characteristics and acceptability during refrigerated storage of clarified apple juice supplemented with *Lactobacillus paracasei* ssp. *paracasei* and oligofructose in different package type. *LWT-Food Sci Tech* 2015;**63**:415-422.

Pitino I, Randazzo CL, Cross KL *et al.* Survival of *Lactobacillus rhamnosus* strains inoculated in cheese matrix during simulated human digestion. *Food Microbiol* 2012;**31**:57–63.

Ranadheera CS, Prasanna PHB, Vidanarachchi JK. *Fruit Juices : Types, Nutritional Composition and Health Benefits*. Elder KE (ed.). New York: Nova Science Publishers, 2014.

Rivera-Espinoza Y, Gallardo-Navarro Y. Non-dairy probiotic products. *Food Microbiol* 2010;**27**:1–11.

Sánchez NB, Sánchez GM, Salas-Salvadó J. New scientific evidence on the benefits of yoghurt. 2016:1–38.

Sanders ME, Marco ML. Food formats for effective delivery of probiotics. *Annu Rev Food Sci Technol* 2010;**1**:65–85.

Sheehan VM, Ross P, Fitzgerald GF. Assessing the acid tolerance and the technological robustness of probiotic cultures for fortification in fruit juices. *Innov Food Sci Emerg*

Technol 2007;**8**:279–84.

Shori AB. Influence of food matrix on the viability of probiotic bacteria: A review based on dairy and non-dairy beverages. *Food Biosci* 2016;**13**:1–8.

Sultana K, Godward G, Reynolds N *et al.* Encapsulation of probiotic bacteria with alginate–starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt. *Int J Food Microbiol* 2000;**62**:47–55.

Tamminem M, Salminen S, Ouwehand AC. Fermentation of carrot juice by probiotics: viability and preservation of adhesion. *Int J Biotechnol Wellness* 2013;**2**:10–5.

Vasudha S, Mishra HN. Non dairy probiotic beverages. *Int Food Res J* 2013;**20**:7–15.

Vizoso Pinto MG, Holzapfel WH, Franz C *et al. Lactobacillus* spp. with in vitro probiotic properties from human faeces and traditional fermented products. *Int J Food Microbiol* 2006;**109**:205–14.

Chapter 4

General conclusions and recommendations

4.1 General Conclusions

- Acid tolerance was similar for non-, freshly- and old- adapted probiotic cells. Bile tolerance was not improved by the adaptation process as non-adapted cells exhibited better survival in bile than both adapted cultures. Therefore, the process of adaptation as well as long term storage had no bearing on acid resistance of the strains, however, it negatively impacted bile tolerance. Although the bile salt hydrolase activity (BSH) was induced by fresh adaptation, this did not translate to better bile tolerance as BSH negative non-adapted strains still survived better in bile salts.

- Antimicrobial activity of probiotics against *E. coli* and *S. aureus* was similar except for *L. plantarum* whose old-adapted cells had significantly lowered inhibition activity against *E. coli* compared to non- and freshly adapted cells. This indicated that the production of antimicrobial compounds by *L. plantarum* were negatively impacted by cold-storage.

- The three different cultures of probiotics exhibited the same antibiotic susceptibility profiles, indicating that adaptation and subsequent cold storage had no impact on sensitivity to antibiotics. But, in the case of *L. plantarum* and *B. longum* Bb46 further studies are required to assess whether the deviations that occurred were as a result of gene transfer or not.

- SEM revealed changes in the morphology of both adapted cultures for *Bifidobacteria* and *L. acidophilus.* Stress adaptation also altered the cell surfaces properties of the probiotics. It resulted in higher hydrophobicity in *L. plantarum, B. longum* LMG 13197 and *B. bifidum* LMG 11041, but lowered hydrophobicity for *L. acidophilus* and *B. longum* Bb46. Auto-aggregation was low overall and further diminished by both adapted cultures overtime. Since morphology, aggregation and CSH are linked to adherence, there is a high likelihood that adaptation and long term storage will influence the way that strains adhere to mucosal and epithelial surfaces.

- Storage in food matrices allowed adapted cells to survive better than non-adapted *L. plantarum* cells throughout storage periods. Demonstrating how the process adaptation enhances survival and viability during storage. The low acidity of the all the foods was

most likely the reason for the losses in cell viability during storage, however, the decreased viability was not below the recommended levels for probiotics. Thus, the food matrices used are still suitable candidates for delivery of stress adapted *L. plantarum*.

- Under simulated GIT conditions, the freshly adapted cells survived better in the foods. The juices enabled the *L. plantarum* to survive better in SIF, while the yoghurt provided better survival in SGF. Overall, carrot juice was better at providing protection against viability losses during both storage and passage through simulated GIT conditions. Carrot juice would therefore be the most favourable matrix for incorporating multi-stress adapted *L. plantarum.*

- The enhanced survival of freshly-adapted *L. plantarum* in simulated GIT reiterates the benefit of stress adaptation for improved stability and viability. However, the differences that occurred between old- and freshly adapted strains shows that long term storage can alter acquired enhancements. Therefore, other alternatives must be explored to store *L. plantarum* that will not negatively affect survival in simulated GIT.

4.2 Recommendations for future work

- Since multi-stress adaptation enhanced certain properties in specific strains, a combination of probiotics can be used to create a formulation that can have all the probiotic properties boosted at the same time, where one strain may lack a certain beneficial property another will possess it. This way more dynamic probiotic products can be manufactured.

- The adherence of strains to CaCo-2 cell line should also be investigated, to determine whether long-term storage will negatively influence the strains' adherence abilities.

- Multi-stress adapted strains were incorporated into juices in this study, and previously in yoghurt as food vehicles through which the probiotics can be made available to consumers. Other potential food vehicles such as traditional African beverages, confectionery, cheese, ice cream can be assessed for their suitability for delivery of stress adapted probiotics. This way the probiotic industry can expand their market.

- Different methods such as encapsulation have been used to improve viability of probiotic cultures. The multi-stress adapted can be encapsulated in different encapsulating matrices to determine which will result in improved stability of the multi-stress adapted probiotics both in products during storage as well as under simulated gastrointestinal conditions.

- It is not known expression of which genes is affected by the multi-stress adaptation process. Differential gene expression between non-adapted and multi-stress adapted probiotics can be determined, which will also serve to elucidate the mechanism of action of the stress adapted strains.

- The efficiency of cell free supernatant of multi-stress adapted strains for control of microbial biofilms in the food industry, such as those formed by *Listeria monocytogenes*, can be investigated. It is envisaged that due to potential differential gene expression in these strains, the potency of the antimicrobial compounds released into culture media may be stronger than that of non-adapted strains.

- Since in this study the stress adapted cells were preserved by freezing subsequent to adaptation, future studies can also investigate alternative culture preservation methods that will not have negative effects on structural and functional properties of stress adapted probiotic cultures.