

**Pre-adaptation of selected probiotic strains to multiple stress factors: consequent effect
on their stability and probiotic properties**

by

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DECLARATION

I declare that the dissertation ‘**Pre-adaptation of selected probiotic strains to multiple stress factors: consequent effect on their stability and probiotic properties**’ 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.

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PUBLICATIONS

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Moloko G Mathipa and Mapitsi S Thantsha. (2015). 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 Pathogens* 7: 5

LIST OF ABBREVIATIONS

BSH: Bile salt hydrolase

Cys- HCl: L- cysteine Hydrochloric acid

CFU/ ml: Colony forming units per millilitre

DNA: Deoxyribonucleic acid

F6PPK: Fructose- 6- phosphate phosphoketolase

FDA: Food and Drug Administration

GC: Glycine: Cytosine content

GIT: Gastrointestinal tract

HCl: Hydrochloric acid

IBS: Irritable Bowel Syndrome

LAB: Lactic acid bacteria

MRS: De Man Rogosa Sharpe

NaCl: Sodium Chloride

NaOH: Sodium Hydroxide

OD: Optical Density

RNA: Ribonucleic acid

rpm: Revolutions per minute

spp.: Species

TDCA: Taurodeoxycholate

v/v: Volume per volume

WHO: World Health Organization

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SUMMARY

Pre-adaptation of selected probiotic strains to multiple stress factors: consequent effect on their stability and probiotic properties

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Department : Microbiology and Plant Pathology

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The interest in the use of probiotics has escalated in the last decade, this is consequent to an increase in the number of studies showing that these microorganisms have beneficial effects on the host's health. Probiotics can be administered in different ways, as capsules (pharmaceuticals) and also incorporated in different food products. In the definition of probiotics, it is highlighted that for them to be beneficial, they have to be administered alive and in adequate numbers. Nevertheless, there have been a number of problems associated with the number of viable cells of probiotics, with reports that viable numbers decline drastically on exposure to various stresses including those that prevail in the GIT. This raised an interest in the probiotics research, focusing on the techniques that can yield stress resistant or tolerant probiotics. These techniques are aimed to increase the number of the surviving cells after the exposure to technological and gastrointestinal stress factors. There has, therefore, been an increase in the studies focusing specifically on how to adapt the probiotic cells to different stress factors. The mechanism of pre- adaptation or cross- protection has been one of the most studied areas. With these mechanisms, researchers pre- expose probiotics to different stress factors so that they can survive better when they are later exposed to the same stress factor. Pre- adaptation of probiotics to multiple stress factors will therefore offer tolerance to more stress factors.

Taking that into consideration, the present study aimed at determining whether probiotic cells that have been pre- exposed to multiple stress factors (acid, bile and temperature) will have better tolerance to different gastric and intestinal conditions when compared to non- adapted cells. The first part of the research followed a stepwise stress adaptation mechanism for six probiotics (*Bifidobacterium bifidum* LMG 11041, *B. longum* LMG 13197, *B. longum* Bb46, *Lactobacillus acidophilus* La14 150B, *L. fermentum* and *L. plantarum*). The results obtained show that the stability of the probiotic cells improves when the cells are further adapted to more stress factors. After the probiotics were exposed to stress factors, the tolerance of these probiotics towards acid and bile was investigated. These are the stress factors the probiotics encounter through their GIT following their consumption. The acid and bile tolerances of the stress exposed cells were higher than those of the cells that were not exposed to stress factors. After sequential exposure of the cells to the simulated gastric and intestinal conditions, viability of the three Lactobacilli cells and *B. bifidum* LMG 11041 were higher than their non- adapted counterparts. The bile salt hydrolase (BSH) activity and the antibiotic profiles of the probiotics remained unchanged. From these results it was evident that multi- stress pre- adaptation of probiotics increases the chances of survival for these probiotics in the gastrointestinal tract, without negatively affecting their antibiotic sensitivity profile or their ability to produce the enzyme BSH, which is one criterion used for selection of probiotics, specifically those with cholesterol lowering properties.

The observed better survival of multi- stress pre- adapted cells when exposed to simulated gastrointestinal conditions raised an interest in another study that was used in the treatment of diseases using probiotics, the use of multiple probiotics. This part of the study aimed to determine first the survival of multiple cells when exposed to acid and bile, and then investigating their ability to inhibit growth of enteric pathogens, specifically *Staphylococcus aureus* and *Escherichia coli*, when used individually as single- or multiple- stress adapted cells, combinations of multi-stress adapted cells and comparing them to a combination of the non- adapted cells. A cocktail containing *L. plantarum*, *L. fermentum* and *B. longum* Bb46 and the one containing all the six adapted cells survived better in 2% bile and pH 2, respectively. Interestingly, for both the acid and bile tolerance studies, a cocktail containing all the six non- adapted cells was the least resistant. In the antipathogenic tests, a combination containing *L. plantarum*, *B. longum* Bb46 and *B. longum* 13197 inhibited *S. aureus* better and combination containing all the six stress adapted cells inhibited *E. coli* better. It was evident that although

the stress adapted single cells inhibited both pathogens, there was an increase in the inhibition when the stress- adapted combinations were used. In all cases the combination containing all the six non- adapted cells was the least effective of all cocktails in inhibition of *E. coli* and *S. aureus*.

The results of this study revealed that multi- stress pre- adapted probiotics survive better than the single stress adapted cells and above all, the use of non- adapted cells. This was even demonstrated in the use of combinations, where the stress adapted combinations had better results than the non- adapted combinations. This study is of importance to consumers, food industries, pharmaceutical and the probiotic industry as a whole. This study shows the increase in surviving cells after the exposure to stress factors. This information can be used in the production of different products. For an increase in the number of surviving cells, they can use the pre- adaptation technique before the production of any products. The pharmaceutical industry can also apply the mechanisms of using multi- stress pre- adapted cells for their treatment of different diseases. The pre- adaptation of probiotic cells to multiple stress factors will be beneficial to the consumer because they will be getting the adequate number of live cells when they ingest probiotic products.

INTRODUCTION

Probiotics, defined as ‘live microorganisms that when administered in adequate amounts confer a significant health benefit on the host’ (FAO, 2003), represent a new and effective alternative to traditional prophylactic and therapeutic regimes in a variety of clinical settings (Sleator and Hill, 2007). There are several criteria that microorganisms must meet to be considered a probiotic. Criteria such as surviving in a low pH environment and being capable of surviving contact with bile, adhering to intestinal epithelial cells and stabilizing intestinal microflora by having the ability to multiply fast and colonize the gastrointestinal tract either permanently or temporarily. Probiotics must be non-pathogenic to the host and they must survive in feedstuffs and in the production and packaging of the product, and they must also be able to confer beneficial effects on multiple hosts (Tomasik and Tomasik, 2003).

Probiotic bacteria are becoming increasingly popular as food cultures, in parallel with a heightened awareness of their contribution to good health (Stanton *et al.*, 2001). It has been recommended that foods containing such bacteria, referred to as probiotic functional foods, should contain at least 10^7 live microorganisms per g or per ml (Ishibashi and Shimamura, 1993, Corcoran *et al.*, 2006). At the end of their shelf life, the product need to maintain a cell viability of at least 10^6 colony forming units (cfu) per g to confer the desired health benefits (FAO/WHO, 2003). This is very important in the production of probiotic foods and supplements. To include probiotics in food products, the production process should take into consideration their presence, therefore modifying the processes to meet the probiotics requirements (Dave and Shah, 1997).

Currently, fermented milks and other dairy products represent the largest group of probiotic carriers, although a larger variety of forms are also being explored for such purposes (Mattila-Sandholm *et al.*, 2002). The history of probiotics provides an explanation as to why dairy products form the largest segment of marketed probiotics. The growth of probiotics during the production of fermented foods can lower process costs and increase the adaptation of probiotics leading to enhanced viability (Song *et al.*, 2012). The reason why dairy products are still used

as probiotics foods is that many of them are optimized to some extent for the survival of fermentation organisms (Heller, 2001).

During fermentation process, there is a decrease in the pH, leading to inhibition of probiotic organisms which is a problem that we are facing in the food production. The adaptation of the probiotic strains to different challenges that are faced during production such as the decrease in pH and increase in temperatures is therefore crucial. The challenging thing about probiotics is that they have to survive in large numbers during the production and remain genetically stable during their storage and still confer the beneficial effects (Heller, 2001; Knorr, 1998). However, these microorganisms are nutritionally demanding and are sensitive to different environments. This brings the challenge of maintaining the probiotic viability in the products where they show sufficient number of the viable cells at the time of manufacture, but do not ensure that the same number of cells will be available at any point during the shelf life (Nag and Das, 2012). Many probiotic bacteria have shown to die in the food products after exposure to low pH, oxygen and/or acid after fermentation, during refrigeration, distribution and storage of products and in the human stomach (Shah, 2007; Kailasapathy and Chin, 2000).

The definition of probiotics underlines the importance of having a sufficient number of viable microorganisms throughout the entire shelf-life of the product. With that being the case, there have been several reports showing a relatively poor survival of probiotic strains during most of the technological processes used by the food industry (Charteris *et al.*, 1998, Kailasapathy *et al.*, 2008, Gueimonde *et al.*, 2012) which results in limited use of probiotics. Moreover, in addition to technological stress, once they are ingested, probiotics must overcome biological barriers present in the GIT to reach their place of action and exert their beneficial effects. These barriers include digestive enzymes, acidic pH and bile. In order to withstand the conditions in the GIT and be able to grow and multiply and have beneficial effects these products need to be able to either tolerate or be resistant to stress factors. However most of these microorganisms are reported to be fastidious, nutritionally demanding and very sensitive to environmental conditions (Gueimonde and Sanchez, 2012). Candidate probiotic strains should thus possess traits such as resistance to gastric acidity and bile toxicity. Therefore, strains with increased stress tolerance would open new opportunities for the development of novel products. Several

strategies are under investigation to improve probiotic survival, mainly by using pre-exposure to sub-lethal stresses or selection of derivative strains by stress adaptation.

Various strategies to enhance stability, viability and functionality of probiotics and some of these strategies have been recently reviewed (Betoret *et al.*, 2011; Gueimonde *et al.*, 2012; Sanchez *et al.*, 2012). Most of the approaches that are followed to yield a stress tolerant strain include stress adaptation and cross-protection mechanisms that are usually done by pre-exposing the cultures to sub-lethal stress-treatments of starvation, heat, bile, salts or acidic pH (2, 4) (Sanchez, 2007) prior to being used. Stress response phenomenon of probiotic strains therefore has become very interesting (Schmidt *et al.*, 1999; Teixeira *et al.*, 1994; Lorca *et al.*, 1998). This is where understanding the mechanism of stress may lead to the development of cultures with improved capacity to survive and function under industrial production conditions. Successful results indicating that exposure of probiotics to these stress factors can provide protection against further hostile environmental conditions were reported (Noriega *et al.*, 2004; Champagne *et al.*, 2005; Bergley *et al.*, 2006). Teixeira *et al.* (1995) reported that heat adaptation increased the survival of *Lactobacillus bulgaricus* during heat stress. Sanchez *et al.*, (2007a) reported that pre-adaptation of *Bifidobacterium longum* to low pH increased its ability to regulate internal pH. Study by Sanchez *et al.*, (2007b) reported an increase in the tolerance of *Bifidobacterium animalis* subsp. *lactis* to bile following pre-exposure to low bile concentration; it does this by reducing the deleterious impact of bile on the cell's physiology (Sanchez *et al.*, 2007b). Sheehan *et al.*, (2007) worked on improving the gastric transit, gastrointestinal persistence of *Bifidobacterium breve* and reported an improvement in the gastric transit and intestinal persistence. These positive results indicate that stress adaptation is one mechanism that can be used to increase stability of probiotics. In these studies, the researchers pre- adapted their probiotics to single stress factors and leading to an increase in the activity and the efficacy of these strains. Taking all of that into consideration, this study aimed to extend on stress adaptation research, by investigating the effect of pre-adaptation of probiotics to multiple stress factors on their stability and antagonistic effects against selected enteric pathogens. The specific objectives are:

1. Develop multiple stress adapted probiotics through their subsequent pre-exposure to acid, bile and high temperature.

2. Determine stability and survival of acid-bile-temperature adapted probiotics when they are consequently exposed to these individual stress factors.
3. Investigate the effect of acid- bile- temperature pre-adaptation of probiotics on their stability under simulated gastrointestinal conditions, and on their bile salt hydrolase activity (BSH) and antibiotic sensitivity profiles.
4. Compare antagonistic effects of individual and different cocktails of multi-stress adapted probiotics, as well as their non-stress adapted counterparts against *Escherichia coli* and *Staphylococcus aureus*

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Chapter 1

Review of Literature

1.1.The gastrointestinal tract (GIT)

The GIT of human beings consist of hollow muscular tube that begins in the mouth and extends to the anus including the pharynx, oesophagus, stomach, small intestine and the large intestine (Thibodeau and Patton, 2002). This tube is a home to a vast collection of microorganisms ranging from Eubacteria, Archaea, bacteria, yeast and filamentous fungi, which are collectively referred to as the GIT microbiota (Finegold *et al.*, 1974; Miller and Wolin, 1986; Walter, 2008). This GIT microbiota consists of a diversity of microorganisms, both beneficial and harmful microorganisms (Berg, 1996) which exists together. The microbial diversity is generated through the development of the complex food webs where the product of one microbe becomes the substrate of the other (Day *et al.*, 2003; Ley *et al.*, 2006).

Colonization of microbes in the GIT usually happens during early life (Favier *et al.*, 2002). During colonization, all parts of the GIT become colonized by well- adapted microbes, many of which are acquired from the mother (Ley *et al.*, 2006) either during birth (Favier *et al.*, 2002) or through breast feeding (Fanaro *et al.*, 2003). Colonization of the GIT by different microorganisms is also due to natural selection, where the emergences of ecological specialists that are highly adapted to the niches are favoured (Kassen and Rainey, 2004). Different microorganisms colonize different parts of the GIT for different reasons. Microorganisms such as *Lactobacillus*, *Bifidobacteria*, *Bacteroides*, *Escherichia coli* (Zoetendal *et al.*, 1998; Tannock *et al.*, 2000; Vanhoutte *et al.*, 2004; Scanlan *et al.*, 2006) colonize different parts of the GIT. Different conditions in the GIT play a role in determining which microorganisms can colonize each part; it is due to these different conditions that competition therefore determines the population level in different specific parts (Adlerberth, 1999). The colonization of the GIT by different microorganisms have been intensively studied. In the next subsection, we discuss different microorganisms that colonize the GIT and the factors affecting them in the GIT.

1.1.1. GIT microbiota and factors affecting them in the GIT

1.1.1.1. GIT microbiota

There has been an increase in interest of GIT microbiota, the relationship they have with each other and the effects they have on the human host. We have already mentioned that there are both beneficial and harmful microorganisms present in the GIT, the main function for this is

to prevent microbial dysbiosis (the microbial imbalance) (Percival, 1997). Figure 1.1 shows the distribution of the different microorganisms in the different parts of the GIT. Microbial numbers are restricted in the stomach and the first two thirds of stomach bowel. This is because of the low pH (pH 2) of the stomach contents, toxicity of the bile salts and the relatively swift flow of the digesta through the stomach (Tannock, 1995; Guarner and Malagelada, 2003). Due to low acidity, microorganisms surviving in low acid environment (acidophiles) such as *Lactobacillus* and *Candida* therefore dominate this part of the GIT. The duodenum, proximal ileum and the jejunum houses *Streptococcus* and *Lactobacillus*. The distal ileum, which is the final section of the small intestines usually has a pH between 7 and 8, therefore has a high number of colonisation ($>10^8$) because these pH is favourable to the growth of many microorganisms (Macfarlane and Macfarlane, 2004). This part of the GIT houses microorganisms such as *Clostridium* and *Streptococcus*. The colon, last part of the digestive system, also has a near neutral pH, which results in diverse bacterial cells (10^{11} - 10^{12} cells/g faeces) (Finegold *et al.*, 1983; Simon and Gorbach, 1984; Cummings *et al.*, 1993). *Bacteroides* and *Bifidobacterium* are found inhabiting this part of the human GIT. Considering the fact that there are different microorganisms in different parts of the GIT, in the next section we look into factors that affects the growth and survival of microorganisms in the GIT.

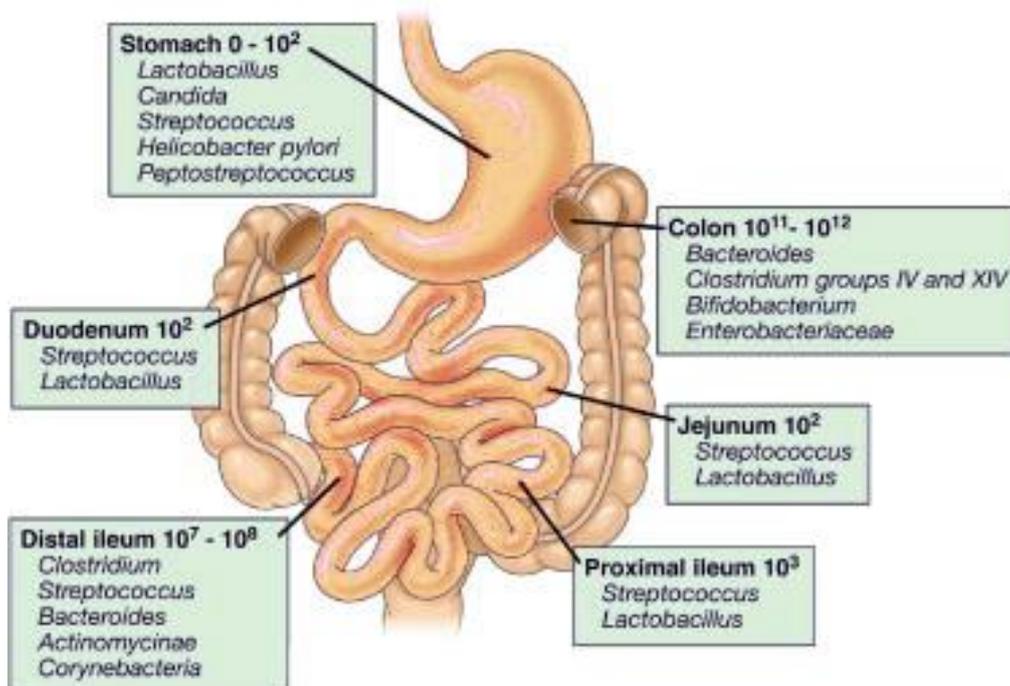


Figure 1.1: The distribution of microorganisms throughout the human GIT (Sartor, 2008)

1.1.1.2. Factors affecting GIT microbiota

The colonization of the human GIT by the different microorganisms is not due to the physicochemical conditions alone, it is also dependent on the type of sugars present. *Lactobacillus* found in abundance in the stomach due to the lower pH break down the simple sugars found in the upper GIT (Vaughan *et al.*, 2005). Complex sugars are found in lower parts of the GIT, where *Bifidobacterium* spp., which ferment complex sugars is found (Ryan *et al.*, 2006). Although some strains of lactic acid bacteria (LAB) can colonize the GIT of the host, some of these bacteria that are ingested from the food are in most cases lost from the GIT within few days after the intake (Lidbeck and Nord, 1993). This loss can be attributed to the fact that some of these microorganisms are not tolerant to the GIT conditions. Lactic acid bacteria and Bifidobacteria strains, are indigenous inhabitants of the digestive tract and therefore, possess the ability to survive in this environment (Durand, 1982; van Nevel and Demeyer, 1988). Despite the conditions in the digestive tract, there are food types that are known to suppress growth of LAB, for example, Ringo *et al.* (1997) reported that linoleic acid inhibits the population levels of *Lactobacillus* spp. This study was further supported by Ringo and Gatesoupe where they showed that dietary fatty acids affect the attachment sites possibly by modifying fatty acid composition of intestinal wall thereby affecting the inhabitation by other microorganisms. Indigenous microorganisms (indigenous inhabitants) grow in the presence of different GIT conditions, low pH and they also are not suppressed by food types reported above. What is it that these microorganisms possess that makes them to be resistant to those conditions? We therefore look into these microorganisms in the next section.

1.2. Probiotics

1.2.1. History and Definition

The history of the use of microorganisms for health benefits dates back to the early ages even in the Bible. Scientists such as Carre (1887), Tissier (1900), and Metchnikoff (1908) started researching the use of microorganisms in the early studies of microbiology. The claim of intake of certain dairy products containing microorganisms such as Lactobacilli as their starter cultures reducing pathogenic bacteria in the human GIT were studied by Metchnikoff (1908). Metchnikoff has showed that *Lactobacillus bulgaricus* is able to eliminate pathogenic bacteria from the intestinal microflora. He suggested that it would be possible to modify the gut flora and replace harmful microbes with useful ones. There was therefore an interest in the kind of

microorganisms that were seen as predominant in humans and therefore administering those microorganisms to humans. *Bifidobacteria*, as it was seen to be predominant in the GIT of breast fed infants, was administered to infants that were suffering from diarrhoea to supersede the putrefactive bacteria that cause the disease (Tissier, 1900). Even though this was confusing, further studies were done by other scientists to understand this study. Regter *et al.* (1921, 1935), Nissle (1916) and Koppeloff (1926) looked into this study and showed that *Lactobacillus acidophilus* can survive in the human gut. In this regard, these microorganisms can therefore be ingested to benefit the host.

The term ‘probiotics’ was first used in 1965. Lilly and Stillwell (1965) used it to describe the substances that were secreted by one microorganism, which stimulates the growth of another. Later Parker in (1974) defined this term as organisms and substances which contribute to the intestinal balance, which is maintaining the balance between the harmful and beneficial microflora in the gut. Fuller in 1989 improved on the definition and defined it as ‘live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance’. In 1992 Havenaar *et al.* broadened this definition and defined probiotics with respect to the host and habitat as ‘a viable mono- or mixed culture of microorganisms which are applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora’. Salminen (1996) improved and broadened the definition even further by defining them as ‘a live microbial culture or cultured dairy product which beneficially influences the health and nutrition of host’. Later in 1996, Schaafsma defined these probiotics as ‘living microorganisms which upon ingestion in certain numbers, exert health effects beyond inherent basic nutrition’ which is very close with the explanation that we are using now which defines probiotics as ‘live microorganisms which when administered in adequate numbers confer health benefit to the host’ (FAO/WHO, 2001).

1.2.2. Probiotic microorganisms

Different strains of bacteria are used as probiotics because they exert different beneficial effects by producing different enzymes; and they have specific capabilities even when used on one host species (Ouwehand *et al.*, 1999). A number of microorganisms are studied and used as

probiotics, strains from *Streptococcus*, *Enterococcus*, *Pediococci*, *Weissella* and *Lactobacillus* (Kurzak *et al.* 1998), but most predominately, LAB and Bifidobacteria spp. are used.

1.2.2.1. Lactic acid bacteria (LAB)

There are different species in this group of bacteria, which are differentiated by characteristics such as type of fermentation, types of cell shapes and their arrangement (Anal and Harjinder, 2007). These species have also adapted to grow under widely different environmental conditions and are therefore widespread in nature (Ringo and Gattessoupe, 1998). They are characterized as Gram positive, non- spore forming (Renault, 2002), usually non- motile bacteria that produces lactic acid as a sole (homolactic) or major (heterolactic) product of fermentative metabolism (Ringo and Gattessoupe, 1998). Members of this group contain both rods (*Lactobacilli* and *Cornobacteria*) and cocci (*Streptococci*) (Stiles and Holzapfel, 1997). Strains of probiotics cultures in this group usually belong to species *Streptococcus*, *Enterococcus*, *Lactobacillus*, *Aerococcus*, *Cornobacterium*, *Leucosostoc*, *Lactococcus* and *Pediococcus* (Adams and Nicolaides, 1997). These LABs are known to be nutritionally fastidious, requiring carbohydrates, amino acids, peptides, nucleic acid derivatives and vitamins for growth (Schleifer *et al.*, 1995, Klein *et al.*, 1998).

Lactic acid bacteria (LAB) have a long history of use by man, either for production of food or food preservation (Mckay and Baldwin, 1990). Due to their use as starter cultures and preservatives, they are also found in milk and dairy products (Sharp, 1981). Members of the *Lactococcus* and *Lactobacillus* are generally “regarded as safe” (GRAS) status (Ashraf and Shah, 2011). This means that these microorganisms can be added into food products, that is, they can also be referred to as food additives. They are widely used as starter cultures in fermented foods (Gibbs, 1987). For example *Lactobacillus delbruekii* subsp. *bulgaricus* and *Streptococcus thermophilus* are used as starter cultures in the production of yoghurt. They are indigenously found in the GIT of various endothermic animals (Tannock *et al.*, 1982; Finegold *et al.*, 1983; Tannock, 1988). Some sea food products also contain probiotics (Maugun and Novel, 1994) and they are also found on some plant surfaces (Keddie, 1959). This group of bacteria are usually found in habitats that are rich in nutrients and are therefore found making part of the microflora in the mouth, intestine and vaginas of mammals (Whittenbury, 1964).

Microorganisms belonging to this group of bacteria are commercially used as probiotics because they are known to acquire an ability to inhibit pathogenic microorganisms, thus preventing diseases and food spoilage and therefore production companies are interested in them. They are also intrinsically beneficial to the human health as they are said to have positive effects on well-being of consumers (Adams, 1999). They are resistant to low pH and/ or bile, they have resistance against antibiotics and also grow at defined temperatures (Fuller, 1989). This means that this group of bacteria will be able to grow in the most adverse conditions, and will therefore reach the colon where metabolism takes place to add the beneficial health effects.

There are currently a number of bacteria belonging to this group that are used as probiotics, *L. delbreuckii* ssp. *bulgaricus*, *L. acidophilus*, *L. casei*, *L. fermentum*, *L. plantarum*, *L. brevis*, *L. lactis* and *L. reuteri*. They have met the criteria of probiotics and they also have nutritional and therapeutic effects. In addition to that, the LAB have other beneficial effects, such as the ability to utilize lactose, bacteriocins production and the ability to inhibit pathogens (Rattanachaiakunsoon and Phumkhachorn, 2010). These probiotics are therefore used differently for different purposes. A study by Majamaai *et al.*, (1995) that tested the ability of *Lactobacillus* GG to shorten the duration of rotavirus induced diarrhoea gave positive results. These probiotics can be used alone (single strains) and they can also be used in combination, depending on the kind of disease that they are being used for.

1.2.2.2. Bifidobacteria

Lactic acid bacteria have been used and studied for a longer time because of their ability to survive in the gastric and intestinal conditions; there has however been an escalating interest in the use of Bifidobacteria strains. They are major constituents of the microbiota that colonize the intestinal tract of animals and humans (Fanaro *et al.*, 2003; Sghir *et al.*, 2000). Bifidobacteria are believed to play an important role in the maintenance of the gut health and well-being by modulating the intestinal microbial balance as well as the immunology and the physiology of the host (Ouwehand *et al.*, 2002). The effects of these group of bacteria are clinically relevant as observed in prevention or alleviation of infectious diarrhoea, protection from carcinogenic activity of the intestinal microbiota, and improvement of inflammatory

bowel conditions (O'May and Macfarlane, 2005; Picard *et al.*, 2005). They are therefore safe for consumption and have since been studied for their use as probiotics.

Bifidobacteria spp. are rod shaped, with high GC, Gram-positive, non-spore-forming, and non-motile and catalase- negative, non- gas producing anaerobic bacteria belonging to the phylum of Actinobacteria (Ishibashi *et al.*, 1997). They are able to ferment glucose to lactic and acetic acids via a metabolic pathway that is characterized by the presence of the enzyme fructose-6-phosphate phosphoketolase (F6PPK) (Ballongue, 2004; Gomes and Malcata, 1999), making them unique when compared to other microbes. They were first isolated in 1900 by Tisser, who described them as pleomorphic rods of different shapes, including curved, short and bifurcated Y shapes, and initially classified as *Bacillus bifidus* communis. They were renamed *Lactobacillus bifidus* before De Vries and Stouthamer in (1967) suggested that they should be reclassified as a distinct genus (*Bifidobacterium*) because of the presence of F6PPK and the simultaneous absence of glucose-6-phosphatase dehydrogenase and aldolase, i.e. the two enzymes present in Lactobacilli (Ballongue, 2004; Cheikhyoussef *et al.*, 2008; Ishibashi *et al.*, 1997). They are an important group of human gut commensal bacteria, accounting for around 3–7% of the microbiota in adults and, according to some reports, up to 91% in new-borns (Ballongue, 2004; Cheikhyoussef *et al.*, 2009). Some strains of *Bifidobacterium* possess traits that have resulted in them being used as probiotics. Some of those *Bifidobacteria* spp. includes *Bifidobacterium adolescentis*, *B. animalis*, *B. bifidum*, *B. infantis*, *B. longum* and *B. thermophilum*.

Bifidobacterium bifidum is tolerant to the acidity of a model GIT system, with only a 20% decrease in numbers as the pH decreased from 5.0 to 1.8 over an 80 min period (Marteau *et al.*, 1997). Xiao *et al.*, (2006) studied the effects of *B. longum* BB536, although not statistically significant differences were detected, nasal symptoms such as itching and blockage, as well as throat symptoms tended to be relieved with the BB536 yoghurt. In an earlier study by Xiao *et al.* (2003) they showed that intake of *Bifidobacterium* supplemented milk showed a significant lowering of the serum concentration of the total cholesterol. The study showed the use of probiotics with no side effects involved. The use of these probiotic cultures is therefore favoured compared to antibiotics treatments. The findings highlight that indeed probiotics cultures have health impacts on people who continuously ingest them.

1.2.3. Health benefits of Probiotics

The use of probiotics in humans has escalated due to their health and clinical benefits. There has been studies conducted to document the effects of probiotics and many scientists came up with different results. Table 1.1, adapted from Ray (1996), shows the health benefits that are associated with LAB, indicating what the bacteria can combat, reduce or stimulate. The ability of these microorganisms to control infections in the intestine caused by the enteric pathogens cannot be underestimated.

Table 1.1: Health benefits associated with lactic acid bacteria (Ray, 1996)

The bacteria can combat	<ul style="list-style-type: none"> • Growth of indigenous microflora in the intestine • Control infections in the intestine by enteric pathogens • Control infections in the urinary tract • Lactose- intolerance
The bacteria can reduce	<ul style="list-style-type: none"> • Cancer/ tumor in colon and other organs • Serum cholesterol and cardiac heart disease
The bacteria can stimulate	<ul style="list-style-type: none"> • Immune system • Bowel movement

Probiotics have been used in clinical practices to prevent and treat diseases like diarrhoeal illnesses, irritable bowel syndrome (IBS) and rotaviruses. Guarino *et al.*, (1997) conducted a study using 100 children who were admitted to hospital with acute diarrhoea. The results of this study their results showed a reduced duration of diarrhoea in children who received *Lactobacillus GG* for three days compared with controls. In another study, Trevis capsules, containing *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Lactobacillus bulgaricus* and

Streptococcus thermophilus were given to 195 Danish tourists in Egypt and these capsules resulted in a protection rate of 39% against diarrhoea (Black and Andersen, 1989). Probiotics are now being used in the alleviation of antibiotic related and clostridial diarrhoea, with varying results. Gotz (1997) demonstrated a reduction in ampicillin induced diarrhoea in 36 patients who received Lactinex, a preparation containing *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*, as compared with controls (who did not receive Lactinex). Black *et al.* (1991) reported that the use of *B. longum* reduced the incidence of ampicillin associated diarrhoea and the time required for recolonization of the intestine. There is evidence that suggests that probiotics may have a role in the management of patients with irritable bowel syndrome (IBS) but more research is required to reveal the exact role of these substances in clinical practice (McNaught and MacFie, 2001). Feeding yoghurt containing *B. longum* during erythromycin treatment has been reported to reduce the time required to recover from rotavirus diarrhoea (Duffy *et al.*, 1993) confirming the health benefits of probiotics. Successful use of Bifidobacteria as a prophylactic against intestinal disorders in general depends on their ability to survive under gastrointestinal conditions, tolerate antibiotic treatment, and compete with, suppress, and eliminate intestinal pathogens (Kheadr *et al.*, 2007). In addition to the general health benefits mentioned earlier, there are other benefits explained later.

1.2.3.1. Alleviation of gastrointestinal disorders

There have been many therapeutic improvements made in fields such as those of antibiotics, but despite that, their negative effects in the GI infections keep increasing, and thus posing major clinical problems (Rolfe, 2000). Most of these clinical problems are a result of microorganisms becoming resistant to antibiotics, making the use of these antibiotics questionable. Probiotics, are usually the target in the intestinal disorders in which specific factors (such as antibiotics, medication, diet or surgery) disrupt the normal flora of the gastrointestinal tract, making the host animal susceptible to disease (Rolfe, 2000).

Some diarrhoeal diseases such as antibiotic- associated diarrhoea (ADD), travellers and rotavirus diarrhoea can be treated with probiotics. One of the well-known symptoms of antibiotic resistance is antibiotic associated diarrhoea, which is said to be a result of antimicrobial therapy (Bartlett, 1992) where pathogenesis is undoubtedly related to the quantitative and qualitative changes in the intestinal microbiota (Nord *et al.*, 1986). A number of probiotics have been used for the prevention and cure of antibiotic- associated diarrhoea. Probiotics such as *Saccharomyces*, *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* with *S.*

boulardii, *E. faecium* and *Lactobacillus* are shown to be clinically effective in preventing antibiotic-associated diarrhoea (Rolfe, 2000). Traveller's diarrhoea as its name states is found in people travelling to foreign countries. Various pathogens that are known to cause this disease, with enterotoxigenic *E. coli* being the most common known pathogen (Rolfe, 2000). Probiotics that have been tested for the prevention of traveller's diarrhoea include *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and *Saccharomyces* (Hilton *et al.*, 1997, Oksanen *et al.*, 1990, Scarpignato and Rampal 1995). Rotaviruses are the main causes of rotavirus diarrhoea that cause infant morbidity and mortality, particularly in developing countries (Majamaa *et al.*, 1995, Middleton *et al.*, 1977). This kind of diarrhoea was usually treated by oral hydration. The use of probiotics for this disease has also been studied. Studies by Isolauri *et al.*, (1994), Kaila *et al.*, (1992) and Majamaa *et al.*, (1995) all showed a promise of treatment using *Lactobacillus*, as it shortened the duration of this type of diarrhoea in children (Isolauri *et al.*, 1991).

1.2.3.1.1. Probiotic effects on irritable bowel syndrome

Irritable bowel syndrome (IBS) is one of the most common gastrointestinal (GI) disorders, and approximately 5–20% of the population is estimated to suffer from it (Drossman *et al.*, 2002; Hillilä and Färkkilä, 2004). It is reported to be caused by the imbalance in the microbiota and thus contribute to GI symptoms via colonic fermentation resulting in the increased formation of gas and an abnormal pattern of short chain fatty acids (King *et al.*, 1998; Treem *et al.*, 1996). In the treatment of IBS, the safety of the therapy is of particularly important, and hence probiotics may be a relevant option (Kajander *et al.*, 2005). The effect of probiotics in IBS is, however, unclear (Madden and Hunter, 2002). In order for the effects to be understood studies were conducted, some of which reported the efficacy of the probiotics (O'Mahony *et al.*, 2005; Nobaek *et al.*, 2000; Brigidi *et al.*, 2001) while other studies claimed that they were not effective (O'Sullivan and O'Morain, 2000, Sen *et al.*, 2002). Symptoms of IBS are very unstable and heterogeneous, therefore most studies towards this have been for a very short time. One study showed that feeding *B. infantis* 35624 alleviates the symptoms of IBS (O'Mahony *et al.*, 2005). This study shows the beneficial effects of the probiotics towards IBS.

1.2.3.2. Antimutagenic and anticarcinogenic effects

The other health effects that the probiotics have are the antimutagenic and the anticarcinogenic effects. The intestinal microbiota produces enzymes such as glycosidase, β -glucuronidase, azoreductase and nitroreductase (Goldin *et al.* 1980, Goldin 1990, Ling *et al.* 1994, Marteau *et*

al. 1990). These enzymes influence carcinogenesis by transforming pre-carcinogens into active carcinogens (Rolfe, 2000), resulting in tumour cells. There have been a number of studies that were carried out to investigate the antimutagenic and the anticarcinogenic effects of probiotics. Results from such studies showed that probiotics inhibit bacteria responsible for conversion of pre-carcinogens into the carcinogens, therefore, they inhibit tumour cell formation and also bind to and/or inactivate the carcinogens (Rowland and Grasso 1975, Orrhage *et al.*, 1994, Rolfe, 2000).

1.2.4. Antimicrobial activity of probiotics

The other beneficial health effect that the probiotics have, is their ability to produce different enzymes in order to combat the pathogens' survival. Due to different diseases being treated with probiotics, one needs to understand the disease first before they can start treating it. The composition of currently-used probiotics varies from those containing a mixture of many strains to those containing one strain only, but the selection of optimal strains has often been largely empirical (Ehrmann *et al.*, 2002). The effects of the probiotics when used singly and when they are used in combination are different. This usually applies when it is known if the disease being treated is homogenous or heterogenous. Heterogenous diseases will be treated with a combination of different probiotics, so that there may be different enzymes produced. This difference between the homogenous and the heterogenous diseases raises a question as to how these probiotics actually work. We discuss briefly instances where single and combination probiotics were used for treatment of diseases.

1.2.4.1. Single probiotics

Probiotics have been used for some time now, and each probiotic strain having a different effect on certain pathogens. In addition to the balancing effect on the microbiota and the immunomodulatory effects, recent studies also suggest that probiotics may influence intestinal motility. Probiotics could influence IBS symptoms by balancing the microbiota. There is some evidence of an imbalanced microbiota in IBS (Malinen *et al.*, 2005; Mättö *et al.*, 2005). *Lactobacillus paracasei* seems also to attenuate postinfective dysmotility in an animal model of IBS (Verdu *et al.*, 2004). *Lactobacillus plantarum* 299v has been shown to be effective in alleviating flatulence (Nobaek *et al.*, 2000) and in reducing abdominal pain and the total symptom score (Niedzielin *et al.*, 2001). A randomised controlled trial in constipated elderly using

Lactobacillus rhamnosus and *Propionibacterium freudenreichii* showed positive results in defecation frequency (Ouwehand *et al.*, 2002). Chmielewska and Szajewska (2010) found that *Bifidobacterium lactis* DN-173 010, *Lactobacillus casei* Shirota and *Escheria Coli* Nissle 1917, used in different randomised controlled trials in constipated men and non-pregnant women, increased the defecation frequency and improved stool consistency (Chmielewska and Szajewska, 2010).

1.2.4.2. Combination probiotics

Studies on the use of combinations of probiotics mostly focused on the treatment and prevention of specific diseases. Various microbial cultures, in different numbers have been combined, bearing in mind the number of viable counts of the probiotics before they were combined. The use of combination probiotics is very important, especially in diseases such as IBS, which is a heterogeneous and most probably multiaetiological condition, thus making the mixture more effective than the single strain (Kajander *et al.*, 2005). Although *Lactobacillus rhamnosus* GG alone was shown to be effective in treating IBS symptoms, in a study conducted by Kajander *et al.*, (2005), it was shown that when it was combined with other probiotics, it alleviated symptoms of IBS. The mixture of *Lactobacillus rhamnosus* GG, *L. rhamnosus* LC705, *Bifidobacterium breve* Bb99 and *Propionibacterium freudenreichii* ssp. *shermanii* JS was more effective in alleviating IBS symptoms. Other trials also showed that probiotic mixtures appeared to modulate the immune responses in a different way when compared to *Lactobacillus rhamnosus* GG alone (Viljanen *et al.*, 2005a; Viljanen *et al.*, 2005b; Pohjavuori *et al.*, 2004).

LGG[®]Extra, a multi-strain probiotic combination, is based on *Lactobacillus rhamnosus* GG (*Lactobacillus* GG, ATCC 53103) combined with three other strains, *L. rhamnosus* LC705 (DSM 7061), *Propionibacterium freudenreichii* subsp. *shermanii* JS (PJS, DSM 7065), and *Bifidobacterium animalis* subsp. *lactis* Bb12 (DSM 15954). This combination of probiotic cultures has been found to be effective in reducing the symptoms of intestinal discomfort, including IBS (Saxelin *et al.*, 2011). VSL#3 is one of the well-known probiotic mixtures which is a combination of *Lactobacillus* strains (*Lactobacillus casei*, *L. plantarum*, *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus*), three strains of Bifidobacteria (*Bifidobacterium longum*,

B. breve and *B. infantis*) and *Streptococcus salivarius* subsp. *thermophilus* (Chapman *et al.*, 2006). Previous studies showed that VSL #3 is effective in the treatment of IBS in both a placebo- controlled trial and in an open uncontrolled 20- day trial study (Brigidi *et al.*, 2001). The effectiveness of another mixture of probiotics, Ecologic[®]Relief (a mixture of *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W52, *Bifidobacterium longum* W108, *Lactobacillus casei* W79, *Lactobacillus plantarum* W62 and *Lactobacillus rhamnosus* W71) was tested for its effectiveness in constipated children and positive results were attained (Bekkali *et al.*, 2007). The use of probiotic mixtures is therefore more preferable than single probiotic cultures for treatment heterogeneous and multiaetiological conditions.

1.2.5. Mechanisms of action

There are still gaps when explaining how probiotics work. There are different mechanisms that have been reported as the ways that the probiotics use to protect the host from microbial imbalance. Literature shows that they have the ability to adhere to the intestinal cells in high quantities (Fuller, 1989; Collins and Gibson, 1999), they can compete for food required for fermentation by the pathogens through secretion of antimicrobial products (Fuller, 1999) and they can also stimulate immunity (Meydani and Ha, 2000). It is believed that different probiotics affect different pathogens differently; therefore specific mechanisms for specific probiotics are still to be studied. Considering that different probiotics uses different mechanisms in the prevention and treatment of the pathogens, we look at different mechanisms that have already being studied. These mechanisms have been adopted from different studies where they were reported to protect the host from intestinal diseases.

1.2.5.1. Production of inhibitory substances

The first mechanism involves production of a variety of substances that are inhibitory to both Gram-positive and Gram-negative bacteria by the probiotic. These inhibitory substances include organic acids, hydrogen peroxide and bacteriocins. Organic acids such as acetic and lactic acids, produced by probiotics, are known to have strong inhibitory effects against Gram-negative bacteria, and they have been considered the main antimicrobial compounds responsible for the inhibitory activity of probiotics against pathogens (Alakomi *et al.*, 2000). Both these acids will eventually lower the pH, making the microenvironment acidic therefore

excluding pathogens that cannot survive acidic conditions. These inhibitory substances may reduce not only the number of viable cells, but may also affect bacterial metabolism or toxin production. Probiotics are known to enhance the mucosal barrier to pathogens and antigen presentation (Walker, 2008). In a study by Mack *et al.*, (1999) it was reported that *Lactobacillus* strains have the ability to stimulate up-regulation of mucous genes in intestinal goblet cells. The activation and secretion of the mucus in the intestines was correlated with the inhibition of pathogenic *E. coli* preventing it from damaging the intestinal tract (Walker, 2008). Most probiotics especially those belonging to the group LAB, produce antibacterial peptides such as bacteriocins, these antibacterial peptides are active against different microorganisms, excluding them from the microenvironment (Nielsen *et al.*, 2010).

1.2.5.2. Blocking of adhesion sites

Ability to adhere to the intestines is one of the pre-requisite for the colonization of probiotics, and is also important for the interaction between the probiotic strains and the host (Juntunen *et al.*, 2001). Adhesion therefore is one of the criteria that is used when selecting for probiotics. Probiotics have the ability to inhibit bacteria by adhering to the adhesion sites on intestinal epithelial surfaces (Conway *et al.* 1987, Goldin *et al.* 1992, Kleeman and Klaenhammer 1982). Consequently, some probiotic strains have been chosen for their ability to adhere to epithelial cells. This becomes important when there are pathogens involved as the ability of these probiotics to adhere to the intestinal mucus blocks the adhesion of the pathogens, thereby preventing infections (Evelyn, 1996). There are secretory and surface proteins that play a role in adhesion of probiotics to the mucous (Goh and Klaenhammer, 2010). Bacteria such as *Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium bifidum* have surface proteins that play a role in blocking of adhesion sites (Kainulainen *et al.*, 2013) These proteins are also responsible in facilitating colonization of the human GIT (Candela *et al.*, 2007).

1.2.5.3. Competition for nutrients

The GIT is well known for its abundance in nutrients, therefore they are easily accessible for the microorganisms present there. One other mechanism probiotics use is their ability to compete for nutrients with pathogens, thus this favours the growth of probiotics rather than the pathogens. Competition is referred to as the mechanisms that is used by certain microorganisms

to either exclude or reduce the growth of other microorganism. This can be achieved through different mechanisms such as creation of hostile microecology, production and secretion of antimicrobial substances and competing for essential nutrients (Rolfe, 1991). This leads to a possible exclusion of the pathogens; this is called competitive exclusion by probiotics (Gatesoupe, 1999). Lactobacilli and Bifidobacteria have been shown to inhibit a broad range of pathogens, including *E. coli*, *Salmonella*, *Helicobacter pylori*, *Listeria monocytogenes* and rotavirus (Chenoll *et al.*, 2011). Probiotics belonging to Lactobacilli and Bifidobacteria genera share carbohydrate-binding specificities with some enteropathogens (Nesser *et al.*, 2000), a characteristic that enables the strains to compete with those pathogens for the specific receptor sites (Fujiwara *et al.*, 2001).

1.2.5.4. Stimulation of immunity

Among all the mechanisms that probiotics use to exclude pathogens from the system, they are also known to possess immunomodulatory effect (Bermudez-Brito *et al.*, 2012). Previous evidence suggests that stimulation of specific and nonspecific immunity may be another mechanism by which probiotics can protect host against intestinal disease (Fukushima *et al.* 1998, Kaila *et al.* 1992, Link-Amster *et al.* 1994, Malin *et al.* 1996, Perdigon *et al.* 1986, Pouwels *et al.* 1996, Saavedra *et al.* 1994). For example, oral administration of *Lactobacillus* GG during acute rotavirus diarrhoea enhances the immune response (Kaila *et al.* 1992). This may account for the shortened course of diarrhoea observed in treated patients. The underlying mechanisms of immune stimulation are not well understood, but involve specific cell wall components or cell layers. *Lactobacillus casei* increased the phagocytic activity of mice (Perdigon *et al.*, 1986), and this was achieved either by absorption of soluble antigen or by the translocation of Lactobacilli through the GIT wall into the bloodstream (Fuller, 1991)

1.3. Challenges in probiotics industry

Probiotics are first incorporated into the starting cultures of the probiotic foods and they then undergo all the production factors, the technological stress factors (Figure 1.2). The production of probiotic products, in pharmaceuticals or in food carriers involves the exposure of the products to different types of stresses that can affect the viability and the functionality of the

probiotic strains (Ruiz *et al.*, 2011). Different foods are produced in different conditions and alternatively stored in different conditions.

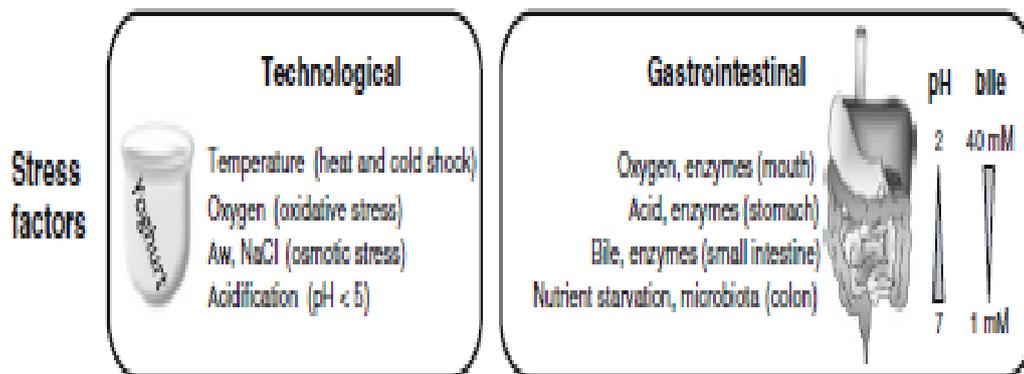


Figure 1.2: The representation of the different types of stress factors that probiotics go through (Gueimonde and Sanchez, 2012)

A major challenge that is faced in the development and production of probiotics is the survival of the cells during processing and storage. Probiotic cultures should be able to withstand food processing and storage conditions encountered during the manufacture of functional foods under industrial conditions (Knorr, 1998; Svensson, 1999). Exposure of the probiotics culture to a sub-lethal stress that include temperatures extremes, such as those encountered during freeze drying or spray drying, and acid exposure, that occur during storage in probiotic fermented food products is also responsible for decline in numbers of viable probiotics (Stanton *et al.*, 2003).

Probiotics are exposed to further detrimental conditions upon their ingestion. Their journey through the gastrointestinal tract also exposes them to unfavourable factors such as acidity, enzymes and bile acids, referred to as gastrointestinal stress factors. The main stress factors encountered under these conditions are further explained below:

1.3.1 Technological stress factors

1.3.1.1. Temperature

Temperature is one of the stress factors that need to be considered during the production and storage of the probiotic products. In most of the production processes, not only are the cells exposed to high temperatures, they are also exposed to low temperatures. With the probiotic cells being exposed to both the high and low extremes, the low temperature is known to compromise cellular integrity, mainly resulting in the reduction in membrane fluidity, increases in the rate of DNA strand breakage, stabilization of RNA and DNA secondary structures which in turn alters transcription, translation and replication (van de Guchte *et al.*, 2002). Most microorganisms are stored as freeze dried cultures, however this method decreases the viability of the microorganisms when compared to other methods such as frozen cultures. Cells are typically frozen at -196°C during freeze-drying, and then dried by sublimation under high vacuum (Santivarangkna *et al.*, 2007) compared to when they are frozen in a freezer which can be as cold as -70°C . The viability improvement of dried lactic acid starter cultures is therefore of increased importance for fermented food industries and starter culture producers (Santivarangkna *et al.*, 2008).

One of the temperature stress factor that has been studied by different researchers is spray drying. The stresses encountered during drying of the probiotic cells during production include extremes in temperatures, from very high temperatures in spray-drying, to very low-temperatures during freeze drying and storage (Mills *et al.*, 2011). During spray-drying, temperatures can be as high as 200°C (Meng *et al.*, 2008) while the exposure time for the cells is extremely short, the integrity of viable bacterial cells can be severely compromised (Mills *et al.*, 2011). There are different factors that are to influence the resistance of bacteria to heat stress and spray drying, and consequently influencing the subsequent recovery of those cells that are sub-lethally damaged (Corcoran *et al.*, 2006). Previous reports have shown that the destruction of bacteria during heat stress and spray drying can not only be ascribed to a thermal effect, but also to a non-thermal drying effect caused by the loss of bound water at the cell surface (Daemen and van der Stege, 1982). Heat stress response in *L. plantarum* has been evaluated (De Angelis *et al.* 2004; Fiocco *et al.* 2007). In their results they reported that the heat resistance of *L. plantarum* was a complex process that related to the general stress responses. Studies focusing on the pre- adaptation of probiotic cells to both high and low

temperature have been done by different researchers. In our current study we focused only on high temperature.

1.3.1.2. Oxygen

The other parameter that affects probiotics viability is oxygen. The microorganisms that are found in the GIT are either anaerobic or microaerophilic and they therefore have no effective scavenging effects against the reactive oxygen species such as oxygen ions and peroxides. *Lactobacillus* spp. and *Bifidobacterium* spp. are the most commonly used microorganisms in the probiotics research that are known to inhabit the human GIT. They are classified as microaerophilic and strictly anaerobic, respectively. Exposure of the probiotics to oxygen causes toxic oxygenic metabolites to accumulate in the cell leading to cell death from oxidative damage (Talwalkar and Kailasapathy, 2004a), a term known as oxygen toxicity. Dairy food products are the most common probiotic food products. They are the widely used as probiotic carriers to the consumers, however their production uses oxygen and hence causing concern to the final bacterial concentration. The presence of these oxygenic environments in the dairy production and storage is believed to have negative effects on the probiotic cultures (Dave and Shah, 1997a, b). For example, during the production of yoghurt, facultative anaerobic *Streptococcus thermophilus* is used to ferment the lactose in the milk to lactic acid, a product that requires plentiful supply of dissolved oxygen in the yoghurt mix (Talwalkar and Kailasapathy, 2004b), however the survival of the probiotic cultures added is of concern. The packaging that is used in the yoghurt industry is easily permeable to oxygen, therefore even during the shelf life of these products oxygen is still dissolving into the final products (Ishibashi and Shimamura, 1993; Miller *et al.*, 2002). This shows that whenever probiotic cultures are added before or after the production of dairy probiotic foods, they are indirectly exposed to oxygen. The resulting oxygen environment in yoghurts or other fermented milks is thought to induce cell death and lead to poor viability of *L. acidophilus* and *Bifidobacterium* spp. (Brunner *et al.*, 1993a; Brunner *et al.*, 1993b; Klaver *et al.*, 1993). Even though the probiotic strains are reported to be susceptible to dissolved oxygen, Meile *et al.*, (1997) reported that this phenomenon was strain dependent and in their study, they were able to isolate a moderately oxygen tolerant species of *Bifidobacterium*, *B. lactis* subsp. *nov.* from fermented milk. Dave and Shah (1997b) reported that there was a better survival of Bifidobacteria over a 35 day period in yoghurt, regardless of the oxygen content and redox potential of the yoghurt. The

survival of Bifidobacteria compared to *L. acidophilus* was better even as the dissolved oxygen of the yoghurt was seen to increase steadily over the shelf life (Miller *et al.*, 2002). The strain dependent susceptibility of the probiotics to oxygen exposure highlights that this factor remains to be considered for probiotic products.

1.3.1.3. Acidification

One of the characteristics of yoghurt is that pH decreases during storage. One of the most important studies in probiotics in food, especially yoghurt is the viability of the cells during storage. Different studies have been done to check for the effect of different starting products in the viability of probiotics during the shelf life. Stijepic *et al.* (2013) reported that when they used cow's milk and soymilk to make yoghurt, cow's milk pH dropped faster than the soymilk one (Famworth *et al.*, 2007). Rasic and Kurmann (1983) reported that the numbers of *B. bifidum* rapidly declined at $\text{pH} \leq 4.3$ when stored in cultured milk for 2 weeks. Sakai *et al.* (1987) demonstrated considerable variation in acid-tolerance for several *Bifidobacterium* species in boiled yoghurt. The change in the pH of the yoghurt is dependent of few factors, the main one being temperature. In low temperatures, the pH values mostly remain the same (Famworth *et al.*, 2007). Most of the dairy products are stored at low temperatures so there will be less changes in pH. Despite the changes in pH, most probiotic cultures are either tolerant or resistant to lower pH. This means that the change in pH will not have significant effect on the number of viable cells in probiotic food.

1.3.2. Gastrointestinal stress factors

With addition to the technological stress factors, bile, acid and oxygen are other types of stresses that probiotics cannot survive; therefore they need to be taken into consideration when production of probiotics is performed. After ingestion, probiotics meet several biological barriers, with the gastric acidity and the high bile salt concentrations in the intestine being the most important ones (Sanchez *et al.*, 2007b). The adaptation of the probiotic cells to stress before use in food processing or gastric transit can be useful for enhancing viability (Corcoran *et al.*, 2006). In order to survive and proliferate within the gastrointestinal tract, probiotics must tolerate several environmental hurdles, including the low pH of the stomach, as well as reduced water activity (a_w) and bile in the upper small intestine. Furthermore, the ability to persist in

the intestine is considered to be a valuable criterion in achieving optimal probiotic efficacy (Vaughan *et al.*, 2002). The two key stress factors encountered by probiotics during their gastrointestinal tract transit are discussed.

1.3.2.1. Gastric acidity

After food ingestion and digestion, the probiotic foods are exposed to different GIT conditions. These different conditions have different effects towards the different probiotics. The stomach has a pH of close to 2, which strongly compromises the viability of the bacteria (Ruiz *et al.*, 2011). Through digestion, the bacteria goes through the stomach, thus they are exposed to the acidic conditions there. The acid tolerance of Bifidobacteria is said to be reduced except *B. animalis* (Kheadr *et al.*, 2007; Masco, 2007). *Bifidobacterium bifidum* was found to be tolerant to the acidity of a model gastrointestinal tract system, with only a 20% decrease in numbers as the pH decreased from 5.0 to 1.8 over an 80 min period (Marteau *et al.* 1997). In a different study done by Lo *et al.*, (2004) a poor survival of Bifidobacteria in pH 2 was reported. A study by Jin *et al.*, (1998) showed that most strains of *Lactobacillus* used were tolerant to acid (pH 2).

1.3.2.2. Bile salts

Bile is composed mainly of bile salts, which are detergent-like biological compounds synthesized in the liver from cholesterol and stored as amino acid conjugates in the gall bladder. During digestion, bile is secreted into the intestine, where it plays a major role in the emulsification and absorption of fats. In addition, bile displays a strong antimicrobial activity by inducing membrane damage and causing oxidative stress to the DNA (Bernstein *et al.*, 1999). Therefore, tolerance to physiological bile salt concentrations is crucial in order for intestinal bacteria to colonize the gut. Sanchez *et al.*, (2007) used *B. animalis subsp. Lactis* IPLA 4549 and its derivative 4549dOx, to test for the adaptation and resistance to bile of this species. They concluded that adaptation and response to bile in *B. animalis subsp. lactis* involves several physiological mechanisms that are jointly dedicated to reduce the deleterious impact of bile on the cell's physiology. Bacteria such as *Lactococcus* (Yokota *et al.*, 2000), *Lactobacillus* (Elkins and Savage, 1998; Elkins and Savage, 2001, Elkins *et al.*, 2003), and

Bifidobacterium (Price *et al.*, 2006) have membranes that take part in the bile resistance mechanisms; by mediating the efflux of the bile salts out of the cells.

1.4.Strategies for improvement of probiotic stability under stressful conditions

As mentioned earlier that probiotics are susceptible to different technological and gastrointestinal stresses, there came a need to work with strains that were either resistant to or could tolerate those stresses so that they can be able to survive and reach the colon in adequate numbers. Stress adaptation is therefore one mechanisms that have been recently studied to enhance the survival and the viability of these probiotics throughout production and their shelf life. There are different studies that are being conducted on probiotics that can survive throughout all these stress factors.

1.4.1. Cell immobilization

Cell immobilization can be defined as the physical confinement of whole cells to a certain defined space while preserving their viability (Karel *et al.*, 1985) is one of the techniques that are used for stress adaptation. There are different techniques that can be used for cell immobilization, these techniques include adsorption or attachment of cells to an inert substrate, self-aggregation by flocculation or use of cross-linking agents and entrapment or encapsulation using polymers (Jen *et al.*, 1996; Kourkoutas *et al.*, 2004). Cell immobilization has been reported to be more advantageous over the use of free cells (Cassidy *et al.*, 1996; Qureshi *et al.*, 2004). In addition to that, other advantages include easy separation of the cells from the products, greater productivity due to high cell concentrations achieved, protection of cells against harsh environmental conditions, possibility of the use of packed columns, reusability of the immobilized cells and prevention of cell washout (Federici, 1993; Park and Chang, 2000; Qureshi *et al.*, 2004; Tripathi *et al.*, 2010; Wang *et al.*, 1997).

1.4.1.1.Adsorption

Adsorption of microbial cells utilizes the natural ability of cells to adhere onto solid supports to form biofilms which can exist as a single layer or can be several millimeters thick (Rezaee *et al.*, 2008; Vucurovic *et al.*, 2008). This is regarded as the easiest method of immobilization,

especially in the case where microbial cells adhere naturally to the surfaces of insoluble materials (Woodward, 1988). This method is usually used in fermentation process. Microbial cells are first inoculated into the bioreactor, then they are allowed to attach to the bioreactor. The advantages of this method include the simplicity and low cost of cell immobilization process. This process also allows for rapid ion exchange (Woodward, 1988). Since the cells have to first be allowed to attach to the bioreactors, this is a disadvantage because it is time consuming. This method also faces serious limitation of extensive cell leakage from the support, making it difficult to obtain cell free effluent for downstream processing (Kosseva, 2011; Zhu, 2007). This method also has a disadvantage that a change in the fermentation medium conditions can alter the degree of adsorption (Bar *et al.*, 1987).

1.4.1.2. Encapsulation

Another technique of cell immobilization is encapsulation or entrapment. This technique has been shown to overcome the drawbacks encountered when they used other techniques such as limited cell loading, cell leakage, low mechanical stability, contamination and mass transfer limitations (Park and Chang, 2000). Encapsulation involves either coating or entrapping microbial cells within a polymeric material to produce beads (1- 1000 μ m) which are permeable to nutrients, gases and metabolites for maintaining cell viability within the beads (Ding and Shah, 2009; John *et al.*, 2011). There are two types of encapsulation techniques; microencapsulation and macroencapsulation (John *et al.*, 2011). Microencapsulation is favoured over macroencapsulation due to lower cell viability and cell death due to hypoxic conditions found in macroencapsulation (McLoughlin, 1994; Christenson *et al.*, 1993). Probiotics are used in different fields including pharmaceutical, agricultural, nutritional and therapeutics (Aghbashlo *et al.*, 2012; Bringas-Lantigua *et al.*, 2012). Microencapsulation is favoured among other immobilization techniques because it has relatively small sized particles, larger specific area for the diffusion of nutrients, in addition to this, microencapsulation also allows for easy separation of cells and minimal cell wash out (Liu *et al.*, 2010; Tan *et al.*, 2011). The end products of microencapsulation have improved tolerance and also protected against stress factors (Park and Chang, 2000). There are different materials and techniques that are used for microbial encapsulation. The selection of materials in encapsulation is dependent on polymer types (Jen *et al.*, 1996) which is responsible for producing stable microspheres. The other advantage of using microspheres is that they are usually produced using water- soluble polymers, therefore, they have a high degree of permeability (Rathore *et al.*, 2013). For

microencapsulation to yield high viability, encapsulation must be performed under mild conditions (Rathore *et al.*, 2013). From the different studies that were done on encapsulation, the most common techniques include extrusion, coacervation, spray- drying and emulsification (de Vos *et al.*, 2010). Among all these techniques listed here, the most important factor is that the methods should be able to produce microspheres that will maintain high cell viability, easy to scale up in fermentation and cause minimal damage to cell integrity (Rathore *et al.*, 2013).

In addition to the above mentioned studies, one study that has been done extensively is the pre-adaptation of probiotics to stress then further study the effects of this mechanism by further exposing the probiotics to the simulated GIT conditions.

1.4.2. Stress adaptation by pre- exposure

The use of different strategies on probiotics strains to enhance their stability, viability and functionality has therefore been studied and reviewed in most recent probiotics work (Betoret *et al.*, 2011; Gueimonde *et al.*, 2012; Sanchez *et al.*, 2012). Before the probiotic bacteria can start to confer their physiological role in the gut, the bacteria themselves are exposed to a number of stresses, therefore, they must be able to withstand this. Most of the approaches that are followed to yield a stress tolerant strain include stress adaptation and cross-protection mechanisms that are usually done by pre- exposing the cultures to sub-lethal stress-treatments of starvation, heat, bile, salts or acidic pH (2, 4) (Sanchez, 2007) prior to being used. It is known that exposure of bacteria to stress factors (heat, bile salts, or acid pH) can provide protection against further hostile environmental conditions (Noriega *et al.*, 2004; Champagne *et al.*, 2005; Bergley *et al.*, 2006). There has been different work done in order to adapt the probiotics to different stress factors and also to test their tolerance after the pre- adaptations. Sanchez *et al.*, (2007) tested the low- pH adaptation of *Bifidobacterium longum*. They reported in their results that pre- adaptation of the *Bifidobacterium longum* to low pH increased their ability to regulate internal pH. In a different study by Sanchez *et al.*, (2007b) they adapted and later tested the tolerance of *Bifidobacterium animalis* subsp.*lactis* to bile. In their results they reported that there was an increase in the tolerance of *Bifidobacterium animalis* subsp.*lactis* to bile, it does this by reducing the deleterious impact of bile on the cell's physiology (Sanchez *et al.*, 2007b). A different study by Sheehan *et al.*, (2007) worked on improving the gastric transit, gastrointestinal persistence of *Bifidobacterium breve*. They reported that there was an

improvement in the gastric transit and intestinal persistence. Stress adaptation is one mechanism that can be used and that has been seen to have positive, significant results. In these studies, the researchers used pre-adapted their probiotics to single stress factors and there was an increase in the activity and the efficacy of these strains. Taking all of that into consideration, we will be looking closely into the stress adaptation of probiotics. We will be adapting the probiotics to multiple stress factors and looking at the effects that they have on further exposure to the same stress factors.

1.5. Conclusions

Probiotics are known for their beneficial effects in hosts, but most importantly, they must be viable in great numbers for them to confer those effects. The adaptation of probiotics to stressful conditions such as higher temperatures, lower acid concentrations and low bile salts concentrations is one of the strategies used to improve stability and viability of probiotic cultures. These studies are also done to increase the survival rate of the cultures during production and also throughout their storage, with a view to increasing their shelf life and survival during their journey through the gastrointestinal tract. The effects of the pre-exposure of the probiotic cultures to stressful conditions is therefore studied to see if there is an increase in the beneficial effects of the probiotics compared to when they are not pre-exposed to those conditions. Previous studies have indicated the potential for this strategy to improve stability and viability of probiotics when exposed to stressful conditions. However, these studies are mostly focused on preadaptation of the probiotic cultures to a single stress factor. Thus, it is not yet well known how preadaptation of the probiotics to multiple stress factors will affect their consequent stability, viability and their functional properties.

Probiotics can either be used singly or combined to form a mixture for better results. It is envisaged that since a single probiotic culture offers positive results, then a mixture of different probiotic cultures should therefore increase the effects. As the pre-exposure of the probiotic to the stressful conditions increases the survival rate of the probiotics, mixing adapted probiotic cultures should therefore enhance the benefits even further. Hence in this study, probiotic cultures were pre-adapted to multiple stress factors, and this was followed by evaluation of their stability during their subsequent exposure to similar stress factors. Furthermore, cocktails

of pre-adapted probiotic cultures were compared to those of their non-stress adapted counterparts in terms of their antibiotic sensitivity profiles, survival in simulated gastrointestinal fluids and their effects against selected enteric pathogens.

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Chapter 2

**Acid- bile-temperature pre- adaptation of
commercial *Lactobacillus* and *Bifidobacterium*
species to enhance their stress tolerance and effect of
stress adaptation on their selected functional
properties**

2.1. ABSTRACT

Probiotics encounter stressful conditions during their production, storage and through their gastrointestinal tract transit upon ingestion. Their success in delivery of health benefits depends on their ability to withstand these conditions; hence development of robust cultures is critical to the probiotic industry. We investigated the effect of multiple stress pre-exposure of probiotics on their stability under simulated gastrointestinal conditions, and on their bile salt hydrolase activity (BSH) and antibiotic sensitivity profiles. Cultures were exposed to pH 2, 2.5 and 3 for 2 h. Survivors of pH 2 were incubated in 1, 2 or 3% bile solutions for 1 h. Cells that showed growth after exposure to 2% bile solution for 2 h were finally inoculated in MRS broth and incubated at 55°C for 2 h. The probiotic cells that survived were then used as stress exposed cultures. Viability of these bacteria after subsequent exposure to simulated gastric (pH 2) and intestinal (pH 6.8) fluids was determined. The BSH activity and antibiotic sensitivity of the adapted strains was compared to those of their non-adapted counterparts. Acid and bile tolerances of most of the stress-adapted cells were higher than of the non-adapted cells. Viability of all the adapted Lactobacilli and *Bifidobacterium bifidum* LMG 11041 were higher after sequential exposure to simulated gastric and intestinal fluids, measured using bacterial counts. However, for both *B. longum* strains, viability of non-adapted cells was higher than for adapted cells after exposure to these fluids. Stress adaptation had no effect on BSH activities of *B. longum* LMG 13197, *Lactobacillus acidophilus* and *L. fermentum* but resulted in the loss of BSH activity of *B. longum* Bb46, *B. bifidum* LMG 11041 and *L. plantarum*. Most interestingly, there were no changes to antibiotic sensitivity profiles of the cultures due to stress exposure. Multi-stress pre-adaptation could be a safe mechanism for enhancing viability of probiotics under unfavourable conditions.

2.2. INTRODUCTION

The human gastrointestinal tract (GIT) is a home to a community of microorganisms, present in great richness and complexity (Ruiz *et al.*, 2011), with the vast majority of microbial communities present in our bodies (10 to 100 trillion) inhabiting our GIT (Backhed *et al.*, 2005). The gut microbiota is estimated to outnumber human somatic and germ cells by a factor of ten (Gill and Guarner, 2004). There are different bacteria, beneficial and harmful, present throughout the GIT, in the different niches from the mouth to the colon. The balance between the beneficial and pathogenic microorganisms plays a role in human health (Clemente *et al.*, 2012) as the imbalance results in dysbiosis. Health effects associated with the beneficial microflora have led to the development of probiotics products. Probiotics are defined as ‘live microorganisms which when administered in adequate amounts, confer a health benefit on the host’ (FAO/WHO, 2002). They play a role in the stabilisation of the intestinal microflora by competition against pathogens (Gibson *et al.*, 2003), reduction of lactose intolerance (de Vrese *et al.*, 2001), prevention of antibiotic-induced diarrhoea (Pochapin, 2000), prevention of colon cancer (Wollowski *et al.*, 2001) and stimulation of the immune system (Isolauri *et al.*, 1991), just to name a few. In order for a microorganism to be referred to as a probiotic; among other criteria, it must exhibit resistance to technological processes used in preparing the vehicle of probiotic delivery and produce antimicrobial substances (Gibson and Roberfroid 1995; Collins and Gibson 1999; Simmering and Blaut 2001).

Probiotics are consumed in the form of functional foods such as milk, yoghurt, yoghurt based beverages and cheese, and also as pharmaceutical preparation e.g. capsules. The probiotic cells can either be added at the beginning or end of the fermentation process. They are used as starter cultures and they therefore undergo all the stress factors during production and storage. The addition of the cells at the beginning of the process, during which they are used as starter cultures, means that the cells can be exposed to different technological stresses such as temperature, oxygen and acidification. After their storage, they are consumed and pass through the GIT where they are exposed to conditions such as low pH and high bile concentrations. These technological and gastrointestinal factors present a significant challenge to the probiotic industry. In order for probiotic cells to confer their beneficial effects to the host, they have to be present in high numbers (Song *et al.*, 2012). Many probiotic bacteria have shown to die in the food products after exposure to low pH during fermentation, oxygen during refrigeration,

distribution and storage of products, and/or acid in the human stomach (Shah, 2007; Kailasapathy and Chin, 2000).

The challenge when working with probiotics is that they have to survive in large numbers during the production and remain genetically stable during their storage and still confer the beneficial effects (Heller, 2001; Knorr, 1998; Svensson, 1999). Most of the strains cannot survive all these conditions as they do not have the traits to do so. Resistant and adapted strains are therefore more desirable to use in this case than the non- adapted strains, leading to the adaptation of strains to stress prior to being used. The adaptation of the probiotic strains to different challenges encountered during their production and administration is therefore crucial for their survival. Previous researchers have reported that the pre- exposure of the probiotic cultures to stressful conditions enhances their stability when subsequently exposed to those stressful conditions (Gueimonde and Sanchez, 2012). According to Teixeira *et al.* (1994), heat adaptation increased the survival of *L. bulgaricus* during heat stress. There is limited knowledge on the effects of pre- adaptation of probiotic cells to more than one stress factor before they are used for multi- strain preparations. Therefore, the current study aimed to enhance the stability of probiotics under simulated gastrointestinal conditions through pre- exposure to acid- bile- temperature.

2.3. MATERIALS AND METHODS

2.3.1. Bacterial cultures

Bifidobacterium bifidum LMG 11041, *B. longum* LMG 13197, *B. longum* Bb46, *Lactobacillus acidophilus* La14 150B, *L. fermentum* and *L. plantarum* glycerol stock cultures from our laboratory were used as test probiotic cultures. *Lactobacillus* spp. were sub-cultured twice in de Man Rogosa and Sharpe (MRS) broth (Merck, South Africa) and *Bifidobacterium* spp. in MRS supplemented with 0.05% v/v L- cysteine hydrochloride monohydrate (MRS- cys- HCl), followed by incubation at 37°C for 72h in anaerobic jars containing Anaerocult A gaspacks. After the final subculturing, the initial concentration of probiotic bacteria present was determined by serially diluting the cultures in ¼ strength Ringer's solution, followed by pour plating onto MRS and MRS-cys- HCl plates in triplicates, for *Lactobacillus* and *Bifidobacterium* spp., respectively. The plates were incubated anaerobically at 37° C for 72 hours. The adapted and the non- adapted cells were normalised to an optical density of 0.2 at 600nm, which is approximately equivalent to 10⁸ cfu/ ml, in the different experiments.

2.3.2. Stress adaptation of probiotics

2.3.2.1. Acid adaptation

Overnight broth cultures were harvested by centrifugation at 3000 gravitational force, g for 15 min using a Mini spin Eppendorf centrifuge. The pellet was resuspended in 1 ml of ¼ strength Ringer's solution (Merck, South Africa). Then 1ml of this culture was added to separate tubes containing 9 ml MRS broth adjusted to pH 2, 2.5 and 3 using 1M HCl. The cultures were then incubated at 37°C and 100 µl subsamples were removed from each flask at 30, 60 and 120 min and transferred to 900 µl MRS or MRS- cys- HCl broth. The suspensions were then serially diluted up to 10⁻⁷ using ¼ strength Ringer's solution. The cultures were recovered by plating them on MRS or MRS- cys- HCl agar plates in triplicates, and incubated anaerobically in anaerobic jars with Anaerocult A gaspacks and Anaerotest strips (Merck, South Africa) for 72 hours. The cells that survived incubation for 120 min at pH 2 were taken as the acid adapted strains and were subsequently used for the bile adaptation process.

2.3.2.2. Bile adaptation

Ten millilitres of the overnight cultures of the acid- adapted strains were aseptically transferred into Falcon tubes containing 1.0, 2.0 and 3.0 % (w/v) bile solutions. The flasks were then incubated anaerobically in a shaking incubator (100 rpm) at 37°C. At 20, 40 and 60 minutes, 1 ml aliquots were harvested and added to 9 ml MRS-cys- HCl broth. The suspensions were then serially diluted up to 10^{-7} using $\frac{1}{4}$ strength Ringer's solution. The surviving cells were then recovered by growing them on MRS or MRS- cys- HCl agar plates, incubated anaerobically in anaerobic jars with Anaerocult A gaspacks and Anaerotest strips for 72 hours. The cells that survived after the exposure to 2.0% bile for 60 min were regarded as acid-bile adapted cells and were used further for the temperature adaptation.

2.3.2.3. Temperature adaptation

Overnight cultures of acid- bile adapted strains grown in MRS or MRS- cys- HCl broth at 37°C in a shaking incubator at 100 rpm were used. One millilitre of the overnight culture was added to 9 ml of fresh MRS/ MRS- cys- HCl broth and the cultures were incubated at 55°C (AccuBlock digital dry bath). One hundred microliters were withdrawn after 30, 60 and 120 minutes and added to 900 μ l MRS or MRS- cys- HCl broth. The suspensions were then serially diluted up to 10^{-7} using $\frac{1}{4}$ strength Ringer's solution. The cultures were recovered by plating in triplicate on MRS or MRC- cys- HCl agar plates in anaerobic jars with Anaerocult A gaspacks and Anaerotest strips at 37°C for 72 hours. The cultures that grew after incubation at 55°C for 120 min were used further as acid- bile- temperature adapted strains. These acid- bile- temperature adapted strains were stored in 20% glycerol (1:1) at -20°C.

2.3.3. SURVIVAL UNDER THE GIT CONDITIONS

2.3.3.1. Acid tolerance

The investigation of the tolerance of the stress adapted cells to acid was done using the method described by Brashears et al. (2003), with minor modifications. Briefly, cultures of the adapted and non- adapted cells of Lactobacilli spp. and Bifidobacterial spp. were grown in MRS or MRS- cys- HCl at 37°C overnight in a shaking incubator at 100 rpm. The cultures were sub-cultured into 10 ml of fresh MRS or MRS- cys- HCl broths adjusted to different pH values (2, 2.5 and 3) with 1M HCl followed by incubation at 37°C in a shaking incubator (100 rpm). Then 100 μ l aliquots were harvested at 60, 120 and 180 minutes, transferred into 10 ml MRS or

MRS- cys- HCl broth. The suspensions were then serially diluted up to 10^{-7} using $\frac{1}{4}$ strength Ringer's solution and 0.1ml of each dilution was pour plated onto MRS or MRS-cys- HCl plates in triplicates. The plates were incubated anaerobically in anaerobic jars with Anaerocult A gaspacks and Anaerotest strips at 37° C for 72 hours. The colonies of the plates containing 30- 300 colonies were counted.

2.3.3.2. Tolerance to bile salts

Tolerance of the probiotic cultures to bile was performed using a method by Tsai et al. (2007) with minor modifications. Briefly, overnight broth cultures of both the adapted and the non-adapted Lactobacilli spp. and Bifidobacterial spp. were harvested by centrifugation at 3000 rpm for 10 min. The pellets were washed in $\frac{1}{4}$ strength Ringer's solution and mixed by vortexing for 30 seconds. Then 100 μ l of the solution was added to MRS or MRS- cys- HCl broth adjusted to 1, 2 and 3% (w/v) bile salts and grown in a shaking incubator at 37° C with the readings taken every 30 min for 3 hours and then after 24 hours. Cultures inoculated in 0% bile were used as controls. The suspensions were then serially diluted up to 10^{-7} using $\frac{1}{4}$ strength Ringer's solution and 0.1 ml of each dilution was pour plated onto MRS or MRS-cys- HCl plates in triplicates. The plates were incubated anaerobically in anaerobic jars with Anaerocult A gaspacks and Anaerotest strips at 37° C for 72 hours. The colonies of the plates containing 30- 300 colonies were counted.

2.3.3.3. Simulated gastric and intestinal conditions

2.3.3.3.1. Preparation of simulated gastric and intestinal fluids

The simulated gastric juice was prepared by briefly suspending 3 g/l of pepsin (Merck, SA) in saline (0.5% w/v) and adjusted to pH 2.0 with 1M HCl. The simulated intestinal fluid was prepared by dissolving 6.8 g monobasic potassium phosphate (Merck, SA) into 250 ml distilled water. Then 77 ml of NaOH (0.2 N) was added and mixed. Five hundred millilitres of distilled water was then added and the solution was mixed by vortexing for 30 s. Then 10 g of pancreatin was added and mixed and the solution was adjusted to pH 6.8 with 1M NaOH or 1M HCl. The volume of the solution was then made up to 1000 ml with distilled water. The solution was the autoclaved and stored and used for the exposure of the probiotics to the gastric and intestinal conditions.

2.3.3.3.2. Exposure to gastric and intestinal conditions

The non- adapted and adapted cultures of *Lactobacilli* spp. and *Bifidobacteria* spp. were grown overnight in MRS broth and MRS- cys- HCl broth, respectively. Aliquots of 1 ml were added to 9 ml of simulated gastric fluid (pH 2) for 2 h at 37 °C. For all the probiotics, 0.1 ml from the simulated gastric fluid solution was withdrawn and added to 0.9 ml of simulated intestinal fluid (pH 6.8). The probiotics were incubated at 37°C for two hours. From the simulated intestinal fluid, after two hours, 100 µl were withdrawn from the tubes and plated in triplicates onto MRS or MRS- cys- HCl agar plates. The plates were incubated anaerobically in anaerobic jars with Anaerocult A gaspacks and Anaerotest strips at 37°C for 72 hours. The colonies of the plates containing 30- 300 colonies were counted.

2.3.3.4. Screening for bile salt hydrolase (BSH) activity

BSH screening medium was prepared by weighing MRS/ MRS- cys- HCl agar, and then adding 0.5% sodium salt of TDCA (Taurodeoxycholate) to it. The agar with the sodium salt of TDCA was added into a calculated volume of distilled water. This was autoclaved together and poured onto sterile petri dishes. Cultures of the non- adapted and adapted *Lactobacilli* spp. and *Bifidobacterial* spp. were grown on MRS/ MRS- cys- HCl overnight. Overnight cultures were streaked (using inoculating loop) onto the BSH screening agar plates in triplicates. Plates were incubated anaerobically in anaerobic jars with Anaerocult A gaspacks and Anaerotest strips at 37°C for 72 hours. The presence of precipitated bile acid around colonies (opaque halo) was considered a positive result.

2.3.3.5. Assay for sensitivity to Antibiotics

The sensitivity of the cultures to antibiotics was determined using the Mastring antibiotic discs (Davies Diagnostics, SA). Eight different antibiotics were used: chloramphenicol 25 µg, erythromycin 5 µg, fusidic acid 10 µg, oxacillin 5 µg, novobiocin 5 µg, penicillin G 1 unit, streptomycin 10 µg and tetracycline 25 µg. Cultures of the adapted and the non- adapted *Lactobacilli* spp. and *Bifidobacterial* spp. were grown in MRS or MRS- cys- HCl. The cultures (100µl) were spread onto MRS or MRS- cys- HCl plates and the antibiotic discs were placed in the middle of the plates. The plates were incubated anaerobically in anaerobic jars with Anaerocult A gaspacks and Anaerotest strips at 37°C for 72 hours in triplicates. The results were obtained by measuring the diameters of the inhibition zones for the sensitivity of the cultures to the antibiotics.

2.3.4. Statistical analysis

Statistical analysis of the difference between the adapted and the non- adapted strains was analysed by using two- way Student *t- test* from the software Statistica v10, where a p- values < 0.05 was considered to be statistically significant and p- values > 0.05 , statistically non-significant.

2.4. RESULTS AND DISCUSSION

2.4.1. Acid- bile- temperature adaptation of probiotic cultures

The use of probiotics is increasing at a very fast rate as their importance is seen throughout the world. However, their sensitivity hinders their use. The definition of probiotics highlights the importance of maintaining high number of viable microorganisms throughout the entire shelf-life of the products into which they are incorporated. These products must contain a number of viable cells shown to be efficacious, which is generally $10^6 - 10^8$ cfu/ ml or g (Charteris *et al.*, 1998). However, a number of reports indicate that there is relatively poor viability of probiotic strains during most of the technological processes used by the food industry (Gueimonde *et al.*, 2002; Dunne *et al.*, 2001); most products therefore, do not contain the required number of viable microorganisms. Poor viability of probiotics stimulated research interest into different methods to protect or improve their viability. The use of different strategies on probiotics strains to enhance their stability, viability and functionality has been studied and reviewed in most recent work on probiotics work (Betoret *et al.*, 2011; Gueimonde *et al.*, 2012). The optimization strategies based on stress adaptation and cross protection mechanisms therefore constitute an attractive option to improve performance and functionality of probiotics (Sanz, 2007). It has been reported earlier that the exposure of probiotics to sub- lethal stress for the enhancement of stress tolerance/ resistance has been found to be highly effective (Lorca and de Valdez, 2001). Taking those studies into consideration, the current study investigated the effect of successive pre-adaptation of probiotic strains to multiple stress factors corresponding to those they encounter during processing and after ingestion, specifically acid, bile and high temperature, on their (probiotics) stability when later exposed to those similar individual factors.

Performance of the six commercial strains of probiotics, Lactobacilli and Bifidobacteria during pre- adaptation to acid is shown in Figure 2.1. The six commercial probiotics were each grown at 3 different pH values and the viable cells were calculated. In pH 2 (Figure 2.1 A) the number of the surviving cells ranged from 6.58 to 7.76 log cfu/ ml. There was a viable cell reductions of 1.15, 1.2, 1.27, 1.30 and 1.4 log cfu/ ml after 120 min incubation for *L. plantarum*, *L. fermentum*, *B. bifidum* LMG 11041, *B. longum* LMG 13197 and *B. longum* Bb46, respectively. When we looked at the survival of the probiotics in pH 2.5 (Figure 2.1 B) and pH 3 (Figure 2.1 C), the viable cells ranged from 6.67 to 7.82 and 6.73 to 7.84 log cfu/ ml, respectively. In pH

2.5 after 120 min incubation viable cell reductions of 0.35, 1.03, 1.16, 1.19, 1.25 and 1.33 log cfu/ ml were recorded for *L. plantarum*, *L. fermentum*, *B. bifidum* LMG 11041, *B. longum* LMG 13197 and *B. longum* Bb46, respectively. Lastly, in pH 3 viable cells reductions of 0.31, 0.66, 1.03, 1.1, 1.14 and 0.27 log cfu/ ml were recorded for *L. plantarum*, *L. fermentum*, *B. bifidum* LMG 11041, *B. longum* LMG 13197 and *B. longum* Bb46, respectively. The best surviving cells in all the pH values were *L. acidophilus* La14 150B. *Lactobacillus acidophilus* La14 150B had final log cfu/ ml of 7.57, 7.65 and 7.69 log cfu/ ml in pH 2, 2.5 and 3, respectively. This means that through the exposure to acid there was viable cell reduction of 0.43, 0.35 and 0.31 log cfu/ ml in pH 2, 2.5 and 3, respectively. In all the pH values, the *Lactobacilli* cells were more tolerant to acid than *Bifidobacteria* cells. Through the exposure of the probiotics to these three different pH values, it was observed that indeed pH 2 was the most extreme condition. The standard pH for the acidity that the bacteria have to be able to survive in is pH 2 (Corcoran *et al.*, 2006). Taking that into consideration, the six commercial strains exposed to pH 2 for 120 minutes were taken as the acid adapted and then they were subcultured for use in bile adaptation study.

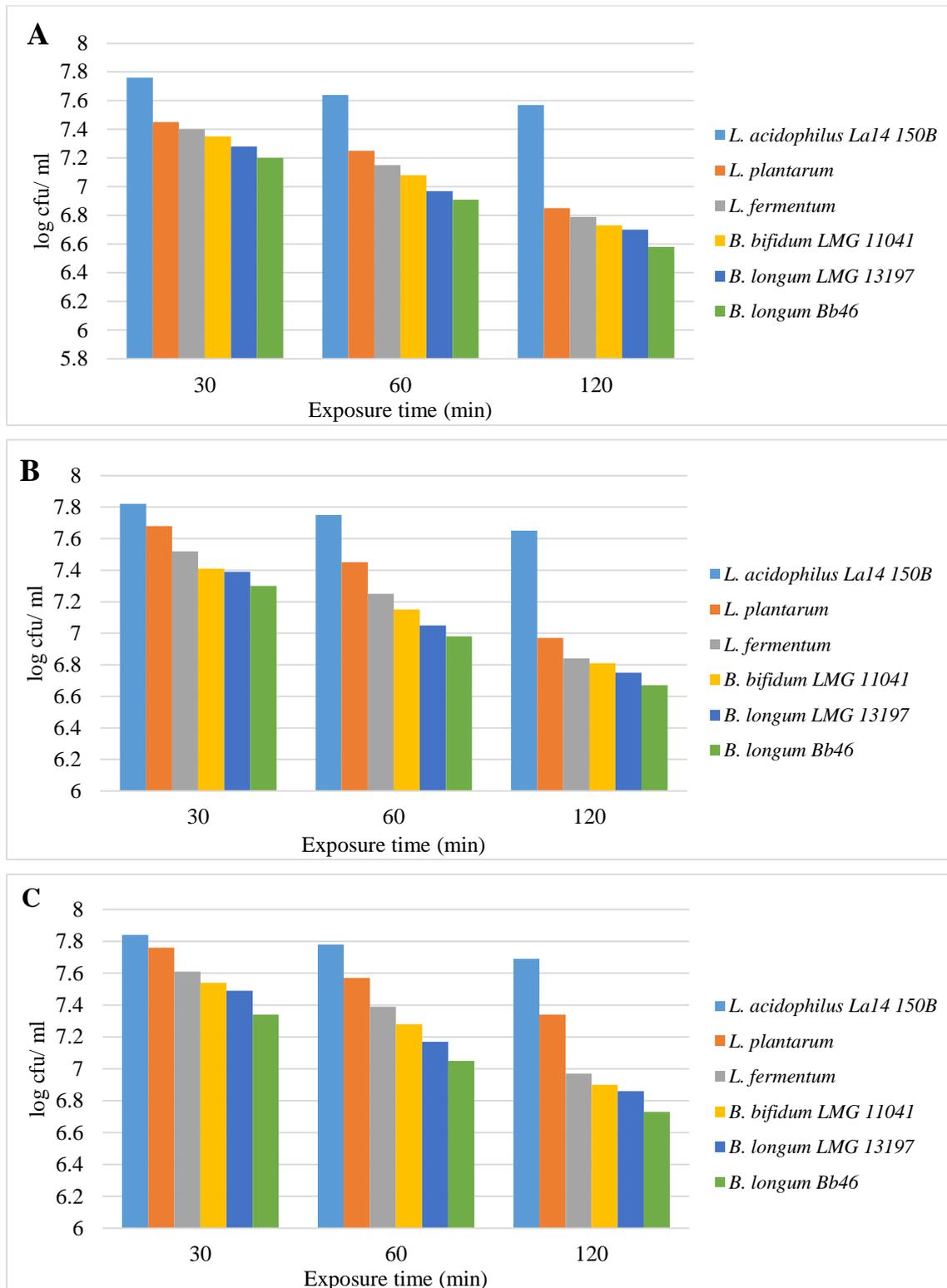


Figure 2.1: The pre- adaptation of the probiotics to acid

A- pH 2, B- pH 2.5, C- pH 3

Not only do probiotics have to be able to survive in low acid environment, they also have to be able to grow in high bile concentration for them to confer health effects on the host. Therefore,

the acid adapted cells were exposed to bile salts to check for their survival in the intestinal conditions. The number of the cells that survived at the different bile salt concentrations over time were calculated for each bacterial culture. From the initial concentration of 10^8 log cfu/ml there was a decrease in the number of surviving cells for all the probiotic cultures. In 1% bile salt concentration after 60 min of incubation (Figure 2.2A), the viable cell numbers ranged from 6.95 to 7.91 log cfu/ml. There was a viable cell reduction of 0.26, 0.35, 0.65, 0.79, 0.88 and 1.05 log cfu/ml for *L. acidophilus* La14 150B, *L. plantarum*, *L. fermentum*, *B. bifidum* LMG 11041, *B. longum* LMG 13197 and *B. longum* Bb46, respectively. In 2% bile salt concentration after 60 min of incubation, the viable cell numbers ranged from 6.72 to 7.87 log cfu/ml. There was a viable cell reduction of 0.38, 0.46, 1.11, 1.19, 1.22 and 1.29 log units for *L. acidophilus* La14 150B, *L. plantarum*, *L. fermentum*, *B. bifidum* LMG 11041, *B. longum* LMG 13197 and *B. longum* Bb46, respectively. Lastly, in 3% bile salt concentration also after 60 min of incubation the viable cell numbers ranged from 6.59 to 7.68 log cfu/ml. There was a viable cell reduction of 0.51, 0.65, 1.25, 1.26, 1.33 and 1.41 log cfu/ml for *L. acidophilus* La14 150B, *L. plantarum*, *L. fermentum*, *B. bifidum* LMG 11041, *B. longum* LMG 13197 and *B. longum* Bb46, respectively.

These results reveal that with an increase in the bile salt concentration, there is a decrease in the viable cell numbers. There was better survival of cells in 1% bile salt concentration than in 2% and 3%. The viable number in the acid adaptation process (pH 2, 120 min) ranged from 6.58 to 7.56 log cfu/ml, while for the bile adaptation (2% bile salt concentration, 60 min) ranged between 6.72 and 7.62 log cfu/ml. It was interesting and worth noting that even though there was a decrease in the viable numbers of the strains during exposure to bile salts for the cultures, reduction for the strains was lower compared to when the original strains were exposed to acid. The observed less reduction in viability is attributed to pre-exposure to acid, which increased stability of strains. The bile salt concentration of 2% represents the most extreme concentration that can be found in the human intestine during the first hour of digestion (Gotcheva *et al.*, 2002; Jin *et al.*, 1998). Taking that into consideration, cells that survived after their exposure to 2% bile concentration for 60 min were regarded as acid- bile adapted cells and were then subsequently used in high temperature adaptation experiments.

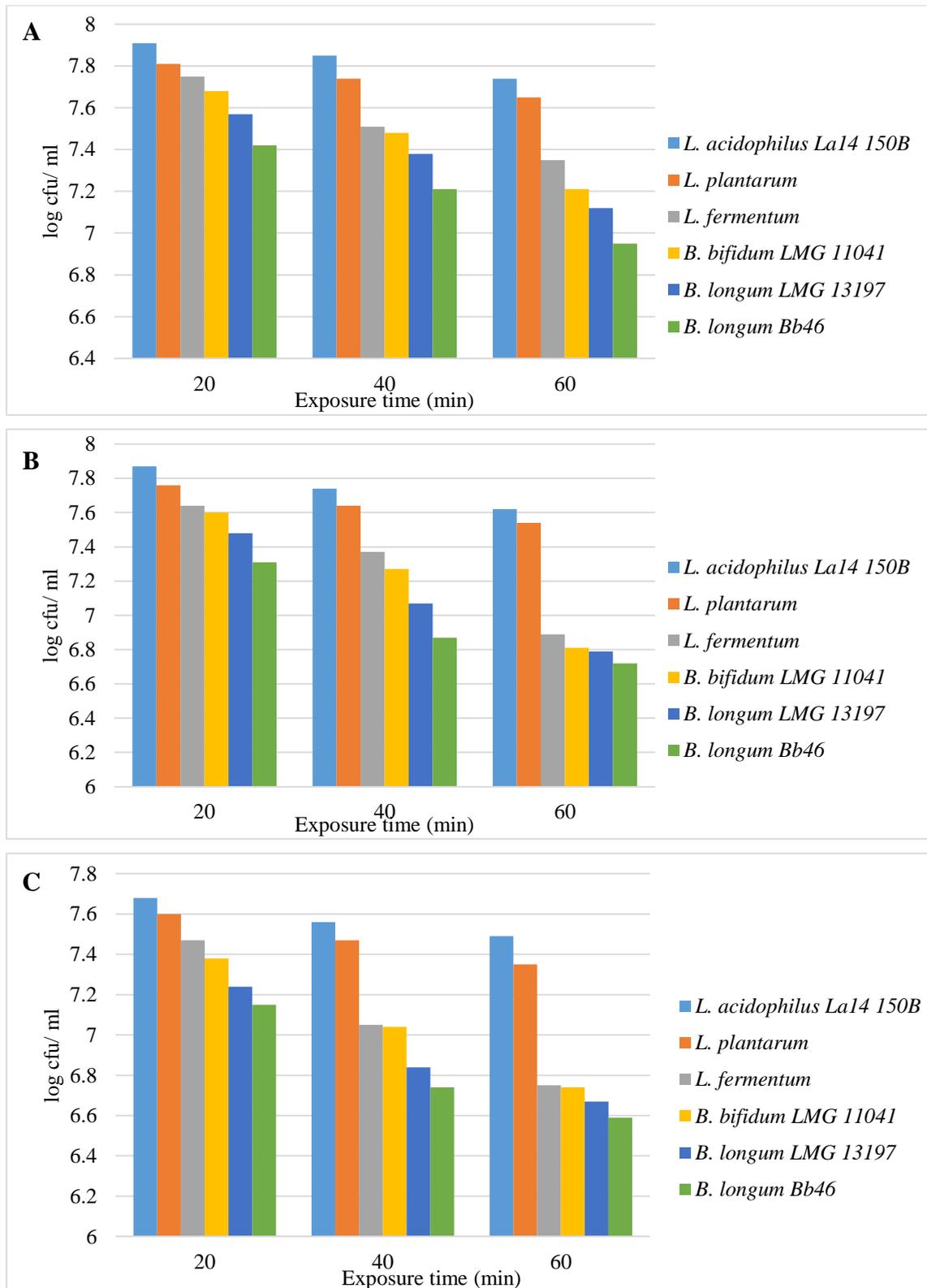


Figure 2.2: The pre- adaptation of the acid adapted probiotics to bile

A- 1% bile solution; B- 2% bile solution; C- 3% bile solution

The probiotics were then incorporated high temperature into the stress adaptation process. The acid- bile adapted cells were incubated at 55°C, this temperature was used to adapt the cells as opposed to spray drying. The viable cells after 30, 60 and 120 min incubation (Figure 2.3) were enumerated. The viable cells ranged from 6.82 to 7.94 log cfu/ ml over time. After 30 min of incubation, there was a viable cell reduction of 0.06, 0.09, 0.11, 0.16, 0.32 and 0.41 log cfu/ml for *L. acidophilus* La14 150B, *L. plantarum*, *L. fermentum*, *B. bifidum* LMG 11041, *B. longum* LMG 13197 and *B. longum* Bb46, respectively. After 120 min incubation there was viable cell decrease of 0.14, 0.24, 0.37, 0.49, 1.11 and 1.17 log cfu/ ml for *L. acidophilus* La14 150B, *L. plantarum*, *L. fermentum*, *B. bifidum* LMG 11041, *B. longum* LMG 13197 and *B. longum* Bb46, respectively, in order from the best to the least surviving strain. There was a decrease in the number of viable cells of all the probiotics over time. There was less decrease after 30 min incubation than after 120 min incubation. The *Lactobacillus* strains survived better than the *Bifidobacterium* strains throughout the whole stress adaptation process, indicating that Lactobacilli are more resistant than Bifidobacteria. This is in agreement with an earlier statement by Sanz (2007) that *Bifidobacterium* strains are highly sensitive strains when compared to *Lactobacillus* strains. Similar to what was observed during acid and bile adaptation studies, reduction in viable numbers after exposure to 55°C was the lowest for acid-bile adapted cells compared to parental cells during acid adaptation, and acid-adapted cells during bile adaptation.

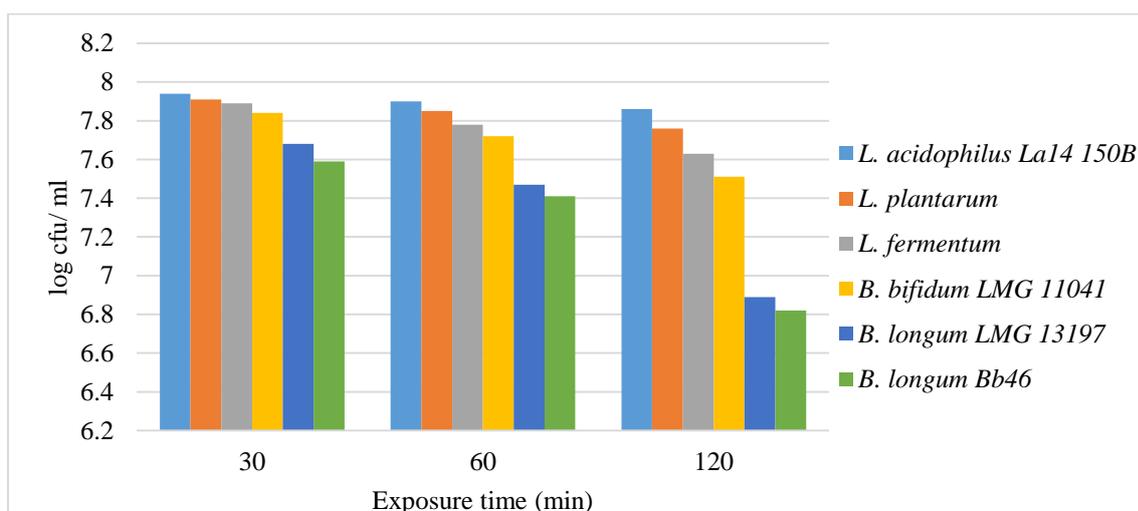


Figure 2.3: The pre- adaptation of the acid adapted probiotics to bile

Previous study to investigate the effect of different single stress factors on the survival of probiotics in the GIT concluded that the stress adaptation to either acid or bile did not show statistical relevant positive effect (Lorca *et al.*, 2002). The results found in Sumeri *et al.*, (2010) showed that when they pre- treated probiotics to temperature (50°C, 30 min) the viability reduction trend was the same as with the non- treated cells. However, further study using combined stress factors, showed that *Bifidobacterium* isolates pre- treated with acid- bile- NaCl showed improved performance when they were later exposed to acid, bile and NaCl conditions. This, therefore, indicated that pre- exposure to combined stress factors had better effects than when using single stress adapted cells. This suggested that multi- stress pre- treatment may be useful to enhance the stability and the functional properties of the probiotics (Nag and Das, 2013). It is for this reason that, in our current study, we further pre- treated the acid- adapted cells to high bile and temperature, to make the cells more robust when exposed to stress later on. It was envisaged that initial stress adaptation process will enhance survival of the probiotics when further exposed to single stress factors. Our results showed a step by step improvement of the survival of the probiotics when they were pre- treated with acid, bile and then temperature. These results therefore demonstrate that probiotic cells pre- exposed of to acid- bile- temperature were more stable than the acid adapted and acid- bile adapted cells. This is confirmation that the multiple stress pre- adapted cells are better to use when compared to the single stress adapted and the non- adapted cells.

2.4.2. The survival of the acid- bile- temperature adapted and the non- adapted cells in acid and bile

Oral probiotic strains experience severe acidic conditions in the stomach, where the pH is close to 2 (Ruiz *et al.*, 2011). After the cells pass through the acidic stomach, they are exposed to bile salts in the intestine, where the normal concentration is around 0.3%, but can range up to the extreme of 2.0% (Gotcheva *et al.*, 2002). Both these factors strongly compromises bacterial viability. Resistance of these strains to acid and bile upon ingestion is therefore crucial in the production of probiotic products (Jamalifar *et al.*, 2010). The survival of the acid- bile- temperature adapted cells in the presence of the different acidic and bile concentrations was studied and then compared to their respective non- adapted cells.

2.4.2.1. Acid Resistance

Figure 2.4 shows survival of the non- adapted and adapted cells in pH 2, 2.5 and 3 over a period of 180 min. From the results, the range of the viable cells in pH 2 was 6.43 to 7.93 and 6.64 to 7.97 log cfu/ ml for the non- adapted cells (Figure 2.4 A1) and the adapted cells (Figure 2.4 A2), respectively. There was a visible reduction of viable cells for both the non- adapted and the adapted cells over time. For the non- adapted cells there was a cell reduction of 0.31, 0.11, 0.21, 0.12, 0.27 and 0.17 log cfu/ ml more than the adapted cells for *B. bifidum* LMG 11041, *B. longum* LMG 13197, *B. longum* Bb46, *L. acidophilus* La14 150B, *L. fermentum* and *L. plantarum*, respectively.

The non- adapted and the adapted cells were further exposed to pH 2.5 and 3 to check for their acid tolerance. In pH 2.5, the viable cell numbers ranged from 6.54 to 7.97 and 6.58 to 7.99 log cfu/ ml for the non- adapted (Figure 2.4 B1) and the adapted cells (Figure 2.4 B2), respectively. After 180 min of incubation in pH 2.5, the non- adapted cells had a viable cell reduction of 0.18, 0.10, 0.04, 0.16, 0.25 and 0.10 log cfu/ ml more than the adapted cells for *B. bifidum* LMG 11041, *B. longum* LMG 13197, *B. longum* Bb46, *L. acidophilus* La14 150B, *L. fermentum* and *L. plantarum*, respectively. In pH 3 viable cell numbers ranged from 6.54 to 7.97 and 6.57 to 7.98 log cfu/ ml for the non- adapted (Figure 2.4 C1) and adapted cells (Figure 2.4 C2), respectively. The non- adapted cells had a viable cell reduction of 0.19, 0.05, 0.03, 0.15, 0.23 and 0.18 log cfu/ ml more than the adapted cells of *B. bifidum* LMG 11041, *B. longum* LMG 13197, *B. longum* Bb46, *L. acidophilus* La14 150B, *L. fermentum* and *L. plantarum*, respectively.

There was a higher cell reduction in pH 2 than there was in pH 2.5 and pH 3. This shows that in acidic environments, the probiotic cells are more sensitive. However, the stress adapted cells survived well than the non- adapted cells in all pH values. Survival of all the acid- bile- temperature adapted cells in acid was significantly higher than the non- adapted, with p- values of 0.0257, 0.0448, 0.0464, 0.0018, 0.0452 and 0.0431 for *B. bifidum* LMG 11041, *B. longum* LMG 13197, *B. longum* Bb46, *L. fermentum*, *L. plantarum* and *L. acidophilus* La14 150B, respectively. In this study there were higher counts for stress adapted strains than the non-

adapted cells confirm that pre- adaptation to stress does provide protection to the cells enhancing their growth.

Other studies have reported the effect of pre- adaptation of various probiotic to different stress factors to enhance their growth when they are further exposed to the stress factors. Previous study by Park et al. (1995) reported that pre-adaptation of *B. breve* cells to pH 5.2 protected them against subsequent lethal pH values of 2.0–5.0. Lorca and de Valdez (2001) reported that *L. acidophilus* pre-exposed to acid (pH 3, 60 min) survived better than the non- acid treated cells. Similarly, Betoret *et al.* (2011) reported that pre- adaptation of *L. acidophilus* to acid stress (pH 5.0, 60 min) was found to increase resistance against subsequent exposure to pH 3. Results obtained in the current study are therefore in agreement with these previous reports. However, contrary to these studies in which the probiotics were pre-adapted to a single stress factor, in this study, the probiotics were pre-adapted to multiple stress factors, namely, acid, -bile and high- temperature to further enhance their growth. Desmond *et al.* (2002) reported that *B. longum* is acid- sensitive and that its acid- adaptation would not enhance its acid tolerance. It was interesting to observe that in our study, after pre- adaptation of *B. longum* cells to multiple stress factors, cells grew in the acidic environment. This suggests that pre-adaptation to multiple stress increases stability of even the sensitive strains better than single stress adaptation. The survival of the adapted cells in high numbers that were observed in our current study indicate that the cells could survive in the acidic stomach, therefore, reaching the areas of beneficial activity (Gotcheva *et al.*, 2002) in adequate numbers which is in accordance with the criterion that cells must be able to survive in large numbers.

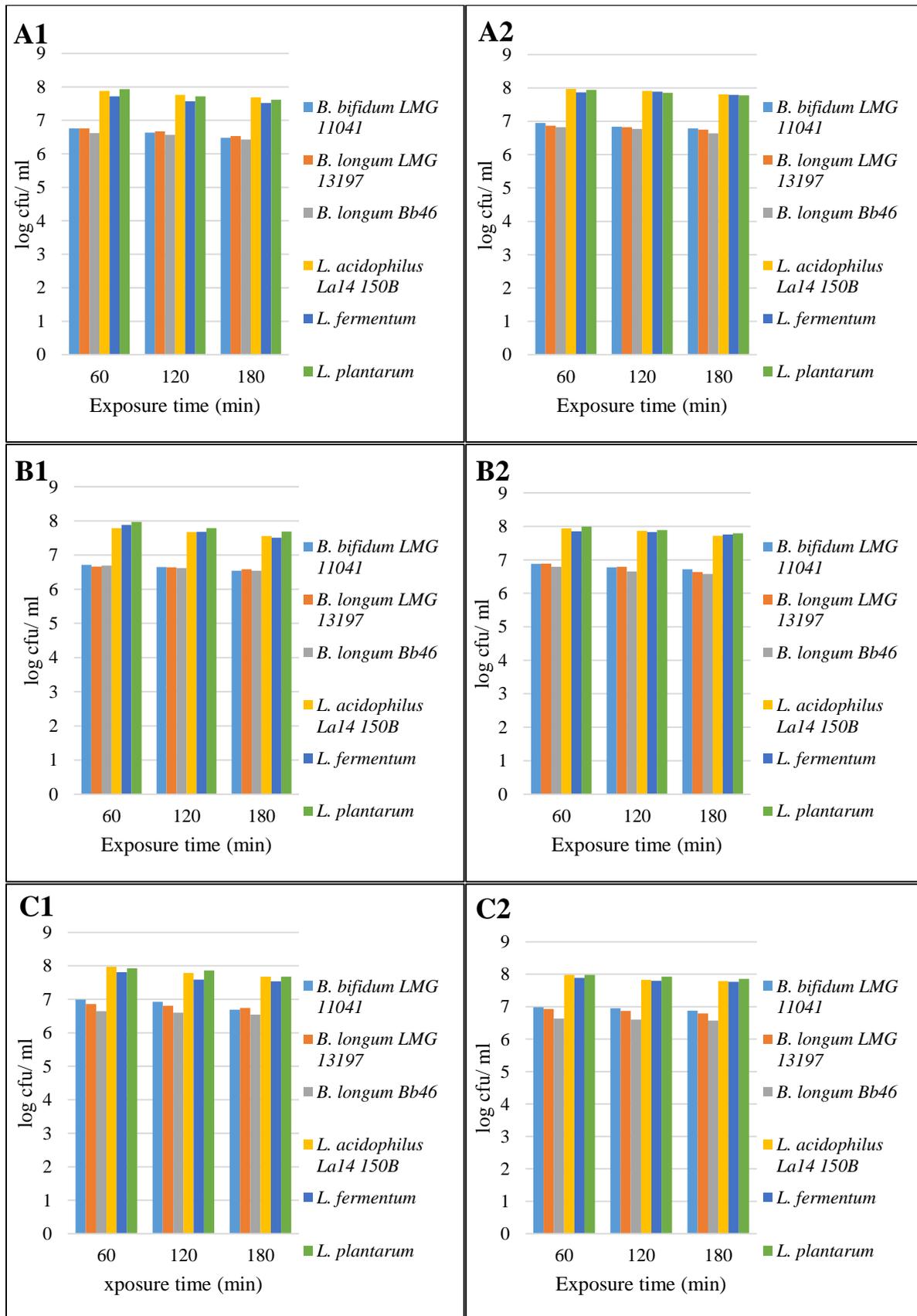


Figure 2.4: The difference log CFU/ ml showing the acid tolerance over time

*A- pH 2 *B- pH 2.5 *C- pH 3

1- Non- adapted 2- Adapted

2.4.2.2. Bile resistance

The ability to survive bile concentrations produced in the human small intestines and to take up residence and multiply in the human large intestine is another important characteristic of probiotics (Collado *et al.*, 2006). Different studies use different bile salt concentrations for bile tolerance studies, with a range of 0.5 to 2.0% (w/v). Our study therefore, looked at the survival of the non-adapted and adapted cells in 0, 1.0, 2.0 and 3.0% bile salts concentration. Figure 2.5 shows the survival of the non- adapted and the adapted cells in the different bile salt concentrations over time. There was an increase in the number of the surviving cells in 0% bile concentrations throughout the whole incubation period. At the beginning of the experiment, the concentration of cells was the same (10^8 log cfu/ ml) for all the probiotics. Viable cell numbers ranged from 8.03 to 8.54 and 8.40 to 9.68 log cfu/ ml for the non- adapted (Figure 2.5 A1) and the adapted (Figure 2.5 A2) cells, respectively. For the adapted cells, there were viable cell increases of 0.77, 0.60, 0.59, 1.14, 0.83 and 1.05 log cfu/ ml more than the non- adapted cells for *B. bifidum* LMG 11041, *B. longum* LMG 13197, *B. longum* Bb46, *L. acidophilus* La14 150B, *L. fermentum* and *L. plantarum*, respectively.

When probiotic cells are grown in the presence of bile salts, there is a decrease in the number of viable cells. In 1% bile salt concentration after 180 min incubation, there was viable cell reduction for both the non- adapted and the adapted cells. The non- adapted cells (Figure 2.5 B1) had viable cell reductions of 0.52, 0.64, 0.64, 0.59, 0.60 and 0.60 log cfu/ ml more than the adapted cells (Figure 2.5 B2) for *Bifidobacterium bifidum* LMG 11041, *B. longum* LMG 13197, *B. longum* Bb46, *L. acidophilus* La14 150B, *L. fermentum*, *L. plantarum*, respectively. When the same non- adapted and adapted cells were exposed to 2% bile concentration, the reduction in viable cell numbers was more than when the cells were exposed to 1% bile concentration. The non- adapted cells (Figure 2.5 C1) had viable cell reductions of 0.5, 0.71, 0.71, 0.56, 0.54 and 0.45 log cfu/ ml more than the adapted cells (Figure 2.5 C2) for *Bifidobacterium bifidum* LMG 11041, *B. longum* LMG 13197, *B. longum* Bb46, *L. acidophilus* La14 150B, *L. fermentum* and *L. plantarum*, respectively. A similar trend was observed when the probiotic cells were exposed to 3% bile salt concentration. For the non- adapted cells (Figure 2.5 D1) at time 180 min, there was viable cell reductions of 0.90, 0.92, 1.02, 0.69, 0.68 and 0.55 log cfu/ ml more than the adapted cells (Figure 2.5 D2) for *Bifidobacterium bifidum* LMG 11041, *B. longum* LMG 13197, *B. longum* Bb46, *L. acidophilus* La14 150B, *L. fermentum* and *L. plantarum*, respectively. The higher the bile salt concentration, the lower the

number surviving. In all cases, the adapted cells had higher log cfu/ ml than the non- adapted cells. Tolerance of the multiple stress adapted cells to bile salts was significantly higher than of the non- adapted ones for all the tested strains at the end of incubation in all bile concentrations, represented by the p- values: 0.04, 0.03, 0.01, 0.03, 0.04 and 0.004 for *B. bifidum* LMG 11041, *B. longum* LMG 13197, *B. longum* Bb46, *L. acidophilus* La14 150B, *L. fermentum* and *L. plantarum*, respectively. In previous different studies, better survival of the adapted cells than the non- adapted cells had been reported (Bezkorovainy, 2001; Kim *et al.*, 2001; Sánchez *et al.*, 2010). The percentage of survival of the bile- adapted *Bifidobacterium* strains was better than their corresponding parental cells when exposed to bile salts in a study by Kim *et al.* (2001). Another study by Sánchez *et al.* (2010) reported that the difference between the *Bifidobacterium* parental and the bile- adapted strain showed statistically significant differences, in favour of the adapted strains.

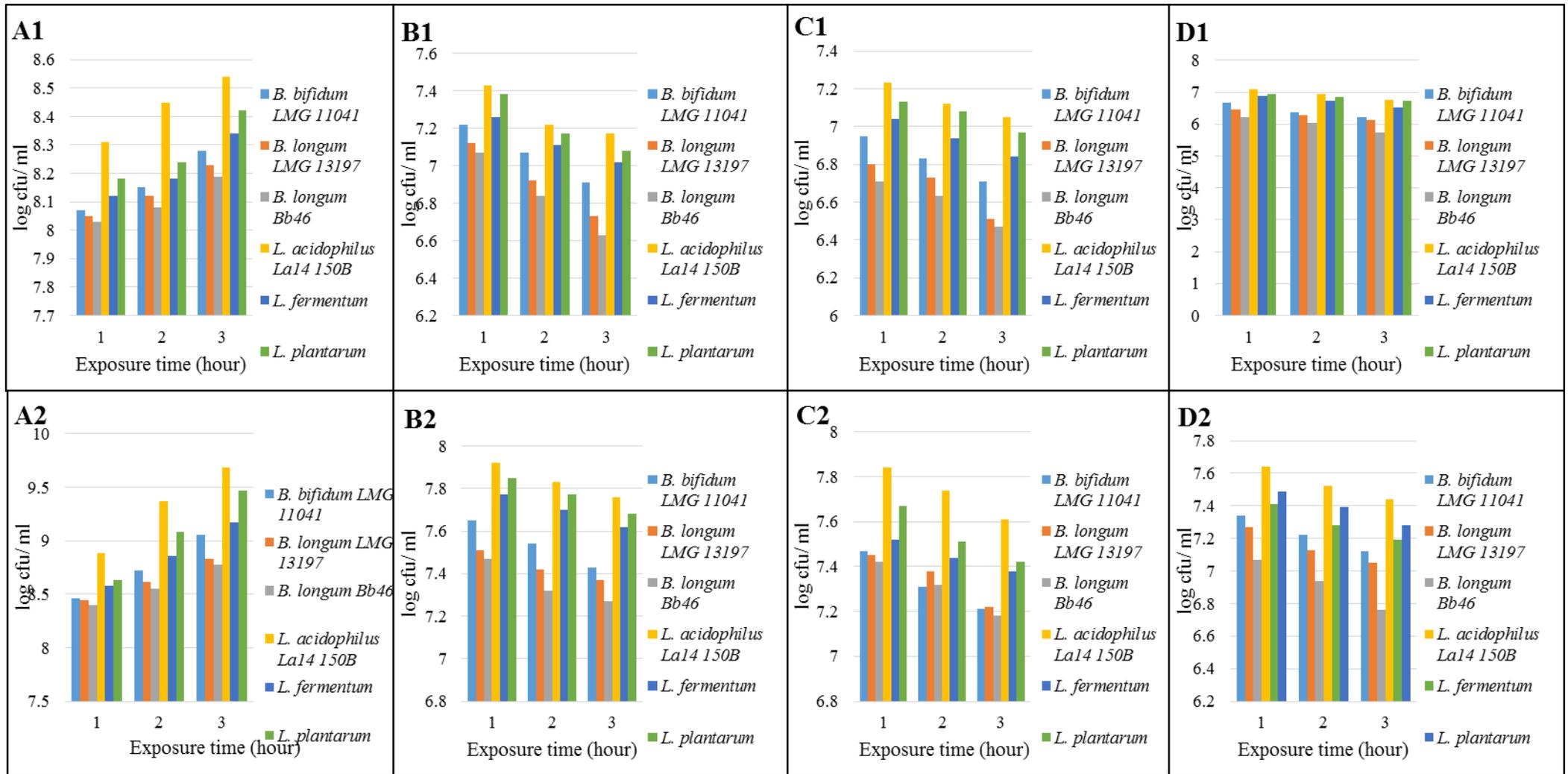


Figure 2.5: The tolerance of the *Bifidobacteria* cells to different bile solutions over time

*A- 0% bile salts *B- 1% bile salts *C- 2% bile salts *D- 3% bile salts

1- Non- adapted 2- Adapted

Overall the Lactobacilli cells survived better than the Bifidobacteria cells in both occasions. The results from Lin et al. (2007) showed that *L. acidophilus* is more resistant when compared with *Bifidobacterium* spp. and in a study comparing two genera of probiotics, they showed that *Bifidobacterium* strains were reported to be more susceptible to viability loss than the Lactobacilli cells (Maus and Ingham, 2004). In a previous study by Gilliland et al. (1984) they maintained *Lactobacillus* and *Bifidobacterium* strains at bile concentrations of 0- 1.5% for 3 hours, and their results showed that survival varied among the strains depending on the bile concentrations and exposure times. Therefore, our results confirm these studies. It is worth noting that in our study the cells were pre- adapted to acid- bile- temperature, not only to one stress factor. When comparing the adapted and the non- adapted cells for their survival in the acid and bile tolerance results, the adapted cells survived better than the non- adapted. Therefore our results proved that the multi- stress pre- adaptation can be used as a safe mechanism to enhance survival of the probiotic to unfavourable conditions.

2.4.3. Survival of probiotics cells after sequential exposure to simulated gastric and intestinal conditions

The first requirement for a probiotic strain is that it must be able to survive the transport to the active site, therefore be able to survive passage through the acidic environment of the stomach (Ruas- Madiedo, 2005). Probiotic cultures need to be able to colonise and survive in the small intestine in order for them to be able to exert positive effects on the health and well-being of the host (Huang and Adams, 2004). Therefore, cultures need to satisfy a criterion entailing their ability to survive the GIT processes, in the stomach and the intestinal tract (Visozo- Pinto *et al.*, 2006). As the two stresses of stomach transit and small intestinal transit might interact and thereby affect the viability of the strains in a synergistic fashion, it is important to evaluate all components (enzymes, low pH, bile salts and food vehicle) in one system, rather than evaluating the effect of each component in separate experiments (Pochart *et al.*, 1992). This study investigated the survival of the non- adapted cells together with their acid- bile- temperature adapted counterparts when sequentially exposed to the simulated gastric and intestinal fluids. The initial concentration for both the non- adapted and the adapted cells was adjusted to 10^8 cfu/ ml, and then the cells were exposed to simulated gastric fluid (pH 2, 2 hours) and sequentially to simulated intestinal conditions (pH 6.8) for two hours at 37°C. At the end of both processes the cells were plated in triplicate and the mean log cfu/ ml of the cells was calculated. The adapted cells had higher log cfu/ ml compared to the non- adapted cells,

except for *B. longum* Bb46 and *B. bifidum* LMG 11041, whose non- adapted cells had higher counts than the adapted cells (Figure 2.6). There was a difference of 1.11, 1.17, 0.91 and 0.53 log cfu/ ml between the adapted and non- adapted cells of *L. acidophilus* La14 150B, *L. plantarum*, *L. fermentum* and *B. longum* LMG 13179, respectively. The difference between the adapted and the non- adapted cells of *B. bifidum* LMG 11041 and *B. longum* Bb46 were 0.03 and 0.01 log cfu/ ml, respectively.

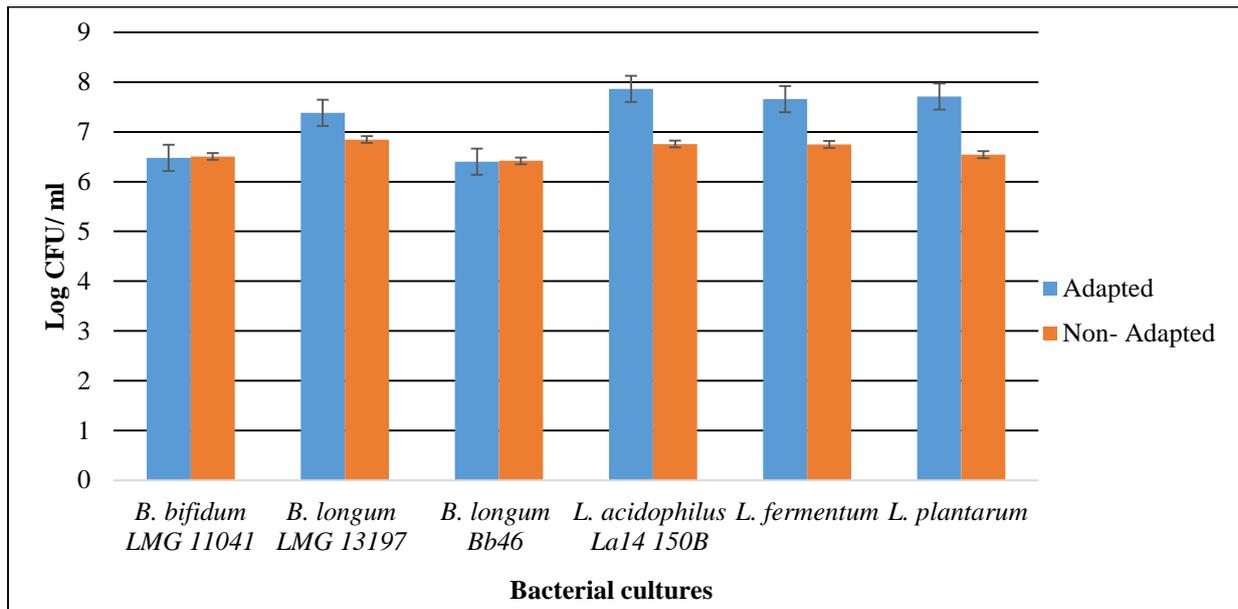


Figure 2.6: The growth of the bacterial culture after the exposure to gastric and intestinal conditions

Drouault et al. (2002) and Berrada et al. (1991) reported that *L. acidophilus* and Bifidobacteria to be more resistant to gastric and intestinal conditions but large differences exist between strains. This was in accordance with our results as both the adapted and the non- adapted cells survived after the sequential exposure to simulated gastric and intestinal fluids. Huang and Adams (2004) tested the survival of the cells in the simulated gastric and intestinal conditions separately. They reported that when the strains were first exposed to gastric conditions, all strains showed progressive reduction in survival while the exposure to simulated intestinal conditions resulted in all strains retaining the same viability. The present study, investigated the final log cfu/ ml after sequential exposure of probiotic cells to simulated gastric and intestinal conditions. The results showed that the survival of probiotics ranged from 6.404 to 7.867 as compared to 6.507 to 6.847 log cfu/ ml for the adapted and the non- adapted cells, respectively. This high number of surviving cells could be attributed to the exposure of the

cells to the intestinal conditions. This confirms that the pre- exposure of probiotic cells does indeed have a positive effect on the survival of the cells. The ability of the cells to survive the gastric and intestinal conditions means that the cells can be used as probiotics, since their survival suggests that they can be delivered to the intestine in high numbers (Ruas- Madiedo *et al.*, 2005). The objective of the current study was to enhance the survival of the probiotic cells after the sequential exposure to the simulated gastric and intestinal fluids through the multi- stress pre- adaptation. In a different study to test for survival of the probiotic cells in the gastric and intestinal conditions, Pochart *et al.* (1992) reported that the survival of *L. acidophilus* and *B. bifidum* cells through the gastric and the intestinal conditions was not significantly different. We used the acid- bile- temperature adapted cells and checked for their survival in the simulated gastric and intestinal conditions There was a significant ($p= 0.047$) increase in the survival of the adapted cells when compared to the non- adapted cells. From our results, the hypothesis that pre- adaptation of the probiotic cells to acid- bile- temperature enhanced the growth of the probiotics under simulated gastric and intestinal conditions, can be accepted.

2.4.4. BSH activity

The main function of the bile salt hydrolase (BSH) is that it is responsible for the bile salt deconjugation. Bile is a yellow green aqueous solution whose major constituents include bile acids, cholesterol, phospholipids and the pigment biliverdin (Carey and Duane, 1994; Hofmann, 1994). The deconjugated bile salts are more readily excreted in the faeces than the conjugated bile salts (de Rodas *et al.*, 1996). The ability of probiotic strains to hydrolyse bile salts has often been included among criteria for the selection of probiotic strains. It has been suggested that the BSH activity should be a requirement in the selection of probiotic organisms with the cholesterol- lowering properties.

Table 2.1: The presence of precipitated bile acid around colonies for the BSH activity

Probiotic cells	Non- adapted	Adapted
<i>B. longum</i> Bb46	-	+
<i>B. longum</i> LMG 13197	-	-
<i>B. bifidum</i> LMG 11041	-	+
<i>L. acidophilus</i> La14 150B	+	+
<i>L. fermentum</i>	+	+
<i>L. plantarum</i>	-	+

+: Precipitate present, -: Precipitate absent

The probiotic cultures, both adapted and non- adapted were streaked onto BSH- media (MRS- 0.5% TDCA salt). This allowed for the identification of the BSH positive and negative strains. Positive results were the plates that either had the cells producing opaque white colonies or had acid precipitation around the active colonies (Dashkevick and Feighner, 1989). The results for the BSH activity of both the non- adapted and the adapted cells are shown in Table 2.1. The non- adapted and adapted cells of the *Lactobacillus* strains tested positive for the BSH activity, with exception of non- adapted cells of *L. plantarum* which tested negative. The adapted and non- adapted cells of *B. longum* LMG 13197 both tested negative for the BSH activity. The adapted cells of *B. longum* Bb46 and *B. bifidum* LMG 11041 tested positive for the BSH activity while their non- adapted cells were negative. This could be attributed to the fact that during the pre- adaptation of the cells to acid- bile- temperature, they acquired factors such as the BSH activity. An increase in the number of surviving cells after their pre- exposure to multiple stress factors was observed. From these two separate experiments it is possible that pre- adaptation has positive effects on probiotics.

BSH activity is a widespread characteristic among *Lactobacillus* spp. (Feighner and Dashkevics, 1988; Vandevoorde *et al.*, 1992). Therefore, our results are in agreement with these results from previous studies. In the present study, the non-adapted cells of the two *Bifidobacterium longum* strains (*B. longum* LMG 13197 and *B. longum* Bb46) both tested negative for the BSH activity. The results therefore were not in agreement with the previous study by Corzo & Gilliland (1999) that reported that BSH was present in *Bifidobacterium longum*. Elevated levels of blood and dietary cholesterol are considered major risk factors for coronary heart diseases and colon cancer (Liong and Shah, 2005). Deconjugation of bile acids can reduce serum cholesterol levels (Reynier *et al.*, 1981). BSH has been reported as beneficial, it has the ability to reduce the serum cholesterol (de Rodas *et al.*, 1996). Since the main function of the BSH is for the deconjugation of bile salt, probiotics harbouring this enzyme are therefore important in hydrolysing of bile salts and therefore lowering the cholesterol levels. The non-adapted cells all tested negative for the BSH activity except *L. acidophilus* La14 150B and *L. fermentum*. The BSH activity of the stress adapted cells was the tested. All the adapted cells tested positive for the BSH activity with the exception of *B. longum* LMG 13197. Therefore, the pre- adaptation of the cells to acid- bile- temperature probably induced their BSH activity. The adapted cells can therefore be very important for lowering the cholesterol in the serum.

This method can be used as a preventative method in diseases associated with high cholesterol. The ability of the probiotic cells to express their BSH activity is crucial and therefore the pre-exposure of the cells to stressful conditions can be used as it allows the cells to express BSH activity.

2.4.5. Antibiotic assay

The resistance of probiotics to antibiotics has been reported by several authors (Bhattacharjee *et al.*, 1988; Pathak *et al.*, 1993; Goni-Urriza *et al.*, 2000; Rhodes *et al.*, 2000). This resistance makes probiotics favourable for promoting beneficial microflora. In our study the resistance/susceptibility of the non- adapted and the adapted cells to different antibiotics was studied. All the cultures, both the non- adapted and the adapted cells were sensitive to oxacillin, penicillin G and streptomycin. However, they all showed some resistance to chloramphenicol, erythromycin, fusidic acid, novobiocin and tetracycline. The zones of inhibition ranged from 6.30 ± 1.53 to 24.3 ± 2.08 for all the tested strains (Table 2.2). The resistance of LAB to antibiotics such as penicillin G, ampicillin, vancomycin, chloramphenicol or ciprofloxacin has been reported in studies by Coppola *et al.* (2005) and Herros *et al.* (2005). Our current study showed that the non- adapted and the adapted cells were susceptible to penicillin G, oxacillin and streptomycin, which was not in agreement with previous literature. Our results also show that *B. bifidum* was susceptible to streptomycin and the penicillin G and resistant to the other antibiotics tested. This was in agreement with a study by Charteris *et al.* (1998) that showed that *B. bifidum* was resistant to streptomycin and fusidic acid and susceptible to penicillins, chloramphenicol and erythromycin. The antibiotic assay of *B. longum*, *L. acidophilus* and *L. plantarum* were tested by Temmerman *et al.* (2002) and in their results for *B. longum* they found that it was susceptible to tetracycline, erythromycin, penicillin G and chloramphenicol. In this study, *B. longum* was only susceptible to penicillin G and grew in the presence of tetracycline, erythromycin and chloramphenicol. However, our results confirmed their results for *L. acidophilus* and *L. plantarum* except in that in their case *L. plantarum* was susceptible to chloramphenicol but not penicillin G, as was found in our results. Even though both the non-adapted and the adapted cells were susceptible and resistant towards the same antibiotics, the zones of inhibition of the adapted cells were larger than those for their non- adapted counterparts. It is desired that the probiotic cells are resistant to antibiotics. This way they will still be able to confer their beneficial effects onto the host. The resistance of the probiotics to antibiotics is also a beneficial effect in case these two are used together. The probiotics will be able to confer the beneficial effects while they also maintain the beneficial microbes in the

human GIT. Our results showed that the probiotic cells were resistant to antibiotics, even after the multi- stress pre- adaptation. This demonstrates that the multi- stress pre- adaptation had a positive effect on the resistance of the probiotic cells.

Probiotic strains	Antibiotics							
	C	E	FC	NO	OX	PG	S	T
	Zone of inhibition (mean diameter (mm) ± SD)							
<i>L. acidophilus</i> N	19.0±2.52	13.0±2.52	16.0±1.00	18.3±3.05	0	0	0	9.30±1.15
<i>L. acidophilus</i> A	22.3±2.31	14.3±1.55	19.3±1.53	17.7±0.58	0	0	0	11.3±15.0
<i>L. plantarum</i> N	21.7±0.58	12.7±0.58	17.3±1.53	15.7±0.58	0	0	0	6.30±1.53
<i>L. plantarum</i> A	23.3±2.08	13.3±1.15	20.3±0.58	16.3±1.52	0	0	0	9.00±1.00
<i>L. fermentum</i> N	21.3±1.53	11.6±1.15	15.6±0.58	14.3±1.53	0	0	0	8.70±0.58
<i>L. fermentum</i> A	26.6±1.53	11.3±1.15	17.3±1.15	16.0±0.00	0	0	0	11.3±1.15
<i>B. longum</i> BB46 N	19.7±1.53	13.3±1.15	16.0±0.00	13.7±0.58	0	0	0	8.70±0.58
<i>B. longum</i> BB46 A	17.7±1.53	12.7±0.58	16.7±0.58	16.7±1.53	0	0	0	10.3±0.58
<i>B. longum</i> LMG 13197 N	18.7±0.58	12.3±0.58	16.7±0.58	15.7±1.15	0	0	0	7.30±0.58
<i>B. longum</i> LMG 13197 A	21.3±1.53	12.7±1.15	18.7±0.58	14.7±0.58	0	0	0	8.30±0.58
<i>B. bifidum</i> LMG 11041 N	19.7±1.15	12.7±1.15	16.3±1.53	13.0±0.00	0	0	0	7.30±0.58
<i>B. bifidum</i> LMG 11041 A	24.3±2.08	14.7±1.53	16.7±1.15	13.7±1.15	0	0	0	10.7±1.15

Table 2.2: The zones of inhibition of the adapted and non- adapted strains by the different antibiotics

A: Adapted, N: Non- adapted. Each value is mean of 3 replicates ± standard deviation (SD); C, Chloramphenicol; E, Erythromycin; FC, Fusidic acid; OX, Oxacillin; NO, Novobiocin; PG, Penicillin G; S, Streptomycin; T, Tetracycline; 0, no zone of inhibition

2.5. CONCLUSION

This study was focused on the stressful conditions that are encountered by probiotics during processing throughout the GIT. The pre- exposure of the probiotics to the stressful conditions in order to increase their robustness was carried out and they were then tested for their survival throughout the GIT conditions. These are the first results that have been obtained for the adaptation of these cultures to acid- bile- temperature and there is no other such adaptation that had been studied to our knowledge. The results of this study showed that for the acid and the bile tolerance, most of the stress- adapted cells were higher than those of the non- adapted cells. The sequential exposure of the cells to the simulated gastric intestinal fluids which showed the survival and the viability of the cells in the low then the high acid concentrations also showed that the adapted cells survived with higher viability than the non- adapted.

The adapted and non- adapted cells were also tested for their BSH activity, no effect on *B. longum* LMG 13197, *Lactobacillus acidophilus* La14 150B and *L. fermentum* but resulted in the loss of BSH activity of *B. longum* Bb46, *B. bifidum* LMG 11041 and *L. plantarum*. What was more interesting in the results was the results for the antibiotic sensitivity profiles, the results were the same for the adapted and the non- adapted cells. With the results that were found, our hypothesis that the pre- exposure of the probiotics to stress enhances their survival when further exposed to single stresses was proved to be true.

Future studies will look more into the incorporation of these adapted cultures into the food and to check for the survival of these cultures during the production of those foods and to also check for the increase in the shelf life of the food.

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Chapter 3

**Survival of multi-stress adapted to GIT conditions,
as well as their antagonistic effects against
Escherichia coli and *Staphylococcus aureus* in
comparison with their non-adapted parental strains**

3.1. ABSTRACT

The use of probiotics in the inhibition of enteric pathogens has been studied and proven to have positive results. The combination of the different probiotic cultures into probiotic cocktails to prevent and treat diseases caused by these pathogens therefore should increase the probiotic effects. The aims of this work were to investigate stability of cocktails of acid- bile- temperature adapted *Lactobacillus* spp. (*L. acidophilus*, *L. plantarum*, *L. fermentum*) and *Bifidobacterium* spp. (*B. longum* Bb46, *B. bifidum* LMG 11041 and *B. longum* LMG 13197) under simulated gastrointestinal conditions and to compare antagonistic effects of individual and different cocktails of multi-stress adapted probiotics against *Escherichia coli* and *Staphylococcus aureus*. From the six acid- bile- temperature adapted cells, 54 different probiotic were prepared. Preliminary experiments were performed and based on results thereof, 10 best combinations were chosen and used in further experiments. A cocktail containing all the six non-adapted probiotic bacteria was used for comparison. Tolerance of the probiotic cocktails to 2% bile concentration, acid conditions (pH 2) and sequential exposure to the gastric and intestinal conditions was teste. In addition, their antibiotic sensitivity was assayed and then their effects on growth of *E. coli* and *S. aureus* were also tested. A combination containing *L. plantarum* + *L. fermentum* + *B. longum* Bb46, survived better than all the other combinations in 2% bile concentration. A combination of *L. acidophilus* La14 150B + *L. plantarum*+ *L. fermentum* + *B. longum* LMG 13197 + *B. longum* Bb46 + *B. bifidum* LMG 11041 best tolerated acid than all the other combinations and survived better after the sequential exposure to gastric and intestinal conditions. A combination containing *L. acidophilus* La14 150B + *L. plantarum*+ *L. fermentum* + *B. longum* LMG 13197 + *B. longum* Bb46 + *B. bifidum* LMG 11041 (non- adapted) was the least surviving in all the simulated gastrointestinal conditions. For the antipathogenic tests, one combination inhibited *S. aureus* better than the other combinations while a combination containing *L. acidophilus* La14 150B + *L. plantarum*+ *L. fermentum* + *B. longum* LMG 13197 + *B. longum* Bb46 + *B. bifidum* LMG 11041 (adapted) best inhibited *E. coli*. All combinations of multi-stress adapted probiotics inhibited the pathogens better than a combination of their non- adapted counterparts. The results for the antibiotic resistance assay showed enhanced resistance of the cocktails to antibiotics. Thus, formulations containing mixtures of multi stress-adapted cells exhibited enhanced antibiotic resistance and synergistic effects against foodborne pathogens.

3.2. INTRODUCTION

Antibiotics are widely used in treating different infections. Such practice has led to an increase in the development of resistance of the bacteria to these antibiotics, thereby leading to fewer treatment options (Džidić *et al.*, 2008). As a result of this emergence of antibiotic resistance, alternative antimicrobial strategies were explored for treatment and prevention of gastrointestinal tract (GIT) infections. Such strategies includes among others, the use of probiotics and their microbial metabolites. It has been revealed in literature that every probiotic is associated with its own specific effect, where most of them are related to their ability to express particular different surface molecules (glycolipids, microbe-associated molecular patterns), or that the products that they secrete interact with the epithelial cells to form barriers in the immune system that lies under the barrier (Marco *et al.*, 2006).

Probiotics are known as bacterial cultures that have mostly been isolated from human intestines (Laiho *et al.*, 2002) and are therefore used to exert beneficial effects in the human GIT. They must have demonstrable benefits to host health and have GRAS (generally recognized as safe) status (Desmond *et al.*, 2004). These probiotic cultures need to have specific desirable traits that can help them in their survival through the production, shelf life and the passage through the GIT. They are mostly taken as supplements (pharmaceuticals) or biotherapeutics (approved drugs with important therapeutic applications) and also in a form of functional foods (Periti and Tonelli, 2002). Some of the health benefits established in numerous clinical trials, which are usually exerted by the probiotics to the human, include prevention and control of GIT infections, immune stimulation, and balancing of the intestinal microflora (Gilliland, 1990; Vaughan and Mollet, 1999).

The introduction and overgrowth of harmful bacteria in the human GIT leads to inflammatory, immunological, neurological and endocrinological problems (Hemaiswarya *et al.*, 2013). Probiotics work against the pathogenic organisms by means of competition; they compete for substrates and space to survive. They first prevent adherence of the pathogenic bacteria to the mucosal cells (Eizaguirre *et al.*, 2002; Mangell *et al.*, 2002) and then release enzymes that kill the pathogens (Fioramonti *et al.*, 2003). Studies have shown that other than the lactic acid that is produced by the lactic acid bacteria (LAB), the most common probiotic cultures, during their transit in the GIT (Bernet-Camard *et al.*, 1997, Van de Guchte *et al.*, 2001; Hemaiswarya *et al.*, 2013).

The use of single bacterial cultures has been studied since the discovery of probiotics and the need to enhance the beneficial effects that these cultures have, has led to introduction of studies of mixtures containing them. Kim *et al.* (2010) studied the effects of a mixture of *B. bifidum* BGN4, *B. lactis* AD011 and *L. acidophilus* AD031 on eczema, and they found that this mixture was an effective approach in preventing the development of eczema in infants at high risk of allergy during the first year of life. VSL #3, a probiotic product containing eight different probiotic bacteria, has been used in different studies and it was shown to have immunodulatory effects (Miele *et al.*, 2008) and it is also effective in preventing pouchitis (Mimura *et al.*, 2004). Another probiotic mixture, Ecological[®] relief, has also been studied in the treatment of constipation during pregnancy (de Milliano *et al.*, 2012), and shown to be a better alternative to laxatives which have side effects such as abdominal pains and diarrhoea (Vazquez, 2008). The use of different probiotic combination cultures are seen to have adverse effects on different diseases. The combination of probiotic cultures in the prevention and treatment of heterogeneous diseases has proven to be more effective than the use of single cultures. Therefore, this study investigated the effect of the pre- adaptation of the probiotics to multiple stresses on their stability under simulated gastrointestinal conditions and the effect of their singular as well as their synergistic antagonistic effect against selected enteric pathogens.

3.3. MATERIALS AND METHODS

3.3.1. Bacterial cultures

Bifidobacterium bifidum LMG 11041, *B. longum* LMG 13197, *B. longum* Bb46, *Lactobacillus acidophilus* La14 150B, *L. fermentum* and *L. plantarum* glycerol stock cultures from our laboratory were used as test probiotic cultures while *Escherichia coli* and *Staphylococcus aureus* were used as select foodborne pathogens for the antipathogenic tests. *Lactobacillus* spp. were sub-cultured twice in de Man Rogosa and Sharpe (MRS) broth (Merck, South Africa) and *Bifidobacterium* spp. in MRS supplemented with 0.05% v/v L- cysteine hydrochloride monohydrate (MRS- cys- HCl), followed by incubation at 37°C for 72 h in anaerobic jars containing Anaerocult A gaspacks. After the final subculturing, the initial concentration of probiotic bacteria present was determined by serially diluting the cultures in ¼ strength Ringer's solution, followed by pour- plating onto MRS and MRS-cys- HCl plates in triplicates, for *Lactobacillus* and *Bifidobacterium* spp., respectively. The plates were incubated anaerobically at 37° C for 72 h. The adapted and the non- adapted cells were normalised to an optical density of 0.2 at 600 nm, which is equivalent to approximately 10⁸ cfu/ ml, in the different experiments.

Escherichia coli and *Staphylococcus aureus* were cultured in Luria Bertani (LB) broth, incubated in an Orbital shaker incubator LM- 530R, 100 gravitational force, g, at 37°C. Their concentrations were determined by plating of subcultures onto Mannitol salt agar (Merck, SA) and MacConkey agar (Merck, SA) plates for *S. aureus* and *E. coli*, respectively. The plates were then incubated at 37°C for 48 h. All the cultures were subcultured twice before their use in experiments.

3.3.2. Preparation of combinations and growth conditions

The six acid- bile- temperature adapted, (stress adaptation described in Chapter 2) and the six non- adapted *Lactobacillus* (*L. acidophilus*, *L. plantarum*, *L. fermentum*) and *Bifidobacterium* cultures (*B. longum* Bb46, *B. bifidum* LMG 11041 and *B. longum* LMG 13197) were used for the preparation of combinations. They were grown overnight in MRS or MRS- cys- HCl broth. The probiotic culture suspensions were prepared for each culture to achieve an optical density of 0.2 at 600 nm (OD₆₀₀). They were then added in equal amounts to make different combinations. There were 54 different combinations from the six cultures. From the 54

combinations, we then tested for their acid tolerance, the bile tolerance and then the subsequent exposure to the gastric and the intestinal conditions as preliminary experiments (Appendix). From there the 10 best tolerant combinations were chosen and a combination of the six non-adapted cells (Table 3.1) and used them for further experiments.

Table 3.1: The ten best probiotic combinations chosen from preliminary trials and control cocktail

Combination number	Probiotic species
24	<i>L. acidophilus</i> La14 150B + <i>L. plantarum</i> + <i>B. longum</i> LMG 13197
25	<i>L. acidophilus</i> La14 150B + <i>L. plantarum</i> + <i>B. bifidum</i> LMG 11041
28	<i>L. acidophilus</i> La14 150B + <i>L. fermentum</i> + <i>B. longum</i> Bb46
30	<i>L. acidophilus</i> La14 150B + <i>B. longum</i> Bb46 + <i>B. bifidum</i> LMG 11041
31	<i>L. acidophilus</i> La14 150B + <i>B. longum</i> LMG 13197 + <i>B. bifidum</i> LMG 11041
32	<i>L. plantarum</i> + <i>L. fermentum</i> + <i>B. bifidum</i> LMG 11041
33	<i>L. plantarum</i> + <i>L. fermentum</i> + <i>B. longum</i> LMG 13197
34	<i>L. plantarum</i> + <i>L. fermentum</i> + <i>B. longum</i> Bb46
35	<i>L. plantarum</i> + <i>B. longum</i> Bb46 + <i>B. longum</i> LMG 13197
59	<i>L. acidophilus</i> La14 150B + <i>L. plantarum</i> + <i>L. fermentum</i> + <i>B. longum</i> LMG 13197 + <i>B. longum</i> Bb46 + <i>B. bifidum</i> LMG 11041 (All adapted)
60	<i>L. acidophilus</i> La14 150B + <i>L. plantarum</i> + <i>L. fermentum</i> + <i>B. longum</i> LMG 13197 + <i>B. longum</i> Bb46 + <i>B. bifidum</i> LMG 11041 (All non- adapted)

3.3.3. Survival under GIT conditions

3.3.3.1. Tolerance to bile salts

Tolerance of the probiotic cultures to bile was performed using a method by Tsai *et al.* (2007) with minor modifications. Briefly, overnight probiotic combination cultures were harvested by centrifugation at 3000 rpm for 10 min. The pellets were washed in ¼ strength Ringer's solution and mixed by vortexing for 30 seconds. Then 100 µl of the solution was added to MRS broth adjusted to 2% (w/v) bile concentration and grown in a shaking incubator at 37°C with the readings taken after 2 hours. The suspensions were then serially diluted up to 10⁻⁷ using ¼ strength Ringer's solution and 0.1 ml of each dilution was pour plated onto MRS agar plates in triplicates. The plates were incubated anaerobically in anaerobic jars with Anaerocult A

gaspacks and Anaerotest strips at 37° C for 72 h. The colonies of the plates containing 30- 300 colonies were counted.

3.3.3.2. Acid tolerance

The investigation of the tolerance of the non- adapted and the stress- adapted cells to acid was done using the method described by Brashears *et al.* (1998), with minor modifications. Briefly, the different probiotics combinations were grown in MRS broth at 37°C overnight in a shaking incubator at 100 rpm. The cultures were sub-cultured into 10 ml of fresh MRS broth adjusted to pH 2 with 1M HCl, followed by incubation at 37°C in a shaking incubator (100 rpm). Then 100 µl aliquots were harvested after 120 min, transferred into 10 ml MRS broth. The suspensions were then serially diluted up to 10⁻⁷ using ¼ strength Ringer's solution and then 0.1ml of each dilution was pour- plated onto MRS plates in triplicates. The plates were incubated anaerobically in anaerobic jars with Anaerocult A gaspacks and Anaerotest strips at 37° C for 72 h. The colonies of the plates containing 30- 300 colonies were counted.

3.3.3.3. Gastric and intestinal conditions

3.3.3.3.1. Preparation of simulated gastric and intestinal fluids

The simulated gastric juice was prepared by briefly suspending 3 g/l of pepsin (Merck, SA) in saline (0.5% w/v) and adjusted to 2.0 with 1M HCl. The simulated intestinal fluid was prepared by dissolving 6.8 g monobasic potassium phosphate (Merck, SA) into 250 ml distilled water. Then 77 ml of NaOH (0.2 M) was added and mixed. Five hundred milliliters of distilled water was then added and the solution was mixed by vortexing for 30 s. Then 10 g of pancreatin was added and mixed and the solution was adjusted to pH 6.8 with 1M NaOH or 1M HCl. The solution was then made up to 1000 ml. Both fluids were sterilized by filtering through a 0.45 µm syringe filter (Millipore). The solution was then autoclaved and stored until measures for the exposure of the probiotics to the gastric and intestinal conditions

3.3.3.3.2. Exposure to gastric and intestinal conditions

The different probiotic cultures were grown overnight in MRS broth. Aliquots of 1 ml were added to 9 ml of simulated gastric fluid (pH 2) for 2 h at 37 °C. After 2 h, 0.1 ml of the solution was withdrawn and added into 0.9 ml of the simulated intestinal fluid (pH 6.8) for 2 h at 37 °C. Then 100 µl were withdrawn from the tubes and plated in triplicates onto MRS agar plates. The plates were incubated anaerobically in anaerobic jars with Anaerocult A gaspacks and

Anaerotest strips at 37°C for 72 h. The colonies of the plates containing 30- 300 colonies were counted.

3.3.4. Antipathogenic tests

Cultures of *E. coli* and *S. aureus* were grown in LB broth overnight at 37°C. Bacterial suspensions were prepared in sterile ¼ strength Ringer's solution for each of the pathogens to achieve an optical density of 0.2 at 600 nm, which corresponds to approximately 1 x 10⁸cfu/ml. The method that was used for the antagonistic tests was adapted from Jamalifar *et al.* (2010) with minor modifications. Briefly, 15 ml of 1 x 10⁸ cfu/ml probiotic combination cultures were added into flasks containing 100 ml LB broth and to that 1ml of 1 x 10⁸ cfu/ml of the pathogen was added. The control flasks did not contain any probiotics. The flasks were incubated in a shaking incubator (100 rpm) at 37°C for 6 h. Hundred microliter subsamples were withdrawn from the flasks hourly, diluted in 900 µl of ¼ strength Ringer's solution, then 100 µl were plated in triplicates onto Mannitol salt agar (Merck, SA) and MacConkey agar (Merck, SA) plates for *S. aureus* and *E. coli*, respectively. The plates were then incubated at 37°C for 24 h.

3.3.5. Assay for sensitivity to antibiotics

The sensitivity of the different probiotic combinations to antibiotics was determined using the Mastring antibiotic discs (Davies Diagnostics, SA). Eight different antibiotics were used: chloramphenicol 25 µg, erythromycin 5 µg, fusidic acid 10 µg, oxacillin 5 µg, novobiocin 5 µg, penicillin G 1 unit, streptomycin 10 µg and tetracycline 25 µg. The different combinations were grown overnight at 37°C in MRS broth. The combinations (100 µl) were spread onto MRS plates in triplicates and then the antibiotic discs were placed in the middle of the plates. The plates were incubated anaerobically in anaerobic jars with Anaerocult A gaspacks and Anaerotest strips at 37°C for 72 h. The results were read by measuring the diameters of the inhibition zones for the sensitivity of the cultures on the antibiotics and then calculating the averages.

3.3.6. Data analysis

Statistical analysis of the difference between the adapted and the non- adapted strains was analysed by using two- way Student t- test from the software Statistica v10, where a p- values

< 0.05 was considered to be statistically significant and p - values > 0.05 , statistically non-significant.

3.4. RESULTS AND DISCUSSION

3.4.1. The survival of the probiotic combinations under simulated GIT conditions

The survival of the probiotics in the different compartments of the GIT is very crucial. It is believed that the probiotic cultures should be able to survive through the GIT and reach the distal part in sufficient numbers (10^6 – 10^7 cells g^{-1}) to exert their beneficial effects (Kurmann and Rasic, 1991). Therefore, the survival of probiotics after the exposure to different stress factors in large numbers is crucial. In this study the survival of the different combinations of the multi-stress adapted cells and a cocktail of non-adapted cells in bile, acid and sequential exposure to simulated gastric and intestinal fluids were compared. Figure 3.1 shows the number of the surviving cells of all the combinations when exposed to these conditions.

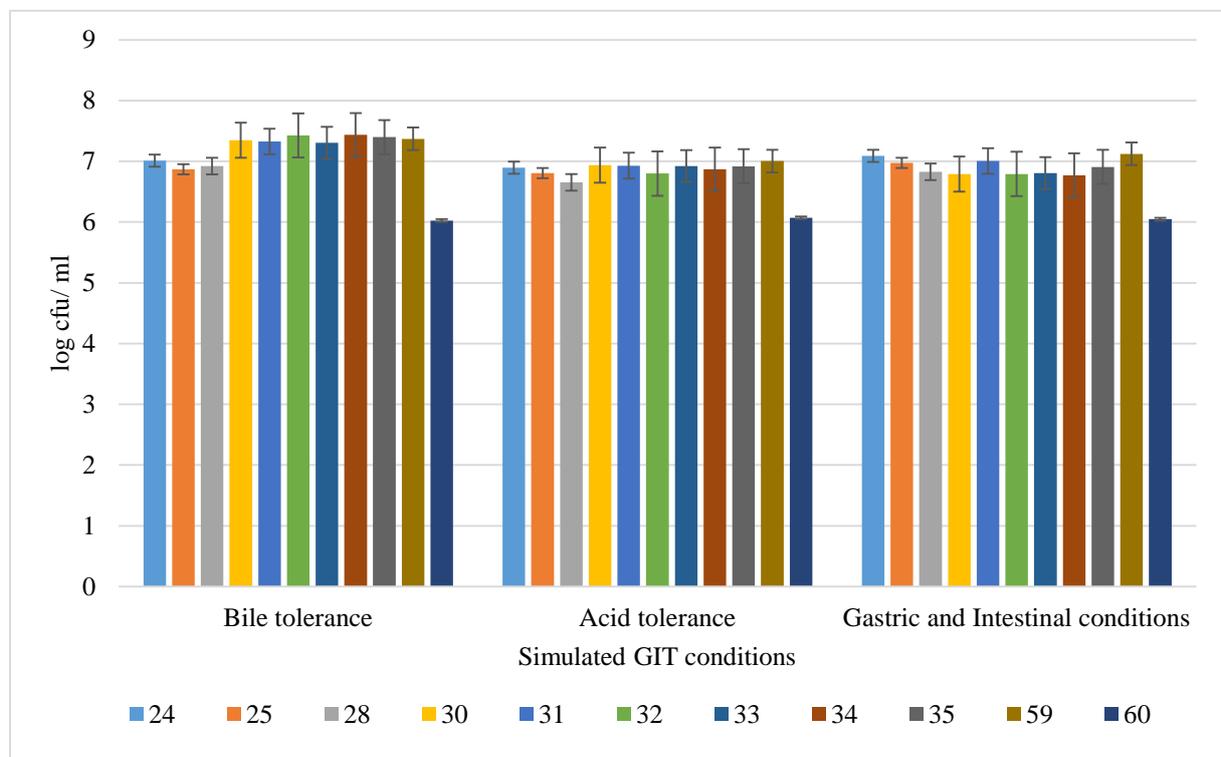


Figure 3.1: The effects of bile, acid and the gastric and intestinal conditions on the growth of the 10 combinations of the multi-stress adapted probiotics and the combination of the non- adapted cells (60)

3.4.1.1. The tolerance of the different probiotic combinations to bile salts

The study first looked at the survival of the 11 different combinations in 2% bile concentration for 2 hours. The bile salt concentration of 2% was used as the standard in the current study as it represents the most extreme concentration that can be found in the human intestine during the first hour of digestion (Jin *et al.*, 1998; Gotcheva *et al.*, 2002). The initial concentration of

all the combinations was adjusted to 8 log cfu/ ml. At the end of incubation, there was a decrease in the number of cells for all the combinations. There was however, less decrease in the viable cells for combination of the stress adapted cells than the combination of the non-adapted cells. The best surviving combination was 34 with a final log cfu/ ml of 7.44, a difference of 0.56 log cfu/ ml from the initial concentration. Combination 60 was the least surviving with a final log cfu/ ml of 6.03, a difference of 1.98 log cfu/ ml from the initial concentration. *Lactobacillus acidophilus* and Bifidobacteria have been reported to be more resistant to bile but large differences exist between strains (Berrada *et al.* 1991; Pochart *et al.* 1992). Since there are large differences between single probiotics, differences between the different probiotic combinations were expected. As expected, there were indeed differences between the combinations. Our results showed that although there was a decrease in the number of the surviving cells for both the non- adapted and stress adapted combinations, the combinations of stress adapted cells survived well than the combination of the six non- adapted cells. This reveals that the use of combinations that are made up of cultures that have been first pre- adapted to stress factors increased the chance of survival compared to when using combinations made up of non- adapted cultures.

3.4.1.2. Acid tolerance of the different probiotic combinations

Probiotics are expected to survive and confer beneficial effects in the most difficult conditions. Van der Gutche *et al.* (2002) mentioned that for probiotic strains need to overcome a wide range of environmental stresses, and one such stress is gastric acidity. Acid tolerance is therefore an important criterion that is used for selecting probiotics. Hill (1990) reported that the human stomach secretes about 3 l/h of gastric juice at a pH of around 2, therefore, it is necessary for probiotic microorganisms to be acid-resistant if they are to reach the colon unaided.

Tolerance of the different combinations to acid (pH 2 120 minutes) was studied. Combination 59 was the best performing combination with a final log cfu/ ml of 7.01, a difference of 0.99 from the initial count of 8 log cfu/ ml. It was followed by the other of stress- adapted cells with the differences of 1.11, 1.19, 1.35, 1.06, 1.07, 1.20, 1.08, 1.13 and 1.08 log cfu/ ml for combination 24, 25, 28, 30, 31, 32, 33, 34 and 35, respectively (Figure 3.1). The non- adapted

combination, combination 60 has a final log cfu/ ml of 6.70, difference of 1.93 log cfu/ ml from the initial concentration.

In a previous study Collado and Sanz (2006), evaluated tolerance of *Bifidobacterium* strains to the same acidic conditions (pH 2). Their results showed that the strains were resistant to acidic gastric conditions. In our previous chapter (Chapter 2) it was reported that the non- adapted cells survived in acid, but their stress adapted counterparts survived better in acid, which was in agreement with report by Collado and Sanz (2006). These results were therefore expected. Therefore, thus present study concludes that the use of stress adapted combinations is better than the use of non- adapted combinations. This study therefore supports that stress adaptation enhances the survival of the probiotics.

3.4.1.3. Survival of different probiotic combinations after subsequent exposure to simulated gastric and intestinal fluids

Smith (1995) reported that after ingestion stays in the stomach for a period of 2 to 4 hours after which the food leaves the stomach and deposited into the small intestines. Hill (1990) and Keele and Neil (1965) both highlighted the need for the probiotic cells to survive in these two compartments of the GIT, the stomach and the small intestine, in order for them to reach the colon and add health benefits to the human host. Therefore this study investigated the effects of these two compartments on survival of the probiotic combinations. From the results (Figure 3.1), the combination of all the six acid- bile- temperature adapted cultures, combination 59, best survived sequential exposure to simulated gastric and intestinal fluids, with a final log cfu/ ml of 7.124, difference of 0.876 log cfu/ ml from the initial concentration. There were differences of 0.91, 1.03, 1.17, 1.21, 1.19, 1.23 and 1.09 log cfu/ ml for combinations 24, 25, 28, 30, 31, 32, 33, 34 and 35, respectively, after subsequent exposure to these fluids. For non-adapted cells, combination 60 had the lowest final log cfu/ ml of 6.048, difference of 1.952 log cfu/ ml than the initial concentration. The cells here were first exposed to low pH than to a more neutral pH, which can lead to a recovery of the cells thereby accounting for the higher final log cfu/ ml values that were found at the end of the experiment. This is adapted from a study by Picot and Lacroix (2004), which reported that there is subsequent recovery of the cells that were previously damaged after the exposure to low pH and bile salt stresses.

Tolerance of single stress adapted cells has been studied, but there is less work that has been done of the probiotic combination. The use of combination of probiotic cultures in the prevention and treatment of heterogeneous diseases has proven to be more effective than the use of single cultures. The results that were obtained for the bile tolerance, acid tolerance and the subsequent exposure to the gastric and intestinal conditions, revealed that the combinations of adapted strains survive better than the combination of the non- adapted strains. These results were substantiated by calculating the statistical difference between the best and the least surviving combinations for bile tolerance ($p= 0.002$), acid tolerance ($p= 0.00002$) and the sequential exposure to gastric and intestinal conditions ($p= 0.004$). It was therefore, concluded that the use of combinations of probiotic cells that have been first pre- exposed to stress is better than using combinations that are made of non- adapted cells.

3.4.2. Antagonistic effects of single and probiotic cocktails on the food pathogens *S.*

aureus* and *E. coli

Antibiotics have always been the drugs of choice for the treatment of bacteria pathogens, but their ineffectiveness against some pathogens (Rakita, 1998), as well as the problem of antibiotic resistance, led to preference for use of alternative treatment strategies. Probiotics have been reported to have the ability to interfere with enteric pathogens and play a role in inducing interruptions of the earlier interactions of the pathogens to the host cells (Resta- Lenert and Barret, 2003). Therefore, the use of probiotics in pathogen inhibitions is favoured more than that of antibiotics. In order to assess how adaptation to stress factors affect the inhibitory activity of the probiotics against pathogens, the inhibitory effect of the stress adapted single probiotics strains was compared to that of cocktails comprising cells of different stress- adapted strains and one containing all non- adapted cells.

3.4.2.1. Antagonistic effects of single and probiotic cocktails on *S. aureus*

The inhibitory effect of multi-stress adapted single strain probiotic against *S. aureus* is shown in Figure 3.2 A. The numbers of *S. aureus* incubated in the absence of probiotics increased throughout the 6 h of incubation from initial count of 8 log cfu/ml to 8.864 log cfu/ml, an increase close to 1 log (0.9) unit. However, when inoculated together with probiotics, the numbers of *S. aureus* decreased in the presence of all strains. *Lactobacillus. acidophilus* La14 150B, the probiotic strain that best inhibited *S. aureus* growth when used alone, reduced the

counts of *S. aureus* from 8.00 to 7.85 log cfu/ ml. *L. acidophilus* La14 150B resulted in 0.15 log cfu/ ml reduction in viable *S. aureus* cells, compared to 0.14, 0.13, 0.12, 0.11 and 0.09 log cfu/ ml for *L. plantarum*, *L. fermentum*, *B. bifidum* LMG 11041, *B. longum* LMG 13197 and *B. longum* Bb 46, respectively.

When comparing inhibitory effects of combinations of probiotics it was interesting to observe that a cocktail containing all the six non-adapted probiotic strains was the least effective in inhibiting growth of *S. aureus*, reducing counts by only 0.07 log cfu/g (Figure 3.2 B). Combination 9 was the cocktail of multi-stress adapted probiotics which best inhibited *S. aureus*, whereby it reduced *S. aureus* counts from 8.00 to 7.52 log cfu/ ml, a difference of 0.48 compared to 0.12, 0.17, 0.36, 0.32, 0.28, 0.40, 0.16, 0.23, 0.43 log cfu/ ml for combinations 24, 25, 28, 30, 31, 32, 33, 34 and 59, respectively. Similar to what was observed for single probiotics, growth of *S. aureus* in the absence of probiotics increased by 1.08 log cfu/ml during incubation period.

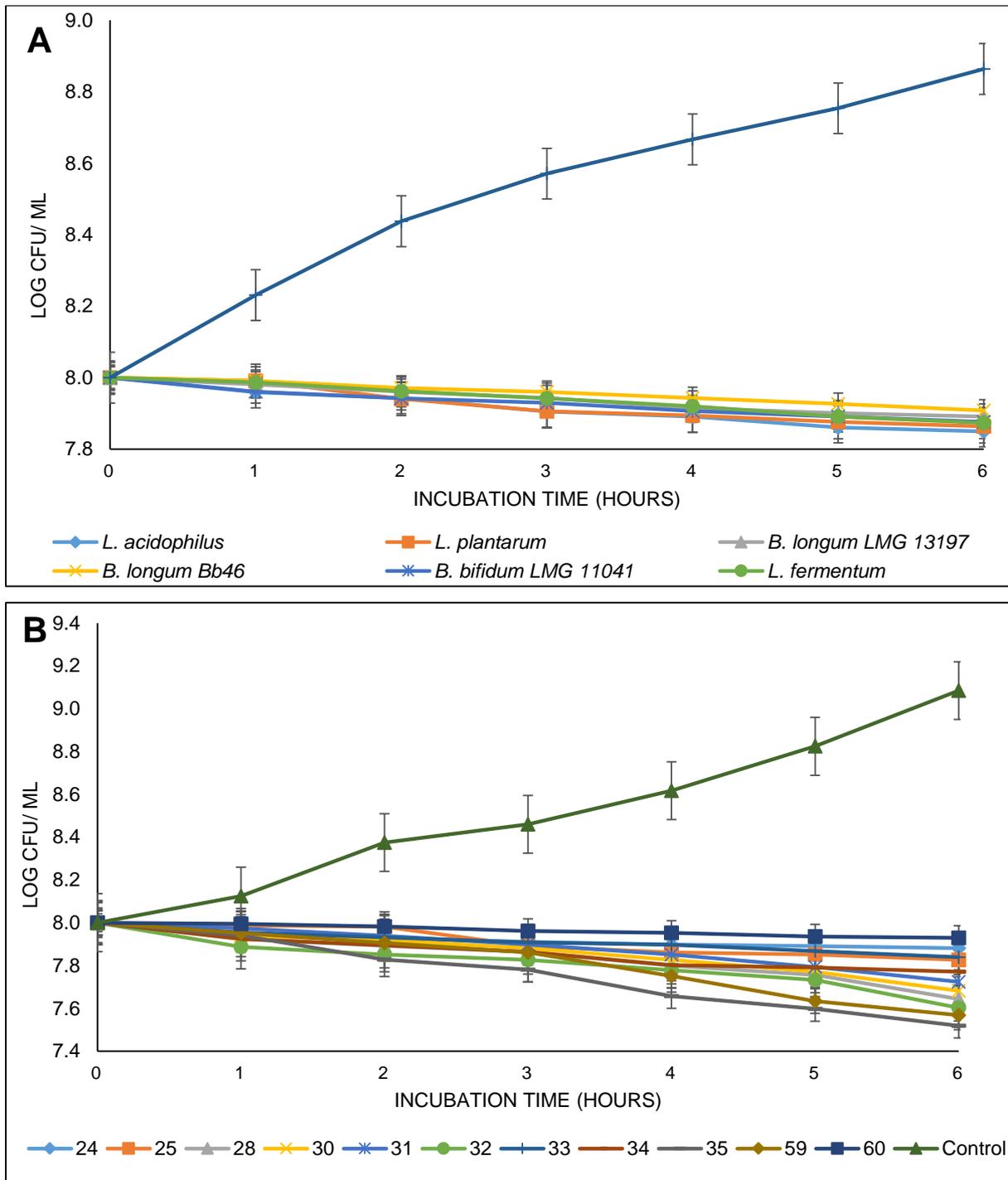


Figure 3.2: The effects of the single (A) and combination (B) probiotics on the growth of the pathogen *S. aureus* over a period of six hours. The control experiment was the pathogen *S. aureus* grown in the absence of probiotics.

3.4.2.2. Antagonistic effects of single and probiotic cocktails on *E. coli*

The study also investigated the inhibition of *Escherichia coli* by single and cocktails of multi-stress adapted probiotic strains (Figure 3.3). Similar to what was observed for *S. aureus*, *L. acidophilus* La14 150B was the most effective in inhibiting pathogen growth, showing a reduction in viable *E. coli* counts by 0.198 log cfu/ ml compared to 0.18, 0.17, 0.16, 0.16 and 0.15 log cfu/ ml for *L. plantarum*, *L. fermentum*, *B. bifidum* LMG 11041, *B. longum* LMG 13197 and *B. longum* Bb 46, respectively (Figure 3.3 A). The control culture increased throughout incubation time by 0.97 log cfu/ ml. Combination 4 reduced *E. coli* better than all the other combinations, reducing viable counts of *E. coli* from 8.00 to 7.49 log cfu/ ml a difference of 0.51 log cfu/ ml compared to 0.24, 0.15, 0.43, 0.47, 0.39, 0.20, 0.27, 0.34 and 0.41 log cfu/ ml for combinations 24, 25, 28, 31, 32, 33, 34, 35 and 59, respectively (Figure 3.3 B). As was observed for *S. aureus*, a cocktail of all the six non-adapted probiotic strains (combination 60) was the least effective in controlling growth of *E. coli*, resulting in 0.14 log cfu/ml reductions in numbers of viable *E. coli* during the 6 h of incubation. Viable numbers of *E. coli* incubated in absence of probiotics increased by 1.34 log cfu/ ml (Figure 3.3 B).

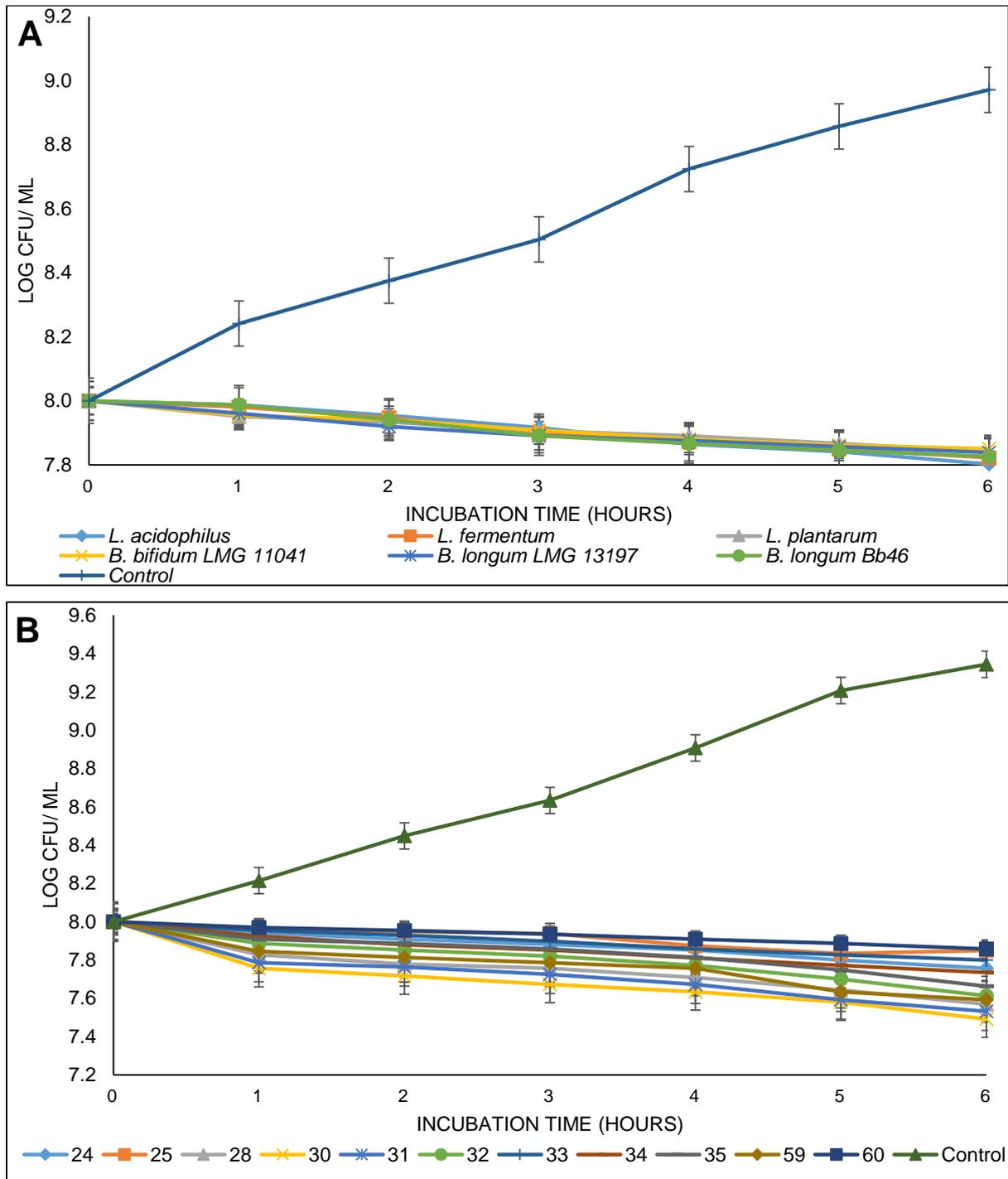


Figure 3.3: The effects of the single (A) and combination (B) probiotics on the growth of pathogenic *E. coli* over six hours. The control experiment was the pathogen *S. aureus* grown in the absence of probiotics .

When single probiotic cultures were used to inhibit the pathogens, *S. aureus* and *E. coli*, the same order of inhibition for both *S. aureus* and *E. coli* was reported. All the Lactobacilli strains were more aggressive and had better inhibitory effects against the tested pathogens than Bifidobacteria, indicating that they maintained their inhibitory effects. Although multi-stress adaptation improved inhibitory effects of Bifidobacteria, in terms of performance they could still not outperform the Lactobacilli. Therefore, from the current study it was concluded that the single Lactobacilli cultures are more aggressive and have better inhibitory effects than the Bifidobacteria culture. Superior inhibitory effects of Lactobacilli than Bifidobacteria have been reported elsewhere (Boudeau *et al.*, 2003; Canzi *et al.*, 2005; Gueimonde *et al.*, 2006), therefore this trend was the same even after pre-adaptation to multiple stresses. The antagonistic effects of the probiotic cells towards the pathogens are mostly related to the ability of the strain to secrete the broad spectrum antimicrobial substances (Gagnon, 2004). Therefore, the results suggest that exposure of the probiotics did not have negative effects on the ability of the probiotics to secrete the antimicrobial substances, a phenotype that is directly linked to pathogen inhibitory abilities of probiotics.

The inhibition of the different stress- adapted combinations compared to the combination of the non- adapted cells was studied. The study aimed to look at ways to enhance the inhibition of the pathogens and also to investigate as to whether the use of stress adapted cells in combinations will have enhanced effect on the pathogen inhibitions. In our results it was evident that cocktails of multi- stress adapted probiotics strains had better pathogen inhibition effects than a cocktail of non- adapted combination. This indicates that pre- adaptation of probiotics to multiple stresses enhanced their antipathogenic effects. The main advantage of using probiotic mixtures is that they have beneficial effects against a wide range of disorders (Drago *et al.*, 1997). This suggests that use of probiotic mixtures can be very important in many clinical models. Collado *et al.* (2006) used the single and combination probiotics to inhibit pathogens from adhering to the human intestinal mucus. They found that all the single probiotics inhibited the pathogens and that not only did their combination probiotics inhibit the pathogens; they also enhanced the inhibition percentages than when the single strains were used. In our study the same results were found, however we only used single stress- adapted cells and their combinations. It was therefore, hypothesized that the use of the multi stress- adapted combination cells have a better effect than either single stress adapted or a combination of the non- adapted cells. The results showed that the use of pre- adapted combination

probiotics enhances the inhibition of the pathogens. The enhancement of the pathogen inhibitions will therefore be useful in the probiotic concept.

3.4.3. Antibiotic assay of the different probiotic combinations

The overwhelming use of antibiotics has played a significant role in the outspread/emergence of antibiotic resistant bacteria (Ashraf and Shah, 2011). The use of antibiotic-resistant probiotic strains is beneficial to people with an abnormal intestinal microflora, with imbalanced or greatly reduced numbers of microbiota due to the administration of various antimicrobial agents (Salminen *et al.*, 1998). The advantages of using probiotics combinations over the single probiotics, especially in their prevention of heterogeneous diseases has already been discussed above. The resistance of the different probiotic combinations to antibiotics was investigated.

The presence of the zone of inhibition indicated the sensitivity to the antibiotics and the absence indicated the resistance of the probiotic strains to the antibiotic. All the probiotic combinations were resistant to streptomycin and oxacillin. Combinations 31, 32 and 35 were resistant to penicillin G while all the other combinations 24, 25, 28, 30, 34, 35, 59 and 60 were resistant. Combinations 24, 31, 32, 59 and 60 (no zones of inhibition) were resistant while combinations 25, 28, 30, 33, 34 and 35 (zones of inhibitions) were sensitive to tetracycline. Combination 24 was the only one resistant to erythromycin. All combinations had different zones of inhibitions for the different antibiotics. Combinations 32 and 34 were the most sensitive to fusidic acid. Combinations 31 was the most sensitive to novobiocin and chloramphenicol while combination 35 was the most sensitive to penicillin G, tetracycline and erythromycin.

Table 3.2: Antibiotic resistance of the different probiotic combinations

Antibiotics	Disc potency (µg)	24	25	28	30	31	32	33	34	35	59	60
		Zone of inhibition (mm)										
Fusidic acid	10	14 ± 2.5	18 ± 0.5	18 ± 1.0	15 ± 0.5	17 ± 1.0	18 ± 1.5	18 ± 0.5	18 ± 1.5	15 ± 1.5	16 ± 1.0	15 ± 0.5
Oxacillin	5	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
Novobiocin	5	15 ± 0.5	16 ± 1.0	15 ± 0.5	15 ± 0.5	18 ± 1.5	13 ± 1.0	12 ± 2.0	11 ± 1.0	12 ± 2.0	14 ± 0.5	15 ± 2.0
Penicillin G	1 unit	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	12 ± 2.0	12 ± 0.5	0 ± 0.0	0 ± 0.0	12 ± 1.5	0 ± 0.0	0 ± 0.0
Streptomycin	10	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
Tetracycline	25	0 ± 0.0	8 ± 1.5	10 ± 0.5	12 ± 1.5	0 ± 0.0	0 ± 0.0	10 ± 2.0	10 ± 1.5	13 ± .05	0 ± 0.0	0 ± 0.0
Chloramphenicol	25	22 ± 1.5	22 ± 1.5	25 ± 0.5	24 ± 2.0	27 ± 2.5	26 ± 0.5	20 ± 2.5	20 ± 0.5	23 ± 1.0	20 ± 0.0	27 ± 1.5
Erythromycin	5	0 ± 0.0	12 ± 0.5	13 ± 1.0	13 ± 1.5	12 ± 2.0	12 ± 0.5	10 ± 2.0	13 ± 0.5	16 ± 1.5	12 ± 1.0	13 ± 2.0

These results represent the means of three independent measurements with standard deviations

As discussed previously, probiotic combinations were used rather than single probiotics due to the advantage of probiotic combinations on heterogeneous diseases. Chapman *et al.*, (2011) supported the use of probiotic combinations than single probiotics. They reported that mixtures are more effective than their component strains. Due to the increasing resistance of most pathogens to antibiotics, the use of these alternative methods are more familiar this days. The antibiotic resistance of probiotics does not manifest a safety concern (Sharma *et al.*, 2014). Zhou *et al.*, (2005) performed a study on the antibiotic susceptibility of Lactobacilli and Bifidobacteria species and from their results, the both these strains were susceptible to penicillin, erythromycin, novobiocin, chloramphenicol, streptomycin and tetracycline and sensitive to fusidic acid. In this study, combinations of probiotics were however resistant to all these antibiotics. Therefore, probiotic mixtures are more resistant to antibiotics than their single strains. Even though Lactobacilli and Bifidobacteria spp. were used as the single strains to come up with the different combinations, the combinations were still more resistant to antibiotics than single strains as reported in other studies.

3.5. CONCLUSIONS

This study investigated the effect of single and their combinations against selected enteric pathogens. In simulated GIT conditions and the antibiotic assay, the combinations of stress-adapted probiotics survived well than the combination of non- adapted probiotics. For the antagonistic effects, probiotic combinations inhibited pathogens more than the single probiotics and also that the combinations of stress- adapted cells inhibited the same pathogens better than the combination of non- adapted cells.

Probiotic combinations may have better effects than the single probiotics and that the combinations that are prepared from stress- adapted cells are better than those that are composed of non- adapted cells.

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Chapter 4

General Conclusions and Recommendations

General conclusions

- Acid and bile tolerances of most of the stress-adapted cells were higher than of the non-adapted cells. Viability of all the adapted Lactobacilli and *Bifidobacterium bifidum* LMG 11041 were higher after sequential exposure to simulated gastric and intestinal fluids. For the two *B. longum* strains, viability of non-adapted cells was higher than for adapted cells after exposure to these fluids.
- Stress adaptation had no effect on bile salt hydrolase activities of *B. longum* LMG 13197, *Lactobacillus acidophilus* and *L. fermentum* but there was loss of BSH activity for *B. longum* Bb46, *B. bifidum* LMG 11041 and *L. plantarum*. A cocktail containing multi-stress adapted *L. plantarum* + *L. fermentum* + *B. longum* Bb46, survived better than all the other combinations in 2% bile concentration.
- A combination containing multi-stress adapted *L. acidophilus* La14 150B + *L. plantarum* + *L. fermentum* + *B. longum* LMG 13197 + *B. longum* Bb46 + *B. bifidum* LMG 11041 best tolerated acid than all the other combinations and survived better after the sequential exposure to gastric and intestinal conditions. A combination containing all the non-adapted was the least surviving in all the simulated gastrointestinal conditions.
- For the antipathogenic tests, combination 35 inhibited *S. aureus* better than the other combinations while combination containing *L. acidophilus* La14 150B + *L. plantarum* + *L. fermentum* + *B. longum* LMG 13197 + *B. longum* Bb46 + *B. bifidum* LMG 11041 (adapted) showed the highest inhibition of *E. coli*. A combination containing non-adapted cells was the least effective in inhibiting the two pathogens.
- Results indicated that it may be advantageous to use formulations of stress adapted cells than the non-adapted ones as they will be more tolerant to unfavourable conditions, while offering enhanced protection to consumers against enteric pathogens.

Recommendations and future work

- It would be necessary to investigate the survival of the multi-stress adapted probiotics in different food products such as African fermented beverages and dairy products to determine whether they remain viable throughout the shelf life of such products, since it is known that post-acidification that occurs during storage of such products is one of the factors resulting in decline of viable probiotic cultures in products.
- It still remains unknown whether the observed phenotypic characteristics in multiple stress-adapted probiotic cultures is transient or permanent. Future studies can look closely at stress-adapted cultures to determine whether the induced changes are maintained by such cultures. This study can be further extended to looking at different culture preservation methods to determine which method will best preserve the stability and hence the properties of probiotic cultures induced by stress adaptation.
- Understanding the molecular mechanism of multiple stress adaptation can advance the use of molecular techniques for production of robust probiotic cultures, allowing a more targeted approach, whereby specific genes identified as those whose expression is modified by exposure to stress factors, will be directly modified using recombinant DNA techniques.
- Microencapsulation is another mechanism that is widely used for improvement of viability of probiotic cultures in products during storage as well as during gastrointestinal transit. It will be interesting to investigate whether stability of stress adapted cells under these conditions can be enhanced further through their microencapsulation in food based coating materials, together with prebiotics. This will allow for production of even more stress tolerant synbiotic preparations.

APPENDIX

I. The combination preparations

Table 1: The six initial probiotic cultures that were used for making the different probiotic combinations

1. <i>L. acidophilus</i>
2. <i>L. fermentum</i>
3. <i>L. plantarum</i>
4. <i>B. longum</i> Bb46
5. <i>B. bifidum</i> LMG 11041
6. <i>B. longum</i> LMG 13197

Table 2: The double combinations prepared from the six initial probiotic cultures

7. <i>L. acidophilus</i> + <i>L. plantarum</i>	12. <i>L. plantarum</i> + <i>L. fermentum</i>	17. <i>L. fermentum</i> + <i>B. longum</i> LMG 13197
8. <i>L. acidophilus</i> + <i>L. fermentum</i>	13. <i>L. plantarum</i> + <i>B. longum</i> Bb46	18. <i>L. fermentum</i> + <i>B. longum</i> Bb46
9. <i>L. acidophilus</i> + <i>B. longum</i> Bb46	14. <i>L. plantarum</i> + <i>B. longum</i> LMG 13197	19. <i>B. longum</i> Bb46 + <i>B. bifidum</i> LMG 11041
10. <i>L. acidophilus</i> + <i>B. longum</i> LMG 13197	15. <i>L. plantarum</i> + <i>B. bifidum</i> LMG 11041	20. <i>B. longum</i> Bb46 + <i>B. longum</i> LMG 13197
11. <i>L. acidophilus</i> + <i>B. bifidum</i> LMG 11041	16. <i>L. fermentum</i> + <i>B. bifidum</i> LMG 11041	21. <i>B. longum</i> LMG 13197 + <i>B. bifidum</i> LMG 11041

Table 3: The triple combinations from the six initial probiotic cultures

22. <i>L. acidophilus</i> + <i>L. plantarum</i> + <i>L. fermentum</i>	32. <i>L. plantarum</i> + <i>L. fermentum</i> + <i>B. bifidum</i> LMG 11041
23. <i>L. acidophilus</i> + <i>L. plantarum</i> + <i>B. longum</i> Bb46	33. <i>L. plantarum</i> + <i>L. fermentum</i> + <i>B. longum</i> LMG 13197
24. <i>L. acidophilus</i> + <i>L. plantarum</i> + <i>B. longum</i> LMG 13197	34. <i>L. plantarum</i> + <i>L. fermentum</i> + <i>B. longum</i> Bb46
25. <i>L. acidophilus</i> + <i>L. plantarum</i> + <i>B. longum</i> LMG 13197	35. <i>L. plantarum</i> + <i>B. longum</i> Bb46 + <i>B. longum</i> LMG 13197
26. <i>L. acidophilus</i> + <i>L. fermentum</i> + <i>B. bifidum</i> LMG 11041	36. <i>L. plantarum</i> + <i>B. longum</i> Bb46 + <i>B. bifidum</i> LMG 11041
27. <i>L. acidophilus</i> + <i>L. fermentum</i> + <i>B. bifidum</i> LMG 11041	37. <i>L. plantarum</i> + <i>B. longum</i> LMG 13197 + <i>B. bifidum</i> LMG 11041
28. <i>L. acidophilus</i> + <i>L. fermentum</i> + <i>B. longum</i> Bb46	38. <i>L. fermentum</i> + <i>B. longum</i> Bb46 + <i>B. longum</i> LMG 13197
29. <i>L. acidophilus</i> + <i>B. longum</i> Bb46+ <i>B. longum</i> LMG 13197	39. <i>L. fermentum</i> + <i>B. longum</i> Bb46 + <i>B. bifidum</i> LMG 11041
30. <i>L. acidophilus</i> + <i>B. longum</i> Bb46 + <i>B. bifidum</i> LMG 11041	40. <i>L. fermentum</i> + <i>B. longum</i> LMG 13197 + <i>B. bifidum</i> LMG 11041
31. <i>L. acidophilus</i> + <i>B. longum</i> LMG 13197 + <i>B. bifidum</i> LMG 11041	41. <i>B. longum</i> Bb46 + <i>B. longum</i> LMG 13197 + <i>B. bifidum</i> LMG 11041

Table 4: The quadruples combinations that were combined from the six initial probiotic cultures

42. <i>L. acidophilus</i> + <i>L. plantarum</i> + <i>B. longum</i> Bb46 + <i>B. bifidum</i> LMG 11041
43. <i>L. acidophilus</i> + <i>L. plantarum</i> + <i>B. longum</i> LMG 13197 + <i>B. bifidum</i> LMG 11041
44. <i>L. acidophilus</i> + <i>L. plantarum</i> + <i>B. longum</i> LMG 13197 + <i>B. longum</i> Bb46
45. <i>L. acidophilus</i> + <i>L. plantarum</i> + <i>L. fermentum</i> + <i>B. bifidum</i> LMG 11041
46. <i>L. acidophilus</i> + <i>L. plantarum</i> + <i>L. fermentum</i> + <i>B. longum</i> Bb46
47. <i>L. acidophilus</i> + <i>L. plantarum</i> + <i>L. fermentum</i> + <i>B. longum</i> LMG 13197
48. <i>L. acidophilus</i> + <i>L. fermentum</i> + <i>B. longum</i> Bb46 + <i>B. bifidum</i> LMG 11041
49. <i>L. acidophilus</i> + <i>L. fermentum</i> + <i>B. bifidum</i> LMG 13197 + <i>B. bifidum</i> LMG 11041
50. <i>L. acidophilus</i> + <i>L. fermentum</i> + <i>B. bifidum</i> LMG 13197 + <i>B. longum</i> Bb46
51. <i>L. acidophilus</i> + <i>B. longum</i> Bb46+ <i>B. longum</i> LMG 13197 + <i>B. bifidum</i> LMG 11041
52. <i>L. acidophilus</i> + <i>B. longum</i> Bb46 + <i>B. longum</i> LMG 13197 + <i>B. bifidum</i> LMG 11041
53. <i>L. plantarum</i> + <i>L. fermentum</i> + <i>B. longum</i> Bb46 + <i>B. bifidum</i> LMG 11041
54. <i>L. plantarum</i> + <i>L. fermentum</i> + <i>B. longum</i> LMG 13197 + <i>B. longum</i> Bb46
55. <i>L. fermentum</i> + <i>B. longum</i> Bb46 + <i>B. longum</i> LMG 13197 + <i>B. bifidum</i> LMG 11041

Table 5: The quintet combinations that were made from the six initial probiotic cultures

56. <i>L. acidophilus</i> + <i>L. plantarum</i> + <i>L. fermentum</i> + <i>B. longum</i> LMG 13197 + <i>B. longum</i> Bb46
57. <i>L. acidophilus</i> + <i>L. plantarum</i> + <i>L. fermentum</i> + <i>B. longum</i> LMG 13197 + <i>B. bifidum</i> LMG 11041
58. <i>L. plantarum</i> + <i>L. fermentum</i> + <i>B. longum</i> LMG 13197 + <i>B. longum</i> Bb46 + <i>B. bifidum</i> LMG 11041

Table 6: The sextet combinations of both the stress adapted and the non- adapted probiotic cultures

59. <i>L. acidophilus</i> + <i>L. plantarum</i> + <i>L. fermentum</i> + <i>B. longum</i> LMG 13197 + <i>B. longum</i> Bb46 + <i>B. bifidum</i> LMG 11041 (Adapted)
60. <i>L. acidophilus</i> + <i>L. plantarum</i> + <i>L. fermentum</i> + <i>B. longum</i> LMG 13197 + <i>B. longum</i> Bb46 + <i>B. bifidum</i> LMG 11041 (Non- Adapted)

II. The preliminary experiments

Table 7: The bile tolerance, acid tolerance and the exposure to gastric and intestinal conditions for the combinations made up of two different stress adapted probiotics.

	Bile tolerance (2% , 120 min) (log cfu/ml)	Acid tolerance (pH 2, 120 min) (log cfu/ ml)	Exposure to Gastric (pH 2, 120 min) and Intestinal (pH 6.8, 120 min)conditions (log cfu/ml)
21	6.67	6.67	6.72
20	6.76	6.72	6.75
19	6.94	6.66	6.63
18	6.66	6.76	6.72
17	6.72	6.71	6.67
16	6.72	6.66	6.76
15	6.65	6.65	6.71
14	6.79	6.92	6.72
13	6.70	6.59	6.60
12	6.65	6.60	6.65
11	6.60	6.63	6.72
10	6.16	6.72	6.65
9	6.76	6.78	6.92
8	6.72	6.91	6.66
7	6.63	6.72	6.62

Table 8: The bile tolerance, acid tolerance and the exposure to gastric and intestinal conditions for the combinations made up of three different stress adapted probiotics

	Bile tolerance (2% , 120 min) (log cfu/ml)	Acid tolerance (pH 2, 120 min) (log cfu/ ml)	Exposure to Gastric (pH 2, 120 min) and Intestinal (pH 6.8, 120 min) conditions (log cfu/ml)
41	5.51	6.50	5.65
40	5.57	5.60	5.60
39	6.62	6.65	5.65
38	6.00	6.16	5.66
37	5.56	6.51	5.56
36	5.52	5.65	5.52
35	7.43	6.60	6.62
34	7.30	6.66	6.55
33	7.42	6.52	6.60
32	7.40	6.55	6.55
31	7.32	6.52	6.54
30	7.37	6.65	6.55
29	6.63	6.55	6.65
28	7.52	6.54	6.50
27	6.54	6.65	6.66
26	6.51	6.51	6.54
25	7.01	6.71	6.77
24	7.01	7.00	7.02
23	6.67	6.67	6.61
22	6.70	6.66	6.63

Table 9: The bile tolerance, acid tolerance and the exposure to gastric and intestinal conditions for the combinations made up of four different stress adapted probiotics

	Bile tolerance (2% , 120 min) (log cfu/ml)	Acid tolerance (pH 2, 120 min) (log cfu/ ml)	Exposure to Gastric (pH 2, 120 min) and Intestinal (pH 6.8, 120 min) conditions (log cfu/ml)
55	5.53	6.55	6.20
54	6.54	5.15	6.53
53	6.55	5.16	6.51
52	6.55	6.55	6.63
51	5.50	6.55	6.50
50	5.53	6.60	6.45
49	5.52	6.56	6.55
48	6.61	6.65	5.25
47	6.66	5.64	5.26
46	6.61	5.65	5.25
45	6.56	6.63	6.56
44	6.23	5.52	6.62
43	6.56	5.60	6.55
42	5.61	6.62	5.62

Table 10: The bile tolerance, acid tolerance and the exposure to gastric and intestinal conditions for the combinations made up of five different stress adapted probiotics

	Bile tolerance (2% , 120 min) (log cfu/ml)	Acid tolerance (pH 2, 120 min) (log cfu/ ml)	Exposure to Gastric (pH 2, 120 min) and Intestinal (pH 6.6, 120 min)conditions (log cfu/ml)
58	5.23	5.27	5.40
57	5.07	5.01	5.15
56	5.34	5.27	5.62

Table 11: The bile tolerance, acid tolerance and the exposure to gastric and intestinal conditions for the combinations made up of six different stress adapted (59) and non- adapted (60) probiotics

	Bile tolerance (2% , 120 min) (log cfu/ml)	Acid tolerance (pH 2, 120 min) (log cfu/ ml)	Exposure to Gastric (pH 2, 120 min) and Intestinal (pH 6.6, 120 min)conditions (log cfu/ml)
60	5.02	5.70	5.01
59	7.22	7.77	7.21