

***IN VITRO* ASSESSMENT OF THE PROBIOTIC PROPERTIES
OF LACTIC ACID BACTERIA ISOLATED FROM SORGHUM**

MAHEWU

By

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Abstract.

Fermentation of cereal grains is associated with beneficial microorganisms, referred to as probiotics. Probiotics are live microorganisms which confer health benefits to host organisms when consumed in adequate amounts. For a probiotic microorganism to exert its beneficial effects, it has to survive transit through the gastrointestinal tract (GIT) and colonise the host gut. Ability to survive simulated GIT transit *in vitro*, can be used to assess probiotic properties of bacteria isolated from fermented foods. The present study evaluated the *in vitro* probiotic potential of lactic acid bacteria (LAB) isolated from sorghum based *mahewu*. Nineteen LAB were isolated from different red and white sorghum *mahewu* batches and characterised using their biochemical properties. The LAB were then screened for ability to tolerate simulated gastric juice conditions. Each isolate was tested for ability to grow and remain viable in de Man Rogosa and Sharpe (MRS) broth adjusted to pH 2 and pH 3, MRS broth with 0.3 % bile, simulated gastric juice and for antimicrobial activity against *Escherichia coli* (ATCC 1129). For the tolerance to simulated gastric transit assays, cells from an overnight culture of each isolate were centrifuged at 5 000 rpm for 5 minutes, washed twice in Phosphate Buffered Saline (PBS) at pH 7.2 and inoculated into the test broth. The inoculated test broth was then incubated at 37°C for three hours. Tolerance was determined by evaluating viable counts in an aliquot of the inoculated test broth before incubation and after three hours of incubation. Growth of the isolates in the test broth was also evaluated by measuring optical density (O.D) of the culture at hourly intervals over the three hours at 620 nm using a spectrophotometer. For evaluation of antimicrobial activity of the isolates against *E. coli*, the culture supernatant of each isolate was used. The culture supernatant was prepared using 48 hour MRS broth cultures of each of the isolates. The 48 hour cultures were centrifuged at 10 000 rpm for ten minutes to pellet the bacteria cells. The culture supernatant of each isolate was then added to wells on Muller Hinton agar plates on which *E. coli* had been spread. The plates were incubated at 37°C for 24 hours. The results of the study indicated that LAB isolated from *mahewu* generally had good probiotic potential, 84.2 % of the isolates had all the probiotic properties tested for in the study. All the isolates remained viable after exposure to MRS broth adjusted to pH 3 for three hours at 37°C. Tolerance to pH 2 however, varied significantly ($p < 0.05$), 89.5 % ($n=19$) of the isolates showed tolerance but a reduction in percentage growth compared to growth in the control MRS broth at pH 6.5. Most of the isolates, 84.7 % showed ability to tolerate MRS broth containing 0.3 % porcine bile, the rest of the isolates were not able to grow in the presence of the bile. The tolerance of the isolates to the presence of bile differed significantly ($p < 0.05$), indicating that the mechanisms and ability to tolerate bile salts differ among LAB. All the isolates were able to inhibit growth of *E. coli*. The sizes of the zones of inhibition varied considerably. This was probably due to differences in the types and amounts of antimicrobial compounds produced by each isolate. Overall, the results indicated that the LAB isolates possess desirable *in vitro* probiotic properties. The levels of tolerance to simulated gastric conditions and antimicrobial activity of the isolates varied considerably, indicating that tolerance mechanisms might be dependent on the specific characteristics of each isolate. Generally, the different LAB isolates could be good candidates for further studies, with *in vivo* tests to validate their potential.

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Declaration.

I, Kanganwiro Mugwanda, declare that the thesis/dissertation, which I hereby submit for the degree of Master of Science in Biotechnology at the University of Zimbabwe, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution. I further declare that all sources cited or quoted are indicated by means of a comprehensive list of references.

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List of Abbreviations.

ANOVA	Analysis of Variance
BSH	Bile Salt Hydrolases
CFU	Colony Forming Units
FAO	Food and Agricultural Organisation
GI	Gastrointestinal
GIT	Gastrointestinal tract
GRAS	Generally Regarded As Safe
IL	Interleukin
Ig	Immunoglobulin
LAB	Lactic Acid Bacteria
MRS	de Man Rogosa and Sharpe
OD	Optical Density
Th	T helper cells
TNF	Tumour Necrosis Factor
PBS	Phosphate Buffered Saline
WHO	World Health Organisation
ZOI	Zone of Inhibition

Chapter One: Introduction

1.1 Overview

Cereals are an important source of dietary nutrients worldwide, providing more food energy than any other type of crops (Sarwar *et al.*, 2013). They have multiple beneficial attributes that can be exploited to develop novel cereal foods or cereal ingredients. Additionally, cereals can be used as fermentable substrates for growth of probiotic microorganisms and as encapsulation materials for the probiotics in order to enhance their stability (Charalampopoulous *et al.*, 2002).

The use of cereal foods in some applications is limited because cereals generally have a low protein content, deficiency in certain essential amino acids, low starch availability and have anti-nutrients and digestive enzyme inhibitors (Chavan and Kadam, 1989; Blandino *et al.*, 2003). Fermentation of cereal grains is one simple and economical method that may be used to remove these undesirable components and enhance the nutritive value of cereal foods (Simango, 1997).

Traditional fermented foods prepared from cereals such as maize, rice, wheat, barley, rye or sorghum are common in the world, particularly in the tropics and in Africa (Marsh *et al.*, 2014). Fermentation of these cereal grains is associated with beneficial microorganisms, referred to as probiotics, which have been shown to confer health benefits to the human body (Tannock *et al.*, 2000). Probiotic bacteria have a number of benefits, including that they inhibit the growth of harmful bacterial, promote good digestion and boost immune function of the human host. (Patricia *et al.*, 2002; Helland *et al.*, 2004).

Worldwide, the demand for consumption of health promoting foods is growing rapidly due to the increased awareness of consumers on the impact of food on health (Marsh *et al.*, 2014). Foods containing probiotic microorganisms in particular, could provide antimicrobial compounds that can be used to address the problem with antibiotic resistance in pathogenic

bacteria (Kalui *et al.*, 2009). Probiotic therapy can be used in the prevention and treatment of different gastrointestinal and urogenital infections, and therefore provide an alternative to the use of antibiotic drugs (Suskovic *et al.*, 2010).

Most probiotic foods that are currently available are dairy based (Granato *et al.*, 2010). Dairy products are popularly used to deliver probiotic microorganisms because they have good compatibility with the microorganisms and attractive sensory profiles (Mohammadi and Mortazavian, 2011). Consumers' preference are however, changing and inclining more with plant based dietary supplements, which usually have minimal or no cholesterol content (Lamsal and Faubion, 2009). A relatively high cholesterol content, lactose intolerance and allergy to milk proteins are some of the drawbacks related to the use of probiotic dairy products for a large percentage of consumers (Granato *et al.*, 2010). An estimated 70 % of the world population are lactose intolerant (Mattar, 2012). As a result, probiotic fermented cereal foods are increasingly becoming important.

Non-dairy probiotic beverages have the advantage that do not contain dairy allergens, have a low cholesterol content and are suitable for vegans (Prado *et al.*, 2008). Cereal based fermented beverages in particular, have the advantage that they have a higher content of some essential vitamins than milk and have significant amounts of beneficial bioactive compounds (Charalampopoulous *et al.*, 2002). Additionally, fermented cereal foods contain water-soluble fibres such as β -glucan, oligosaccharides and resistant starch, and thus have been suggested to fulfil the prebiotic concept (Shah, 2001; Andersson *et al.*, 2001).

In Zimbabwe, fermented beverages are commonly prepared using cereals such as maize (*Zea mays*), sorghum [*Sorghum bicolor* (L.) Moench], bulrush millet (*Pennisetum typhoideum*) or finger millet (*Eleusine coracana*) malt (Gadaga *et al.*, 1999). Sorghum is indigenous to Africa and it grows well in semi-arid regions where other cereals such as maize, wheat and rice cannot grow well (Haussamann *et al.*, 2000). Fermentation of sorghum could provide both probiotic

and whole grain benefits, such as phytochemicals and other bioactive components. Sorghum grain oligosaccharides can function as prebiotics, which have been shown to increase levels of beneficial bacteria in the large intestines, thereby improving gut health (Al-Sheraji *et al.*, 2013). Prebiotics are resistant to human digestive enzymes but serve as food for probiotics thereby promoting their growth and activity (Cummings *et al.*, 2001).

Traditional fermented cereal beverages such as sorghum based *mahewu* are poorly studied, there is little evidence linking them to proposed positive effects on human health. The proposed health benefits of fermented cereal beverages could be a result of direct interaction of ingested live microorganisms with the host, the probiotic effect or indirectly, as a result of interaction of the host and ingested microbial metabolites produced during fermentation, the biogenic effect (Stanton *et al.*, 2005). Ideally, the proposed health claims should be backed by scientific evidence in the form of replicated, randomised, controlled *in vivo* and *in vitro* trials (Pineiro and Stanton, 2007). This study will focus on *in vitro* assessment of potential probiotic attributes of sorghum based *mahewu*.

1.1.1 Justification of the study

There is increasing demand for the development of probiotic foods that are plant-based and can be consumed by lactose and gluten intolerant people. Zimbabwean indigenous fermented foods remain under-utilised in this regard, particularly fermented cereal beverages such as *mahewu*. The probiotic potential of sorghum based *mahewu* needs to be evaluated in order for it to be utilised as a synbiotic product or used as a source of new probiotic formulations.

1.1.2 Objectives of the Study

The main objective of this study was to evaluate the potential of fermented sorghum *mahewu* as a probiotic food.

1.1.3 Specific objectives

The specific objectives of this study were to:

1. Isolate, characterise and quantify Lactic Acid Bacteria (LAB) present before and at the end of the fermentation of *mahewu*
2. Evaluate the probiotic properties of the isolated LAB according to FAO/WHO guidelines, 2001.

Chapter Two: Literature Review

2.1 Probiotics definition

The term probiotic is derived from the Greek words ‘pro’ and ‘bio’, meaning ‘for life’. Probiotics are live microorganisms which, when administered in sufficient numbers, confer a health benefit to the host (FAO/WHO, 2001). This definition, assumes that the microorganisms contained in a probiotic product remain viable in transit through the gastrointestinal tract (GIT) and are fully able to grow and colonise the colon upon arrival. Another assumption is that they remain viable in sufficient numbers to confer the intended health benefit (Fredua-Agyema and Gaisford, 2014). There is no global agreement on a minimum number of bacteria per unit contained in a probiotic product necessary for functionality. It is however generally accepted that probiotic products should have a minimum concentration of 1×10^6 colony forming units (CFUs) per millilitre or gram at the time of consumption (Lourens-Hattingh and Viljoen, 2001).

2.1.1 History of probiotics

The protective nature of microorganisms, particularly lactic acid bacteria (LAB) contained in fermented foods has a long history (Lamsal and Faubion, 2009). Live bacterial cultures have been consumed as part of the human diet for centuries, in the form of fermented milk, cereals and vegetables, without knowledge of the functional ingredients or how they exert their effect (Kolida *et al.*, 2006). One of the earliest reports of probiotic intake was the ingestion of sour milk by nomads over 2000 years ago (Kolida *et al.*, 2006). At the beginning of the 20th century, Elie Metchnikoff (Nobel Prize winner), working at the Pasteur Institute, suggested that the dependence of the intestinal microflora on the food consumed by the host could allow intentional modification of the flora and replacement of harmful microbes with beneficial microbes (Morelli, 2000). He made this suggestion after observing differences in resistance of patients to fatal cholera. Metchnikoff attributed the good health and longevity of Bulgarian peasants to the ingestion of kefir, a fermented milk drink (Ranadheera *et al.*, 2010). He isolated

a bacterium from the kefir and named it *Bulgarian bacillus*, which is currently known as *Lactobacillus delbrueckii* ssp *Bulgaricus* (Morelli, 2000).

In 1917, Nissle, a physician isolated a strain of *Escherichia coli* from the only healthy soldier in a group suffering from infectious diarrhoea (Sonnenborn and Schulze, 2009). He hypothesised that the strain of *E.coli* had the ability to suppress other pathogenic bacteria and therefore was responsible for protection from diarrhoea (Kolida *et al.*, 2006). Such observations on the benefits of ingesting live microorganisms provided the foundations upon which the probiotic concept was based and developed.

2.1.2 Selection criteria for potential probiotic microorganisms

A number of criteria have to be met for a microorganism to be characterised as a probiotic (Dunne *et al.*, 2001). The criteria are strain-specific and have been designed with special attention to *in vitro* assays, which are used to perform a preliminary selection of probiotic microorganisms (Morelli, 2000). The preliminary selection is followed by *in vivo* studies in which healthy volunteers are dosed with strain(s) that performed well *in vitro* assays (Dunne *et al.*, 2001). Possible considerations in the selection process include:

- i. Origin of the strain.

It has been suggested that the best probiotic strains are those of human origin (Morelli, 2000). Generally, it is thought that probiotic microorganisms isolated from the human gastrointestinal (GI) tract are safer for human consumption and may be more adapted to colonising the large intestine (Kolida *et al.*, 2006). Probiotics of human origin are thought to have a better chance of out-competing resident bacteria and of establishing at a numerically significant level in their new host (Morelli, 2000). Certain commercially available probiotics are however not of human origin (Dunne *et al.*, 2001).

ii. Ability of the strain to survive during gastric transit

In order to reach the intestine, ingested probiotic strains must survive the passage of the upper gastrointestinal tract (Kolida *et al.*, 2006). The acidity in the stomach provides a barrier to the entrance of bacteria into the gut (Morelli, 2000). Traits such as resistance to gastric acid, bile salts, and proteolytic enzymes are selected for (De Vuyst *et al.*, 2008). Survival of gastric transit by a probiotic is dependent on tolerance to GIT conditions during a transit time of one to four hours.

iii. Ability of the strain to adhere to intestinal epithelial cells

Adherence to intestinal epithelial cells is considered to be important for colonisation of the GI tract (Morelli, 2000). Consequently, it is thought necessary for the functionality of probiotic microorganisms (Dunne *et al.*, 2001). Colonisation of probiotic bacteria to epithelial cells in the intestine has been shown to prevent the adherence of certain pathogens. The probiotic bacteria and pathogens compete for receptor sites at the intestinal surface (de Vries *et al.*, 2006). The competition for these receptors reduces the capacity of the intestinal surface for colonisation by enteropathogens and thus protects the host from infection. In a study by Shornikova *et al.*, (1997), *L. reuteri* was successfully used to reduce the duration of rotavirus induced diarrhoea in hospitalised infants. In a separate study carried out using *L. bulgaricus*, which does not adhere to and colonise the GIT, there was no reduction in the duration of rotavirus diarrhoea. This indicates that adhesion to the host GIT epithelial cells is important in the probiotic prolonging its colonisation of the GI tract and exerting its beneficial effects. Adhesion of probiotics to host epithelia also plays a significant role in stimulating the immune system thereby enhancing the natural immune response (Yokokura *et al.*, 1986).

iv. Ability of the strain to tolerate bile salts

Bile acids are synthesized in the liver and secreted into the duodenum in a conjugated form (Dunne *et al.*, 2001). An estimated 500-700 ml/day of conjugated bile are secreted into the duodenum from the gall bladder after food intake by an individual (Hofmann and Roda, 1984). Therefore, exposure to bile is a serious challenge for commensal and foreign bacteria. Conjugated bile salts undergo chemical modifications in the colon, one such modification is deconjugation by microorganisms. Conjugated and deconjugated bile acids have antimicrobial activity against both Gram negative and Gram positive bacteria (Dunne *et al.*, 2001). Gram positive bacteria are reported to be more sensitive to the deleterious effects of deconjugated bile salt (Floch *et al.*, 1972; Begley *et al.*, 2005).

One of the suggested mechanisms by which intestinal bacteria cope with toxic bile concentrations is by hydrolysing the bile. Bile salts hydrolysis is catalysed by intracellular enzymes called bile salt hydrolases (BSHs). A number of BSHs have been identified and characterised in probiotic bacteria, and ability of probiotic strains to tolerate bile salts has often been included among the criteria for probiotic strain selection. Gilliland *et al.*, 1984 showed that bile resistance could differ among members of the same species of enteric lactobacilli and that the difference could account for variation in the ability of different strains to colonise the intestinal tract of calves. Excessive microbial BSH activity however, has been suggested to have potentially detrimental health effects. Therefore, it is as yet not completely clear whether BSH activity is in fact a desirable trait in potential probiotic bacteria (Begley *et al.*, 2005). The genera commonly used as probiotics however, do not have the capacity to dehydroxylate deconjugated bile salts (Ahn *et al.*, 2003). The majority of the products of the breakdown bile salts may therefore be precipitated and excreted in faeces (Takahashi and Morotomi, 1994).

v. Safety of the strain.

For an organism to be considered as a probiotic it must be certified as safe. This generally means that the organism must be non-pathogenic, free of transferable antibiotic resistance determinants, have no history of association with diseases such as infective endocarditis or GI disorders and be non-mucin degrading (Saarela *et al.*, 2000). Each potential probiotic strain should undergo safety assessment by characterisation and thorough evaluation of safety *in vitro*, followed by *in vivo* studies.

Probiotic bacteria strains must be chosen using accurate selection criteria in order to survive the transition through the gastrointestinal tract and successfully colonise it for a sufficiently long period to achieve the desired healthy effect (Morelli, 2000). In addition to *in vitro* and *in vivo* tests for efficacy of probiotics to human health, a number of technological criteria have to be met if the probiotic is to be commercialised (Dunne *et al.*, 2001). A potential probiotic should have the following; ability to proliferate in the probiotic delivery medium, good sensory profiles, ability to remain viable during processing and stability in the product during storage (Saarela *et al.*, 2000). The ability of probiotic microorganisms to remain viable and active in the host GIT are thought to be essential for their optimal functionality. Some studies have however suggested that non-viable probiotic microorganisms can have beneficial effects on the host, such as immune modulation and carcinogen binding (Ouweland and Salminen, 1998; Salminen *et al.*, 1999).

2.1.3 Methods for *in vitro* evaluation of potential probiotics.

A number of techniques have been suggested for assaying for potential probiotic microorganisms. The FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food (2002) summarised some of the commonly used tests for study of probiotic strains. Table 2:1 shows some of the methods.

Table 2:1 Main currently used *in vitro* tests for study of probiotic strains.

Ability to resist or tolerate gastric acidity
Resistance to bile salts
Ability to hydrolyse bile salts
Ability to adhere to mucus and/or human epithelial cells and cell lines
Antimicrobial activity against potentially pathogenic bacteria
Ability to reduce adhesion of pathogenic bacteria to epithelial surfaces
Resistance to spermicides (for probiotics for vaginal use)

Source: (FAO/WHO, 2002).

The main parameters of the *in vitro* assays are summarised below.

- i. Tolerance to gastric acidity

The stomach secretes over two litres of gastric juice daily, with pH as low as 1.5 (Morelli, 2000). The low pH of the stomach and the antimicrobial action of pepsin are suggested to provide a barrier to the entry of bacteria into the intestinal tract (Holzapfel *et al.*, 1998). There is no specific protocol for the screening of acid tolerance of potential probiotic strains. Most *in vitro* assays commonly used, select strains that tolerate low pH values (Papadimitriou *et al.*, 2015). *In vitro* assays for tolerance to gastric acidity are typically based on exposing the cells of the test bacteria to a pH between 2 and 3 for varying lengths of time. The exact methods employed vary considerably. The common method is the use of acidified de Man, Rogosa and

Sharpe (MRS) broth. Some studies however, use acidified MRS agar or buffers with no nutrients such as phosphate buffered saline (PBS) or HCl solutions. The length of exposure of the isolates to the acidic conditions varies from one to 24 hours. Considering the short time that food is in the stomach, three hours is suggested to probably be the most relevant time.

A similar experimental set ups using alkaline conditions have reflected that that most probiotic bacteria are resistant to alkaline conditions. For this reason, acid tolerance tests are still used to allow simple, routine screening of large numbers of bacterial strains for probiotic attributes (Papadimitriou *et al.*, 2015).

ii. Bile salt tolerance

Microorganisms that survive the acidic conditions in the stomach enter the duodenum in a stressed state. The microorganisms have to deal with adverse conditions of the small intestine such as the presence of bile salts. The transit time of food through the small intestine is generally between one and four hours (Smith, 1995). Bile salt-resistant lactic acid bacteria can be selected by testing their ability to grow and survive in the presence of various concentrations of bile salt (Gilliland *et al.*, 1984 and Chung *et al.*, 1999). The physiological concentration of bile salts in humans is around 0.3 % (Dunne *et al.*, 2001). Concentrations of bile salts ranging from 0.15 to 0.3 % have been recommended as suitable for selecting probiotic bacteria for human use (Goldin and Gorbach, 1992). Gilliland *et al.* (1984) reported that 0.3 % is considered to be a critical concentration for screening for resistant strains. *In vitro* testing for bile tolerance of lactic acid bacteria usually employs MRS broth to which bile salts have been added. Bile tolerance assays can be carried out using human, porcine and bovine bile. The concentrations of bile used in various studies typically ranges from 0.3 to 7.5 % and survival has been assessed after periods ranging from 1 to 48 hours (Dunne *et al.*, 2001). *In vitro* bile salt tolerance was shown to correlate well with *in vivo* gastric survival (Conway *et al.*, 1987).

In vivo validation of survival through the human stomach is more difficult to obtain. *In vitro* assays of the inhibitory effect of bile acids on the growth of probiotic strains provide a relatively simple method to perform these tests.

iii. Bile salt hydrolase (BSH) activity.

BSH activity can be qualitatively determined on agar plates supplemented with bile salts. Commonly, the bile salt, taurodeoxycholate and CaCl₂ are added to the growth media. BSH activity is indicated by presence of a white halo around colonies of the test bacteria (Begley *et al.*, 2006).

iv. Adherence to mucus and/or human epithelial cells and cell lines.

In vitro tests screening for bacteria that adhere to the GIT epithelial cells are commonly carried out using human epithelial cell lines. Some *in vitro* tests are carried out using immobilized molecules obtained from the intestinal mucus (Jensen *et al.*, 2012).

v. Antimicrobial activity.

The production of antimicrobial compounds such as organic acids by bacteria is one of the properties used to screen for probiotic properties (Dunne *et al.*, 2001). Bacteria that are able to colonise the gut and produce antimicrobial compounds could have bactericidal and/or bacteriostatic effects against pathogenic bacteria (Shah, 2007). This property is important for effective competitive exclusion of pathogenic microorganisms (Ouweland and Salminen, 1998).

2.1.4 Mechanism of action of probiotic bacteria.

A number of beneficial effects resulting from the ingestion of probiotic microorganisms have been suggested. The mechanisms through which probiotics exert their effects on the host include:

i. Stimulation of host immune system.

Probiotic bacteria have been shown to stimulate both non-specific (innate) and specific (adaptive) immunity thereby improving host immunity to enteropathogens (Gill *et al.*, 2009). Stimulation of the host has a number of immunomodulatory effects. The exact mechanisms of immunomodulatory activities of probiotics are however not completely understood.

Modulation of immune responses by probiotic bacteria is thought to be a result of their interaction with intestinal epithelial cells which initiates cascading immune responses. Binding of the probiotic cells stimulates epithelial cells to produce cytokines which play a role in the stimulation and regulation of the immune response (Lammers *et al.*, 2003). The immunochemicals involved in the immunomodulation by probiotic bacteria have been studied both *in vitro* and *in vivo* using a number of models. Schultz *et al.*, 2003 observed that the stimulation of human peripheral blood mononuclear cells with *Lactobacillus rhamnosus* GG *in vitro* resulted in the production of interleukin 4 (IL-4), IL-6, IL-10, tumour necrosis factor alpha (TNF α). The production of these Th2 cytokines resulted in development of B cells and immunoglobulin isotype switching required for the production of antibodies. A study by Halper *et al.*, 2003 showed that *L. acidophilus* supernatant stimulated the proliferation of macrophages and lymphocytes *in vitro*. The supernatant was studied *in vivo* and it resulted in chemotaxis and promoted proliferation of inflammatory cells.

Stimulation of specific and non-specific immune responses by probiotic bacteria could justify their potential use for therapeutic and prophylactic treatment of infections and carcinogenesis (Gardiner *et al.*, 2002).

ii. Production of antimicrobial metabolites.

Probiotics increase the production of antimicrobial compounds in the host gut. This effect can be achieved by stimulating host epithelial cells to produce antimicrobial peptides (Gogineni *et*

al., 2013). Intestinal epithelial cells can be stimulated to produce antimicrobial peptides called defensins and cathelicidins. These peptides have been shown to display antimicrobial activity against pathogenic bacteria, fungi and some viruses. Probiotics can affect other microorganisms directly by releasing peptides, referred to as bacteriocins (Gogineni *et al.*, 2013). Bacteriocins are proteinaceous antimicrobial compounds that are active against other bacteria (Bowdish *et al.*, 2005). *Lactobacillus acidophilus* has been shown to produce two compounds, bacteriocin lactacin B and acidolin. Acidolin was shown to inhibit enteropathogenic organisms and viruses. Some antimicrobial metabolites produced by probiotic bacteria are strain specific. A unique bacteriocin, bifidocin B, is produced by *B. bifidum* NCFB 1454 and is active towards Gram-positive bacteria. Additionally, probiotic bacteria produce antibacterial substances such as ammonia, hydrogen peroxide, organic acids and bacteriocins which inhibit the growth of enteropathogenic microorganisms (Gogineni *et al.*, 2013; Fuller, 1989)

Probiotic microorganisms may also produce molecules which interfere with quorum sensing in bacteria. Quorum sensing is a chemical signalling mechanism by which pathogenic bacteria communicate with each other and the surrounding environment in order to successfully colonise the host (Gogineni *et al.*, 2013). Interference with quorum sensing signalling inhibits pathogen activity. A study by Medellin-Pena *et al.*, 2007 demonstrated that *Lactobacillus acidophilus* secretes a molecule that inhibits the quorum sensing signalling or directly interacts with bacterial transcription of *E. coli* O157 gene. This prevents colonisation of the host gut by the pathogen.

iii. Competitive exclusion of pathogenic microorganisms.

Probiotics play a role in maintaining the normal intestinal microflora by competitive exclusion of invading pathogens (Sengupta *et al.*, 2013). In order for bacteria to colonise the host GIT, they bind to receptors that are expressed on surface of intestinal epithelial cells. Binding of bacteria to host GIT epithelia receptors is competitive, this means that probiotic bacteria can competitively exclude pathogenic bacteria (Gogineni *et al.*, 2013). *L. rhamnosus* R0011 and *L. acidophilus* R0052 were shown to inhibit infection of intestinal cells by *E. coli* by reducing their adhesion (Sherman *et al.*, 2005).

iv. Improved barrier function.

Epithelial cells of the intestine are in constant contact with enteric microorganisms. They provide a first line barrier to the entrance of enteropathogens into the host blood system (Sengupta *et al.*, 2013). The intestinal barrier is a major defense mechanism used to maintain epithelial integrity and to protect the host organism from the microorganisms that enter the gut. Defences of the epithelial barrier include a mucus layer, the epithelial junction adhesion complex, secretion of antimicrobial peptides and secretion of immunoglobulin A (IgA).

Probiotics are suggested to improve the epithelial barrier function by inducing production of mucin, increased secretion of IgA, enhancing tight junction function and preventing programmed epithelial cell death (apoptosis) (Gogineni *et al.*, 2013; Sengupta *et al.*, 2013). *Lactobacillus rhamnosus* GG was shown to prevent cytokine-induced apoptosis in human and mouse intestinal epithelial cell models by inhibiting tumour necrosis factor (TNF) (Yan and Polk, 2002).

- v. Production of enzymes and other beneficial substances into the intestines.

Probiotic bacteria may alter metabolism of the host organism by increasing digestive enzyme activity and decreasing bacterial enzyme activity. They have been suggested to produce enzymes such as beta galactosidase and carbohydrase. Collington *et al.*, (1990) studied the effect of a mixture of multiple probiotic strains of *Lactobacillus* spp. and *Streptococcus faecium* on the small intestine of piglets. The probiotics were observed to promote significantly higher carbohydrase enzyme activities. Probiotic microorganisms colonising the intestine may secrete the enzyme resulting in increase in intestinal amylase activity. Additionally, probiotics microorganisms may alter the pH of the gut and modify its flora to promote increased activity of intestinal enzymes and digestibility of nutrients. Summary of the mechanisms by which probiotics exert their effects on host organisms are shown in Figure 2.1.

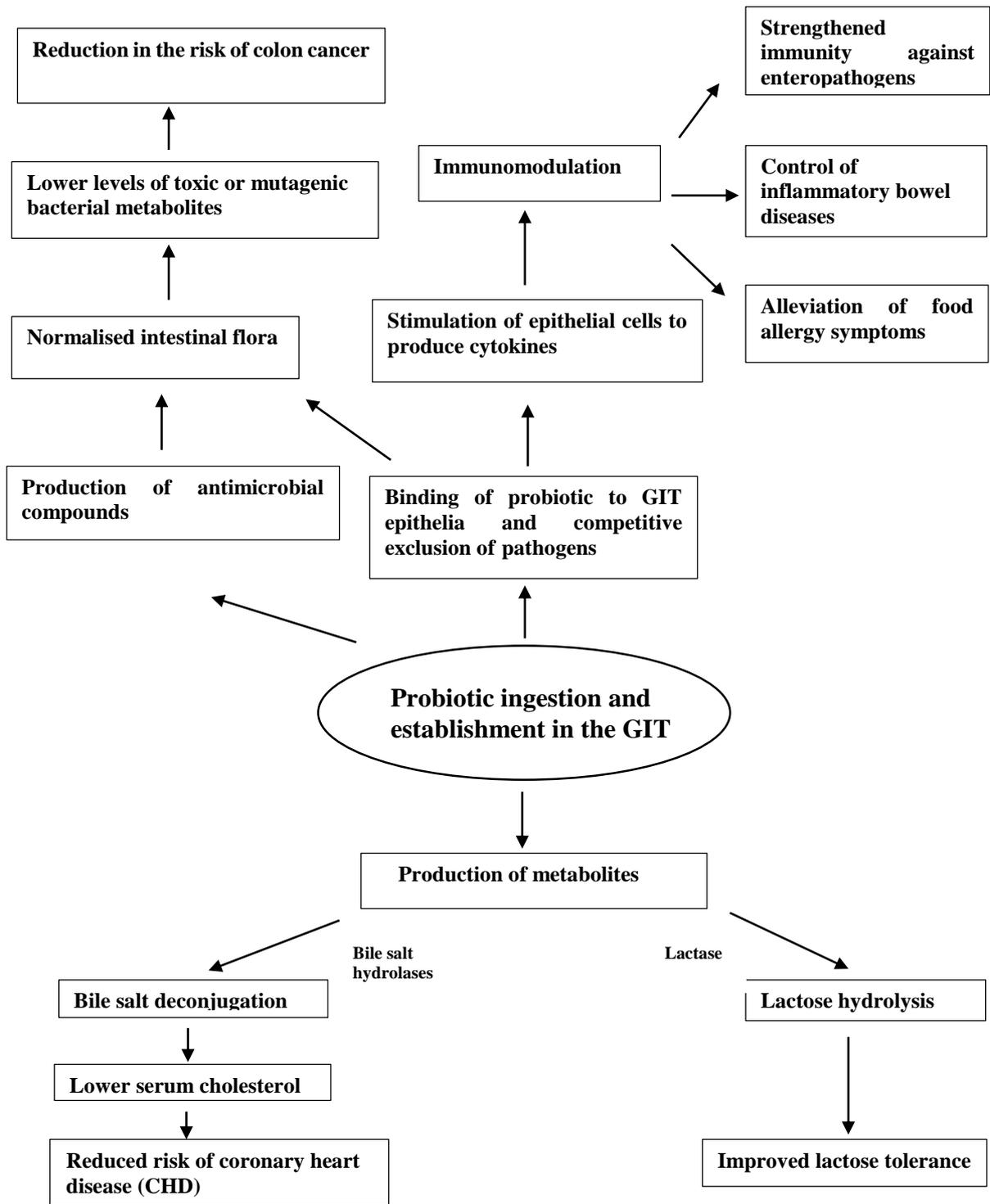


Figure 2.2 Summary of the mechanisms by which probiotics exert their effects on host organisms.

2.1.5 Health benefits of probiotic bacteria

A number of beneficial health effects have been linked to the consumption of probiotics. Some of the proposed health effects have been demonstrated using both *in vivo* and *in vitro* trials. The proposed health benefits of probiotics are thought to be strain-specific (Playne, 2002).

Table 2.2: Summary of some of the proven and suggested benefits of different probiotic strains to human health.

Health benefit	Details	Reference(s)
Treatment of rotavirus diarrhoea	Rotavirus diarrhoea is caused by rotavirus. <i>Lactobacillus rhamnosus</i> GG was effective in treating the diarrhoea in human trials and reduction of the duration of diarrhoea by about 50 % in several controlled trials	Shah, 2007
	<i>L. casei</i> Shirota, <i>B. infantis</i> , <i>S. thermophilus</i> were shown to be effective in prevention and treatment	Saavedra <i>et al.</i> , 1994
Treatment of antibiotic associated diarrhoea (AAD)	<i>L. rhamnosus</i> GG and <i>S. boulardii</i> have been used in successful treatment by oral administration	Armuzzi <i>et al.</i> , 2001
Treatment of Helicobacter pylori infection	<i>L. acidophilus</i> , <i>L. reuteri</i> and <i>L. casei</i> Shirota can inhibit <i>H. pylori</i> both <i>in vitro</i> and <i>in vivo</i>	Franavilla <i>et al.</i> , 2008
Treatment or reduction of lactose intolerance	Lactose intolerance is congenital deficiency of the enzyme β -galactosidase, resulting in inability to digest lactose <i>L. acidophilus</i> and <i>L. reuteri</i> improves maldigestion symptoms in intolerant patients <i>B. longum</i> improves digestion of lactose	Jiang <i>et al.</i> , 1996
Treatment of atopic dermatitis	Atopic dermatitis is an allergic skin condition Oral administration of whey formular supplemented with <i>B. animalis</i> subsp <i>lactis</i> Bb12 or <i>L. rhamnosus</i> GG significantly alleviated clinical symptoms of atopic dermatitis	Isolauri <i>et al.</i> , 2000
Inflammatory bowel disease	<i>Lb. rhamnosus</i> GG has been used successfully in the treatment of colitis	Biller <i>et al.</i> , 1995

2.1.6 Microorganisms currently used as probiotics

Probiotic products available on the market commonly contain strains of the *Bifidobacterium*, *Lactobacillus* and *Streptococcus* genera and yeasts of the *Saccharomyces* genus (Fijan, 2014). *Lactobacillus* spp. and *Bifidobacterium* spp. are the most commonly used as probiotics because they have a long history of safe use in food fermentation and are constituents of the normal intestinal microflora. They have been conferred with the generally regarded as safe (GRAS) status (O'Sullivan *et al.*, 1992). Most commercially available probiotic formulations contain *Lactobacillus* spp. as opposed to *Bifidobacterium* spp. The use of lactobacilli in probiotic formulations is preferred because they are more aero-tolerant and therefore more stable in food products (Crittenden and Playne, 1996). Table 2.3 shows a summary of some of the microorganisms currently used as probiotics.

Table 2.3: Some microorganisms currently used as probiotics.

Genera	Probiotic species	Details of commercial use	Reference(s)
Lactobacilli	<i>Lactobacillus acidophilus</i>	Used in most yoghurt preparations	Anuradha and Rajeshwari, 2005
	<i>Lb. plantarum</i> 299v	Probi AB, Sweden	
	<i>Lb. casei</i>	<i>Lb. Casei Shirota</i> , (Yakult Honsha Co. Ltd., Japan)	
	<i>Lb. johnsonii</i> LA-1 <i>Lb. reuteri</i>	Nestlé BioGaia AB, Sweden	Kolida <i>et al.</i> ,2006 Anuradha and Rajeshwari, 2005
Bifidobacteria	<i>Lb.bravis</i>	Used in yogurt fermentation	
	<i>Lb. rhamnosus</i> GG	Valio Ltd, Finland	Kolida <i>et al.</i> ,2006
	<i>Bifidobacterium bifidum</i> <i>Bif. lactis</i>	Vita Fresh, Mevgal S.A Marketed as <i>Bifidus</i> BL by CERELAC® Infant Cereals, Nestlé, Zimbabwe	Kolida <i>et al.</i> ,2006
Enterococci	<i>Enterococcus faecium</i>	Used in fermented food products and supplements	Anuradha and Rajeshwari, 2005
Saccharomyces	<i>E. faecalis</i>		
	<i>Saccharomyces boulardii</i>	Marketed as a dietary supplement	Anuradha and Rajeshwari, 2005
Streptococcus	<i>S. thermophilus</i>	Marketed under the trade name Biopot by Onken	

2.1.7 General Characteristics of Probiotic Lactic acid bacteria (LAB).

LAB are a group of bacteria that produce lactic acid as their main fermentation product. Typical LAB are Gram-positive, catalase-negative and facultative anaerobes. The main LAB genera are *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus* (Salminen *et al.*, 1998). Although phylogenetically different, bacteria belonging to the *Propionobacterium* and *Bifidobacterium* genera have been grouped under LAB. LAB are fastidious, acid-tolerant, and strictly fermentative. The LAB genera can be further divided into two major groups based on whether their fermentative pathway is homofermentative or heterofermentative (Geurts *et al.*, 1999).

The main characteristics of the major LAB genera are summarised in Table 2.4

Table 2.4: Characteristics and occurrence of the main LAB genera.

Genera	Characteristics	Reference(s)
Lactobacilli	Gram positive rods or coccobacilli which occur singly or in chains Have an optimal growth temperature between 35-40°C The optimum pH for growth is 5.5-6 Homofermentative or heterofermentative metabolism, depending on the species Size ranges from 0.5-0.8 µm across by 2 to 9 µm long	Hammes and Vogel, 1995; Felis & Dellaglio, 2007
Bifidobacterium	Gram positive rods with varying shapes, they are often Y-shaped or clubbed at the end Have an optimum growth temperature for Bifidobacterium is 37- 41°C The optimum pH for growth is 6.5 to 7.0 Possess a unique fructose-6-phosphate phosphoketolase pathway fermentation pathway, the Bifidus pathway Acetic and lactic acid are formed primarily in a 3:2 molar ratio during fermentation	Scardovi, 1986; Shah, 2000
Streptococcus	Gram-positive spherical or ovoid cells, occur in pairs and chains Homofermentative metabolism Sensitive to vancomycin	Hammes and Vogel, 1995 ; Shah, 2000
Leuconostoc	Ovoid cocci, often forming chains Heterofermentative Grow well at 10°C	Hammes and Vogel, 1995; Shah, 2000
Pediococcus	Usually occur in pairs or tetrads Divide along two planes of symmetry Homofermentative Resistant to vancomycin	Hammes and Vogel, 1995 ; Shah, 2000

2.1.8 Importance and occurrence of LAB

LAB are commonly found in carbohydrate rich environments. They occur on plants and materials of plant origin, mucosal membranes of the oral, intestinal, respiratory and vaginal cavities of animals and in fermented foods. Fermented foods are food substrates that are invaded or overgrown by microorganisms (Steinkraus, 2002). The main types of food fermentation include alcoholic, acetic acid, alkali and lactic acid fermentation (Blandino *et al.*, 2003). Lactic acid fermentations are those in which the fermentable sugars are converted to lactic acid by LAB such as *L. mesenteroides*, *L. brevis*, *L. plantarum*, *P. cerevisiae*, *L. bulgaricus*, *L. acidophilus*, *B. bifidus*, *S. thermophilus* and *S. lactis* (Steinkraus, 2002; Mensah, 1997). The LAB produce enzymes, particularly amylases, proteases, lipases which catalyse the hydrolysis of the carbohydrates, proteins and lipids to non-toxic products with pleasant and attractive sensory properties to the human consumer (Steinkraus, 2002).

Lactic acid fermentations are important in food processing because they:

- i. Allow development of a wide diversity of flavours, aromas and textures in food; providing variety to human diets (Nout, 2009).
- ii. Produce bacteriocins, lactic acid and other chemicals which are important in preservation of food. This important in the reducing of the risk of food borne diseases, particularly in the communities where lack of access to refrigeration poses a major barrier to ensuring food safety (Gadaga *et al.*, 1999).
- iii. Enhance food substrates nutritionally with vitamins, protein, essential amino acids (Steinkraus, 2002).
- iv. Detoxify and remove undesirable substance during food fermentation processing. Studies have shown that lactic acid fermentation results in the breakdown and inactivation of mycotoxins without the production of toxic end products (Alberts *et al.*, 2006).

- v. Reduce cooking times and fuel requirements (Steinkraus, 2002).
- vi. Produce prebiotics (Taylor, 2015).

2.2 Lactic acid Fermented foods

Lactic acid fermentation of is one of the oldest technologies used in food processing (Oyewole, 1997). A wide variety of foods are derived from the technology, which is used at household level and small or large scale industrial level. Lactic acid fermentation at household level is usually spontaneous, being brought about by LAB present in the primary food or the starter culture. The technology is common in traditional households in many parts of the world. There are regional differences in the raw materials used, manufacturing practices, desired sensory profiles and acceptable quality level. The differences are a result of factors such as available raw materials, tools or technologies available and cultural practices.

Lactic acid fermented foods can be made using cereals, milk, root crops, and vegetables and to a lesser extend fish and meat (Mensah, 1997). Fermentation of these raw materials has been an important part of the human diet all over the world (Tamang, 1998).

2.2.1 Cereals commonly used in Lactic Acid Fermentations in Africa.

In Africa, lactic acid fermented foods are commonly prepared using cereals, root crops and milk. Cereals are good substrates for lactic acid fermentation because they are carbohydrate rich (Achi *et al.*, 2015). The grains are processed and fermented in various ways to obtain a range of indigenous products such as porridges, breads, couscous, porridges, gruels, pancakes and beverages (Steinkraus, 1996; Taylor, 2003). These foods are commonly used as refreshments, weaning foods and as main meals.

A large proportion of the fermented foods in Eastern and Southern Africa are prepared using the cereals maize, millet and sorghum (Oyewole, 1997). The characteristics of each of these cereals are summarised below.

i. Sorghum

Sorghum is a cultivated grass belonging to the family *Poaceae* (or *Gramineae*). Grain sorghums are classified as *Sorghum bicolor* (L.) Moench and *Sorghum vulgare* (Okorie and Oke, 2003). There are many varieties of *Sorghum bicolor*, with grain colour ranging from white, red, brown and mixed classes (Rooney and Waniska, 2000).

Sorghum is gluten-free, and therefore it is suitable for people with gluten intolerance or coeliac disease. Additionally, it is a potentially important source of nutraceuticals such as antioxidant phenolic compounds and cholesterol-lowering waxes (Taylor *et al.*, 2006). Compared to other cereals, sorghum has the highest content of phenolic compounds reaching up to 6% (w/w) in some varieties (Dicko *et al.*, 2006; Awika and Rooney, 2004). According to Chung *et al.*, (1998) almost all classes of phenolic compounds are found in sorghum. The types and amounts of phenolic compounds vary greatly between and within the species, with cultivation environment and processing technologies. The presence of phenolic compounds in sorghum is generally used as justification to promote consumption of the cereal as a health promoting food.

Some of the phenolic compounds found in sorghum are however, not health promoting, and have been shown to have adverse effects on dietary protein digestibility, digestive enzyme activity and mineral bioavailability (Duodu *et al.*, 2002). This affects the nutritional quality of sorghum foods. The poor digestibility of protein, poor palatability of some varieties and low nutritive value of the grains among other factors undermines the nutritional value and utilisation of the crop (Onyango *et al.*, 2013). Lactic acid fermentation has been shown to improve the digestibility of sorghum proteins, possibly through modification of their structure. Malting of sorghum is known to improve protein quality through proteolysis and transamination. Malting

also results in increased alpha-amylase activity. Foods containing sorghum malt therefore have improved carbohydrate digestibility and palatability. Sorghum foods that combine both malting and fermentation are beneficial in that they have improved nutrient availability and potentially beneficial microorganisms (Onyango *et al.*, 2013).

ii. Millet

Millet is a general category for several species of small grained cereal crops. The most widely cultivated millets are; finger millet (*Eleusine coracana*), pearl millet (*Pennisetum glaucum*), foxtail millet (*Setaria italica*), proso millet (*Panicum miliaceum*), and barnyard millet (*Echinochloa colona*). Pearl millet (*Pennisetum glaucum*) is the most widely grown type of millet. In world cereal crop rankings, millets is among the least important of cereals, contributing less than 2 % of the world's grain annual production. They are however, important in Africa traditional diets (Steinkraus, 1996).

Millets are tolerant to semi-arid conditions and low soil fertility which are prevalent in most regions in Africa, they can be grown in areas where other cereal crops, such as maize or wheat would not survive. They are used in the preparation of traditional fermented beverages, porridges and flat breads.

Their nutrient profile compares well and is even superior to major cereals in terms of energy value, proteins, fat and minerals (Anu *et al.*, 2006). A study by Burton *et al.*, (1972) found that the essential amino acid profile of pearl millet is 40 % richer in lysine and methionine and 30 % richer in threonine than in protein of maize. Another nutritional advantage of millet is that it is a gluten free crop and therefore can be consumed by people who are gluten intolerant. Millets are good sources of phytochemicals such as phenolic acids and phytoestrogens (Osman, 2011). These phytochemicals are said to confer a number of health advantages, phytates for instance are linked to cancer reduction. However, phytochemicals like phytate, polyphenols, oxalates and tannins can have negative effects. These act as antinutrients by forming complexes with

dietary minerals, such as calcium, zinc, iron and calcium, resulting in reduction in bioavailability (Arora *et al.*, 2003).

A number of studies have shown that malting and fermentation of millets can be effectively used for improving nutritional quality of cereal grain by increasing protein content and digestibility and available lysine content (Inyang and Zakari, 2008, Ali *et al.*, 2003). Fermentation has also been found to decrease the concentrations of the following antinutrients: trypsin inhibitory activity, amylase inhibitor, phytate, and tannins (Osman, 2011).

I. Maize

Maize (*Zea mays*) is a member of the family *Poaceae* (or *Gramineae*). Maize is the world's most widely grown cereal, cultivated across a range of climates. Maize can be used to prepare thick porridges, thin porridges for weaning, non-alcoholic fermented beverages and breads (Steinkraus, 1996). The whole maize grain provides mainly carbohydrates and is a good source of many nutrients such as thiamine (Vitamin B1), Pantothenic acid (Vitamin B5), ascorbic acid (Vitamin C). It however contains low protein and antinutrients which may affect nutrient digestion and bioavailability. Fermentation of maize grains has been shown to reduce levels of antinutrients and improve protein digestibility of maize and bioavailability of nutrients (Steinkraus, 1996).

Maize foods may have safety issues due to production of mycotoxins during storage. A study by Chelule *et al.*, 2010 showed that the level of mycotoxins such as aflatoxin B1 and fumonisin B1 are significantly reduced during lactic acid fermentation of maize samples.

2.2.2 Lactic acid fermented foods in Africa with probiotic potential

A number of studies have isolated, characterised and identified LAB from African foods. The type of organisms associated with each type of fermented foods generally depends on parameters such as temperature, inoculum concentration, and nature of cereal and length of the fermentation period (Achi *et al.*, 2015). Table 2.5 summarises the nature of the fermented product and isolated LAB.

Table 2.5: Summary of cereal based lactic acid fermented foods in Africa

Product name	Texture	Area of production	Main Substrate(s)	LAB isolated	References
Ogi	Soft or stiff gel	Nigeria, Benin. West Africa	Maize, Sorghum Or Millet	<i>Lactobacillus plantarum</i> , <i>L. fermentum</i> , <i>Lactobacillus spp</i>	Achi <i>et al.</i> , 2015
Fura	Dumpling	West Africa	Maize, Sorghum Millet	<i>Lactobacillus plantarum</i> , <i>Pedococcus</i> , <i>Leuconostoc</i> , <i>Streptococcus</i> , <i>Enterococcus</i>	Achi <i>et al.</i> , 2015 Owusu-Kwarteng <i>et al.</i> , 2010
Mahewu	Beverage/ gruel	Southern Africa	Maize, Sorghum or Millet	<i>L. delbrueckii</i> , <i>L. bulgaricus</i>	Bvochora <i>et al.</i> , 1999 Mugochi <i>et al.</i> , 2001
Injera	Flat bread/ pancake	Ethiopia	Sorghum, Tef, Maize or Wheat	<i>L. mesenteroides</i> <i>S. faecalis</i> <i>P. cerevisiae</i> , <i>L. brevis</i> , <i>L. plantarum</i> , <i>L. fermentum</i>	Gashe, 1985
Koko or Akasa	Porridge	Ghana	Maize	<i>L. fermentum</i> <i>L. salivarius</i> , <i>P. pentosaceus</i> , <i>P. acidilactici</i> <i>L. paraplantarum</i>	Lei and Jakobsen, 2004
Bushera,	Beverage/ gruel	Uganda	Sorghum, Millet	<i>Lactobacillus</i> , <i>Streptococcus</i> , <i>Enterococcus</i>	Marsh <i>et al.</i> , 2014 Muyanja <i>et al.</i> , 2002
Koko Sour Water	Beverage	Ghana	Pearl Millet	<i>L. fermentum</i> , <i>L. salivarius</i> , <i>Pediococcus</i> spp.	Owusu-Kwarteng <i>et al.</i> , 2010
Togwa	Porridge	Tanzania	Maize, Sorghum or Finger Millet	<i>L. plantarum</i> , <i>L. brevis</i> , <i>L. fermentum</i> , <i>L. cellobiosus</i> , <i>P. pentosaceus</i>	Mugula <i>et al.</i> , 2003

2.2.2.1 *Mahewu*, an African fermented food.

Mahewu is a lactic acid-fermented cereal beverage of Southern Africa (Chavan and Kadam, 1989, McMaster *et al.*, 2005). The term *mahewu* is synonymous with *amahewu* (Ndebele or Zulu), *amarehwu/aramrewu* (Xhosa) and *machleu/mageu* (Sotho) (Holzapfel, 1989). The most commonly used term is *mahewu*. Preparation techniques and materials used in *mahewu* preparation may vary slightly across different ethnic groups.

In Zimbabwe, *mahewu* is prepared from maize or sorghum flour (Mugochi *et al.*, 2001; Bvochora *et al.*, 1999). Although it can be made from sorghum, *mahewu* is almost exclusively prepared using maize (Taylor, 2003). The *mahewu* is prepared by boiling a broth mixture of the maize or sorghum flour and water (1:10 w/v ratio) for about 10-15 minutes to gelatinise the starch in the flour (Bvochora *et al.*, 1999). The cereal base provides fermentable sugars which are utilised by the fermenting microorganisms (Taylor, 2004). The cooked broth is then cooled to about 40 °C and sorghum malt, millet malt or wheat flour is added to the porridge. The malt and wheat flour provide amylolytic enzymes, particular β -amylase and lactic acid bacterium for the spontaneous fermentation process (Gadaga *et al.*, 1999). The slurry is then left to ferment at ambient temperature, typically for 18-24 hours (Simango, 1997; Odunfa *et al.*, 2001). Alternatively, *mahewu* can be prepared by mashing left over thick mealie meal porridge (sadza) into a slurry and fermenting it overnight (Gadaga *et al.*, 1999).

The fermentation of *mahewu* is a spontaneous process carried out by the natural flora of the malt. The predominant microorganism in the fermentation of African *mahewu* is *Lactococcus lactis subsp. lactis* (Steinkraus *et al.*, 1993). Back-slopping is usually practised in the preparation of *mahewu* to increase the concentration of starter micro-organisms. This shortens the fermentation process as the desired pH and organoleptic properties are achieved faster.

Mahewu is typically consumed when it reaches a pH of about 3.5 to 3.9 (Steinkraus, 2002). A clean, sour flavour due to lactate is preferred, and acetic and butyric acid should not be present.

Mahewu is reported to have a shelf life of one to two days. The fermentation process is not stopped during storage, as a result *mahewu* kept for longer may become too sour and develop off flavours (Simango, 1997).

Home production of *mahewu* is widely practised, particularly in rural Zimbabwe. Large scale production is carried out by industrial concerns and mining companies for consumption by their labourers (Mugochi *et al.*, 2001). Industrially produced *mahewu* usually has a pH of 3.5 (Taylor, 2004). The industrial production of *mahewu* differs from the traditional process in that there is the use of thermophilic LAB cultures, usually *L. delbrueckii*, which only produce lactic acid and the extension of the shelf life of the product by pasteurisation and/or chemical preservation (Taylor, 2004). The commercial *mahewu* product also differs from the traditional *mahewu*, it is often artificially flavoured with flavours such as banana, strawberry pineapple, and guava.

Chapter Three: Materials and Methods

3.1 Materials

Sorghum meal (malted and unmalted) used in the study was obtained from Utsanzi[®]. Two brands of unmalted sorghum meal were used in the preparation of sorghum-based *mahewu*, namely, Red Sorghum and White Sorghum. The same brand of sorghum malt was used to prepare *mahewu* using both types of sorghum meal.

3.2 Reagents and chemicals

The media used in the study de Man Rogosa and Sharpe (MRS) Lactobacilli agar, MRS broth, and Muller Hinton agar were obtained from HiMedia Laboratories, India. The porcine bile, pepsin and all the chemicals used to prepare buffer solutions and reagents were obtained from Sigma-Aldrich, Germany, unless otherwise stated.

3.3 Preparation of sorghum *mahewu*

Mahewu was prepared as outlined by Bvochora *et al.*, 1999, with some modifications. A paste was made initially by mixing 250 ml warm water and 25 g unmalted sorghum meal. The mixture was boiled for 10 minutes to gelatinise the starch. After this, 250 ml of cold water was added to the mixture to reduce viscosity and lower the temperature to 40 °C. Subsequently, 13.5 g of sorghum malt was added to the mixture. The mixture was left to ferment in an Erlenmeyer flask for 48 hours at room temperature. After thorough mixing of the fermenting broth, samples were collected for analysis. Collected samples were centrifuged at 2500 rpm for 10 minutes and the supernatant was used as required for analysis.

3.4 Measurement of pH and Titratable Acidity

The pH of the *mahewu* was measured immediately after centrifugation using a pH meter (Crison GLP 21, SA). Measurement was carried out for the first seven hours of fermentation and thereafter at 24 hours and 48 hours.

The method for measurement of titratable acidity of the *mahewu* samples was carried out according to Onyango *et al.*, 2013. Titratable acidity was determined by titration with 0.1 M NaOH to an end-point pH of 8.2, using 0.5 % phenolphthalein as an indicator. The titre volume was noted and used for calculation of titratable acid, which was expressed as percentage lactic acid. The following formula was used to calculate percentage lactic acid:

$$\text{Percentage Lactic Acid} = A * 0.009 * 100 / V$$

Where A= volume of 0.1 M NaOH required for the titration, V= volume of the sample tested and 0.009 is a constant.

3.5 Isolation of Lactic Acid Bacteria (LAB) from sorghum *mahewu*

LAB were isolated from *mahewu* using the spread plate technique. Ten-fold serial dilutions of the centrifuged samples were prepared using sterile peptone water as a diluent. Samples from the last three dilutions (100 µl) were then spread on MRS agar plates and incubated anaerobically using a BBL GasPak System® (Becton, Dickinson and Company, USA) at 37°C for 48 hours. The spread plates were used to estimate cell counts and randomly select LAB for purification.

3.6 Purification of isolates

Pure isolates were obtained from the MRS agar spread plates using the streak plate method as described by Awan and Rahman (2002). Morphologically distinct colonies were selected and allocated an isolate code with a letter component indicating that it was isolated from either red sorghum (R) or white sorghum (W) *mahewu*. The number component of the code indicated the order of selection. Each selected colony was picked with a sterilised loop and streaked on a sterile MRS agar plate using a four-phase streaking pattern. The streaked plates were incubated anaerobically at 37°C for 48 hours. The pure cultures were stored in MRS broth with 20 % sterile glycerol (v/v) at -80°C. The cultures stored at -80°C were revived by sub-culturing at 37°C in sterile MRS broth two times prior to use. MRS agar plates stored at 4°C were used as working stocks.

3.7 Presumptive Identification

LAB were presumptively identified using their morphological and biochemical characteristics. The macroscopic appearance of all the colonies obtained after 48 hours of incubation on MRS agar plates was used for the initial presumptive identification of isolates. The colonies were examined for cultural and morphological characteristics such as size, shape, elevation, colour and texture. The colonies were further characterised using the catalase test and the Gram stain.

Gram positive and catalase negative isolates were further characterised using their sugar fermentation patterns.

3.7.1 Procedure for the Catalase test

The catalase test for detection of catalase producing ability of the each isolates was carried using 3 % hydrogen peroxide (H_2O_2). A drop of 3 % H_2O_2 was placed on a clean glass slide. A loopful culture from each pure colony on MRS agar was then collected using a sterile wooden applicator and mixed in the drop. A positive catalase test was indicated by the production of bubbles. A few tiny bubbles after 20 to 30 seconds were not considered as positive. *Escherichia coli* was used as the positive control for the test.

3.7.2 Procedure for the Gram stain

The Gram staining procedure was carried out with some modifications. A drop of sterile distilled water was placed on a clean slide. A small amount of the test culture was then transferred to the slide using a sterile loop and mixed in. The smear was air dried and heat fixed over a gentle flame. Subsequently, the smear was covered with 1 % aqueous solution of crystal violet for one minute, and washed with distilled water. The smear was then dipped in 5 % solution of $NaHCO_3$ for three minutes to enhance formation of the crystal violet iodine complex. Following this, Lugol's iodine solution was poured on the smear, allowed to sit for two minutes and then rinsed with distilled water. Alcohol (95 %) was then poured gently on the smear for decolourization. The 95 % alcohol was poured on the smear for 10-15 seconds, until the colour of the alcohol running from the slide was clear. The smear was then counter stained with 2 % Safranin solution for 10 seconds. The smear was subsequently washed with distilled water and dried by blotting. The slides were observed under 40 X and 100 X power of objective lens. *E.coli* was used as the negative control for the test.

3.7.3 Sugar fermentation profiles of the isolates

The sugar fermentation profiles of the isolates were determined using a Phenol Red Broth microtiter plate assay. Phenol red broth was prepared using tryptone (10 g), NaCl (5 g) and phenol red (0.018 g) and sterilised by autoclaving. The test carbohydrate was then added to the sterilised medium. Washed cell suspensions were prepared by centrifuging an overnight culture of each isolate at 5000 rpm for 5 minutes and washing the pellet twice in Phosphate Buffer Solution (9 g/L NaCl, 9 g/L Na₂HPO₄·2H₂O, 1.5 g/L KH₂PO₄) adjusted to pH 7.2. The cells were then resuspended in sterile phenol red broth without any carbohydrate and mixed by vortexing. To each well of the microtiter plate, 180 µl and 20 µl of the washed cells were added. The microtiter plates were then incubated anaerobically in a BBL GasPak Jar for 24 hours. The results were scored according to the change in colour of the phenol red broth; a red colour was scored as negative, orange as weakly positive and yellow as positive.

3.8 Evaluation of LAB for probiotic attributes.

Probiotic attributes were evaluated by exposing washed cells of each isolate to the different test solutions, simulating gastric juice conditions.

3.8.1 Preparation of washed cell suspensions

Washed cells for the assay were prepared using a 1 ml aliquot of the overnight culture of each isolate. The cells were collected by centrifugation at 5000 rpm for 5 minutes and washed twice in Phosphate Buffered Saline, pH 7.2. The washed cells were then resuspended in each test solution.

3.8.2 Evaluation of pH tolerance.

An aliquot of the washed cells of each LAB isolate was inoculated (1% v/v) into sterile MRS broth tubes adjusted to pH 2.0, 3.0 and 6.5 (growth control) using 3.0 M HCl and 0.5 M NaOH and incubated at 37°C for 3 hours. Samples (10 µl) were collected from each tube before incubation and after 3 hours of incubation. The samples were inoculated onto MRS agar plates

and incubated anaerobically at 37°C for 48 hours. Subsequent growth of LAB on MRS agar was used to designate isolates as pH tolerant. Growth of the isolates was also monitored by measuring optical density (OD) at 620 nm using a spectrophotometer (Spectronic® Genesys™ Spectrophotometer, USA). OD₆₂₀ was monitored at hourly intervals for three hours. The experiment was performed in duplicate and repeated twice.

3.8.3 Tolerance to simulated gastric juice.

Tolerance to simulated gastric transit was determined following the method described by Charteris *et al.*, (1998). Simulated gastric juice was freshly prepared using 3 mg/ml pepsin derived from porcine mucosa (Sigma) dissolved in sterile 0.5 % NaCl (w/v) adjusted to pH 2.0. The pH of the simulated gastric juice was adjusted using sterile 1.0 M HCl and 0.5 M NaOH. A 0.2 ml aliquot of each washed cell suspension was transferred to an Eppendorf tube and mixed with 0.3 ml of 0.5 % (w/v) NaCl and 1.0 ml of simulated gastric (pH 2.0). For evaluation of tolerance simulated juice, each washed cell suspension was exposed to the simulated gastric juice for three hours. Aliquots of mixture (10 µl) were collected before incubation and after three 3 hours. The aliquots were diluted ten-fold using sterile 0.1 % peptone saline water and spread plated on the MRS agar for the evaluation of viable bacterial counts. Subsequent growth of each isolate on MRS agar after incubation anaerobically for 48 hours was used to indicate tolerance of the isolates.

3.8.4 Investigation of bile tolerance of the LAB isolates.

The assay was carried out according to Jacobsen *et al.*, (1999), with modifications. MRS with 0.3 % porcine bile and normal MRS (0 % bile) were used for the assay. Each solution was inoculated with 1 % (v/v) of washed cells of an overnight culture of the test isolate and cultured at 37°C for three hours. Changes in OD₆₂₀ were measured at hourly intervals for the three hours. The assay was carried out in duplicate.

3.8.5 Evaluation of antimicrobial activity of the LAB isolates.

The antibacterial activity test was performed using the agar-well-diffusion method and *Escherichia coli* (ATCC 11229) as the test microorganism. Antimicrobial metabolite production was achieved by culturing each isolate in MRS broth for 48 hours under the stationary condition. The tubes containing the 48 hour culture were centrifuged at 10 000 rpm for 10 minutes. Each culture supernatant was then transferred to fresh tubes. Evaluation of antibacterial activity of the culture supernatants was carried out on Muller Hinton agar plates. The *E.coli* culture used for the assay was adjusted to 0.5 McFarland's standard and spread on the agar plates using sterile cotton swabs. The plates were allowed to dry and four wells (each 5 mm in diameter) were made in agar plates using a sterile borer. Each well was loaded with 50 µl of isolated bacterial culture filtrate supernatant. The inhibitory activity of MRS broth was determined by adding 50 µl of sterile MRS broth to the agar wells. The plates was incubated at 37°C for 24 hours after which the diameter of the zone of inhibition was measured. The assay was carried out in quadruplicate.

3.8.6 Analysis of results.

The results obtained in the study were computed using GraphPad Prism™ software. Analysis of variance (ANOVA) was used to evaluate the effect of different treatments on the isolates. A P value ≤ 0.05 was considered statistically significant.

Chapter Four: Results.

4.1 pH and percentage lactic acid level of the *mahewu*.

The pH readings and percentage lactic acid level of *mahewu* samples during the first six hours of fermentation are summarised in Figure 4.1.

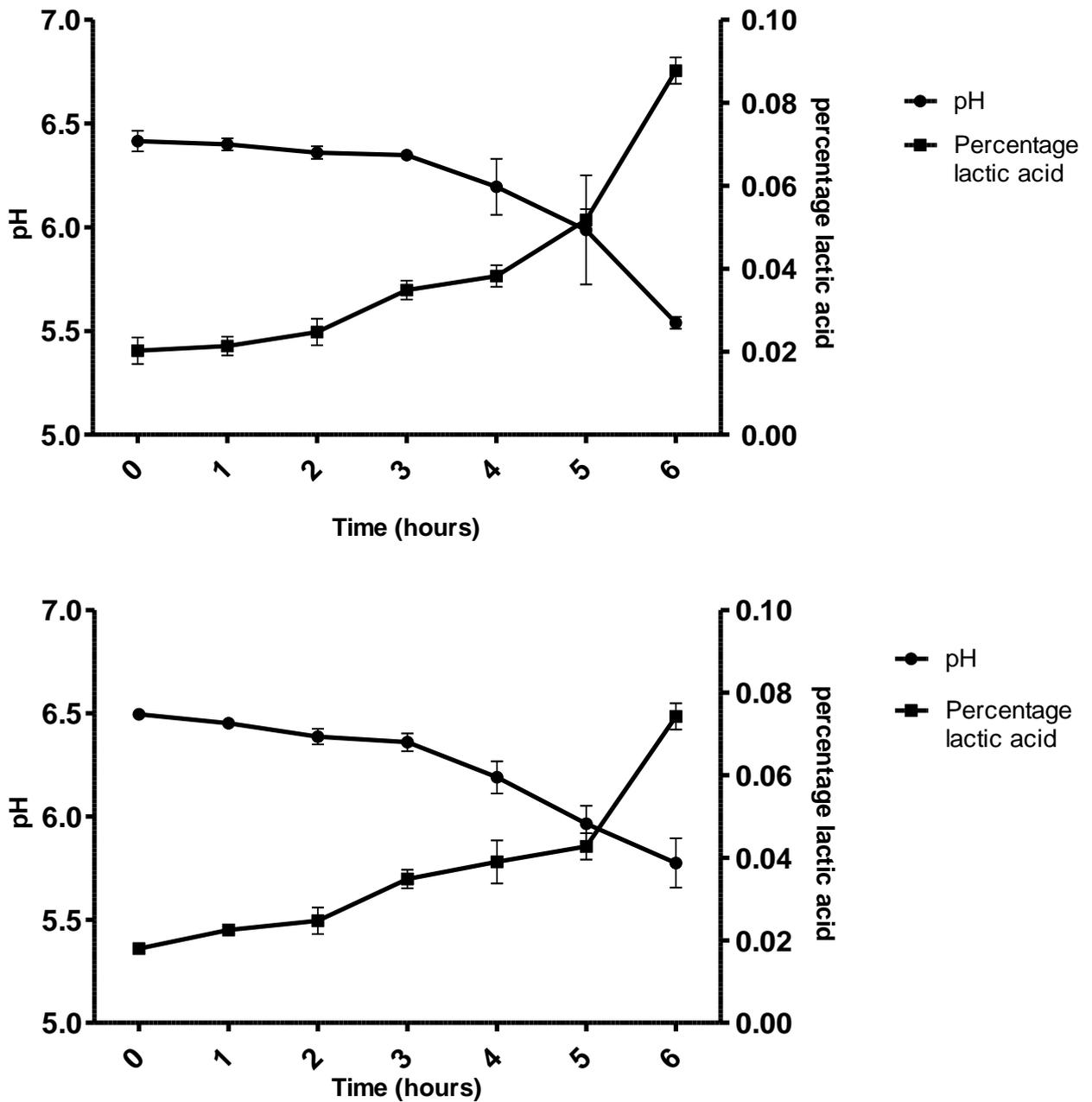


Figure 4.3: pH and lactic acid composition data for red and sorghum *mahewu* samples respectively. pH and percentage lactic acid level were monitored hourly for the first six hours of fermentation. The results were computed as mean \pm standard deviation.

Figure 4.1 shows decrease in pH as the fermentation of both *mahewu* types progressed, with a corresponding increase in percentage lactic acid level. There was no significant difference between changes in pH of red sorghum compared to white sorghum *mahewu* ($p < 0.05$).

4.2 Composition of sorghum *mahewu*.

Table 4:1 Composition of sorghum *mahewu* in terms of pH, percentage lactic acid and total lactic acid bacteria (LAB) counts.

	Red sorghum <i>mahewu</i>			White sorghum <i>mahewu</i>		
Sampling Time (hrs)	0*	24	48	0	24	48
pH	6.342±0.314	3.982±0.172	3.245±0.063	6.450±0.076	3.979±0.110	3.295±0.069
Percentage lactic acid	0.014±0.002	0.111±0.045	0.271±0.045	0.015±0.004	0.139±0.040	0.313±0.063
Log CFU/ml	6.708±1.682	-	9.624±0.889	6.731±0.125	-	11.117±0.112

*Readings are expressed as mean ± standard deviation of ten *mahewu* samples for pH and percentage lactic acid, and for three independent samples for the colony counts.

There was a progressive decrease in mean pH of the sorghum *mahewu* over the 48 hour sampling period, coupled with a corresponding increase in mean percentage lactic acid level and LAB counts. The mean pH of red sorghum *mahewu* was slightly lower than that of white sorghum *mahewu*. Mean percentage lactic acid level and number of LAB was however was higher for white sorghum *mahewu* than red sorghum *mahewu*.

4.3 Isolation of lactic acid bacteria (LAB).

Selected plates showing isolated LAB are shown in Figure 4.2.

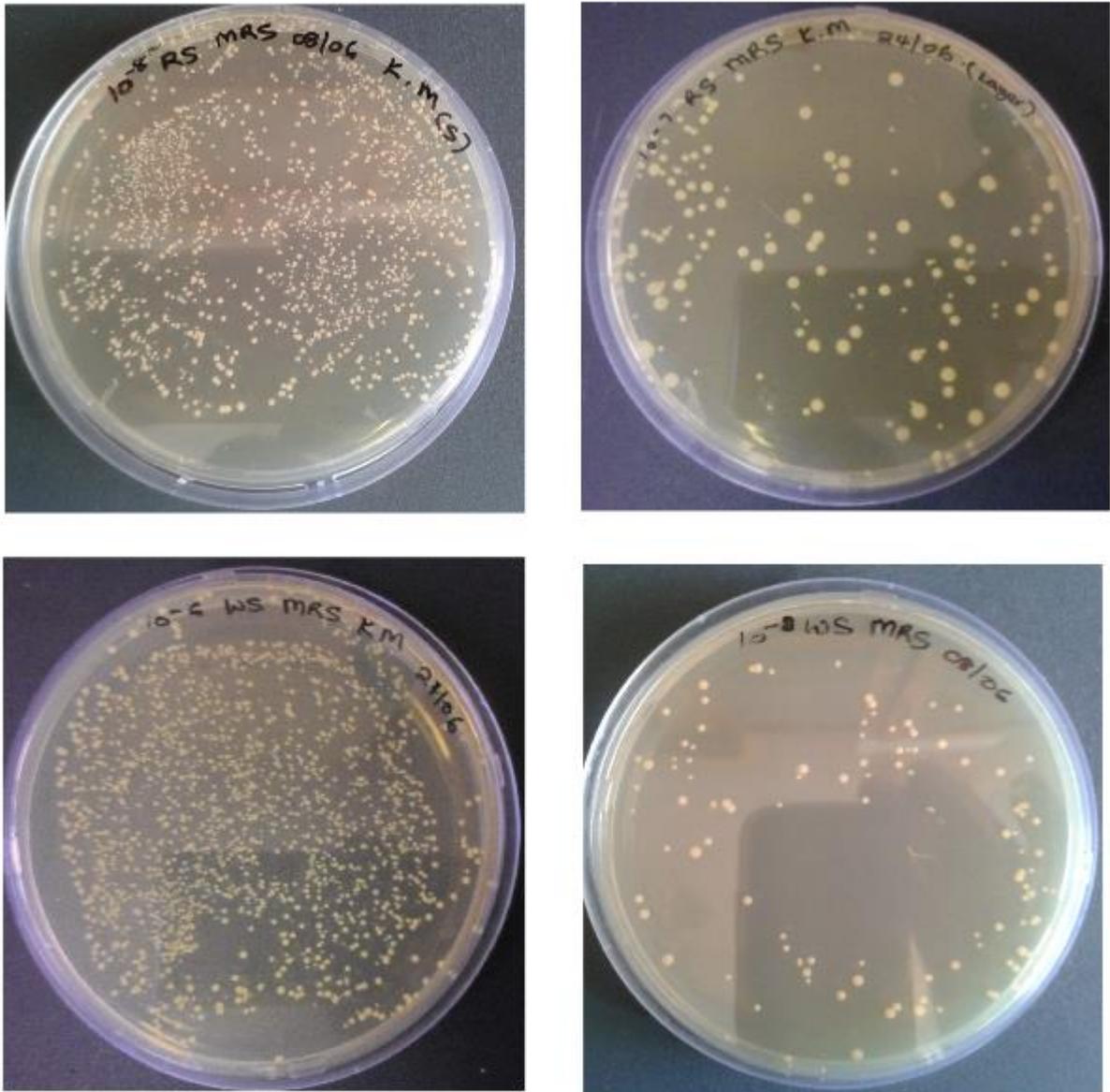


Figure 4.4 A selection of MRS agar plates containing LAB colonies. The LAB were isolated by inoculating a 100 μ l sample of either red or white sorghum *mahewu* onto MRS agar plates and incubating anaerobically for 48 hours.

A number of LAB with varied colony morphology were isolated on MRS agar plates. Isolates with distinct morphological characteristics typical of LAB were selected, allocated an isolate code and streaked on fresh MRS agar plates.

4.4 Streak plates for obtaining pure isolates.

All the isolates selected were purified successfully using the streak plate method. Representative streak plates are shown in Figure 4.3.

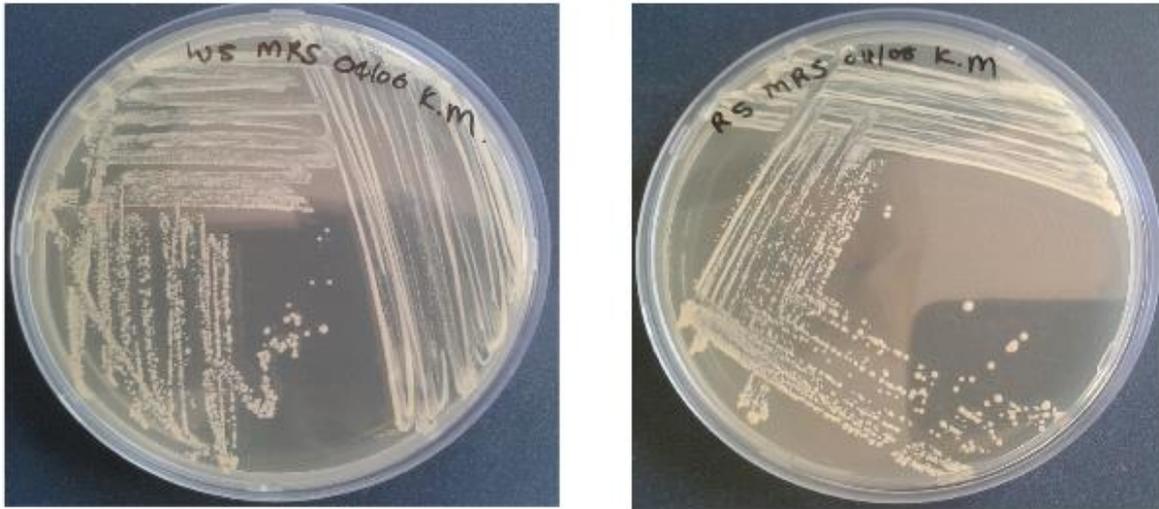


Figure 4.5 Representative streak plates on MRS agar for LAB isolates from red and white sorghum *mahewu*. The isolates were obtained from the MRS agar spread plates and allocated a code, depending on whether they were isolated from red or white sorghum *mahewu* (Rn/Wn). The streaked plates were incubated anaerobically at 37°C for 48 hours.

4.5 Morphological and biochemical characteristics of the LAB

isolates.

The main morphological and biochemical characteristics of LAB isolates are summarised in Table 4.2.

Table 4:2 Morphological and biochemical characteristics of LAB isolates.

Isolate code	Colony morphology	Gram stain	Morphology	Catalase test
R1	Small*, white, circular, convex with an entire margin	+*	Bacilli	-*
R2	Small, white, circular, convex with an entire margin	+	Bacilli	-
R3	Small, white, circular, convex with an entire margin	+	Cocci	-
R4	Small, white, circular, convex with an entire margin	+	Cocci	-
R5	Small, white, circular, convex with an entire margin	+	Bacilli	-
R6	Small, white, circular, convex with an entire margin	+	Bacilli	-
R7	Small, white, circular, convex with an entire margin	+	Bacilli	-
R8	Punctiform, white, circular, convex with a transparent entire margin	+	Coccobacilli	-
R9	Small, white, circular, convex with an entire margin	+	Bacilli	-
R10	Small, white, circular, convex with an entire margin	+	Bacilli	-
R11	Small, white, circular, convex with an entire margin	+	Bacilli	-
W2	Small, white, circular, convex with an entire margin	+	Diplococci	-
W3	Small, white, circular, convex with an entire margin	+	Bacilli	-
W4	Small, white, circular, convex with an entire margin	+	Coccobacilli	-
W5	Small, white, circular, convex with an entire margin	+	Bacilli	-
W7	Small, white, circular, convex with an entire margin	+	Diplococci	-
W8	Small, white, circular, convex with an entire margin	+	Diplococci	-
W9	Small, white, circular, convex with an entire margin	+	Diplococci	-
W10	Small, white, circular, convex with an entire margin	+	Cocci	-
<i>Escherichia coli</i>		-	Bacilli	+

*Small-ranging from <1 mm to 2 mm in diameter, + indicates a positive reaction, - indicates a negative reaction. *Escherichia coli* was used as a control for the catalase test and Gram stain.

The results for the biochemical characterisation (Table 4.2) indicated that all the bacterial isolates were catalase negative and Gram positive. Examination of the cell morphology of isolates showed that, of the 19 LAB isolates there were ten bacilli, two coccobacilli, four diplococci and three cocci shaped LAB.

4.5.1 Sugar fermentation profiles of the isolates.

The sugar fermentation profiles of 19 LAB isolates showing their ability to ferment 11 sugars is shown in **Table 4.3**.

Table 4.3 Sugar fermentation profiles of LAB isolates.

Isolate Code	Shape	Sugar										
		Gal ^a	Fuc ^b	Malt ^c	Suc ^d	Lact ^e	Man ^f	Celbi ^g	Glu ^h	Dex 10 ⁱ	Fruct ^j	Lactl ^k
R1	Bacilli	+*	-	±	+	+	+	+	+	+	+	+
R2	Bacilli	±	-	±	+	+	+	+	+	+	+	+
R3	Cocci	±	-	+	+	+	+	+	+	+	+	+
R4	Cocci	+	-	+	+	+	-	+	+	+	+	+
R5	Bacilli	±	-	+	-	+	-	+	+	+	+	+
R6	Bacilli	+	-	+	+	+	-	+	+	+	+	+
R7	Bacilli	±	-	+	+	+	+	+	+	+	+	+
R8	Coccobacilli	+	-	+	+	+	+	+	+	+	+	+
R9	Bacilli	+	-	+	+	+	+	+	+	+	+	-
R10	Bacilli	±	-	+	+	+	+	+	+	+	+	+
R11	Bacilli	-	-	±	+	+	-	+	+	+	+	+
W2	Diplococci	+	-	±	+	+	+	+	+	+	+	+
W3	Bacilli	-	-	±	+	+	+	+	+	+	+	+
W4	Coccobacilli	±	-	+	+	+	-	+	+	+	+	+
W5	Bacilli	-	±	±	+	+	-	+	+	+	+	+
W7	Diplococci	-	±	+	+	-	-	+	+	+	+	+
W8	Diplococci	-	±	+	+	-	-	+	+	+	+	+
W9	Diplococci	-	±	+	+	+	-	+	+	+	-	+
W10	Cocci	+	±	+	+	-	±	±	±	+	+	+

*+ indicates that the isolate fermented the sugar, ± indicates that the isolate weakly fermented the sugar and - indicates that the isolate did not ferment the sugar. The sugars in full a to k respectively D-galactose, D-fucose, Maltose, Sucrose, Lactose, D-mannose, Cellulobiose, D-Glucose, Dextrin 10, D-fructose and Lactulose.

Characterisation of the sugar fermentation profiles of bacteria revealed that each of the isolates had a unique sugar fermentation profile. All the isolates were able to ferment D-glucose, maltose, cellulobiose and dextrin 10. The other sugars were fermented in a varied pattern. Most of the isolates were able to ferment lactose, lactulose, sucrose and fructose. The least fermentable sugars were fucose and D-mannose.

4.6 Evaluation of probiotic properties of the lactic acid bacteria (LAB).

4.6.1 Tolerance to pH 2

Optical density readings for monitoring of growth in MRS broth adjusted to pH 2 are shown in **Figure 4.4**

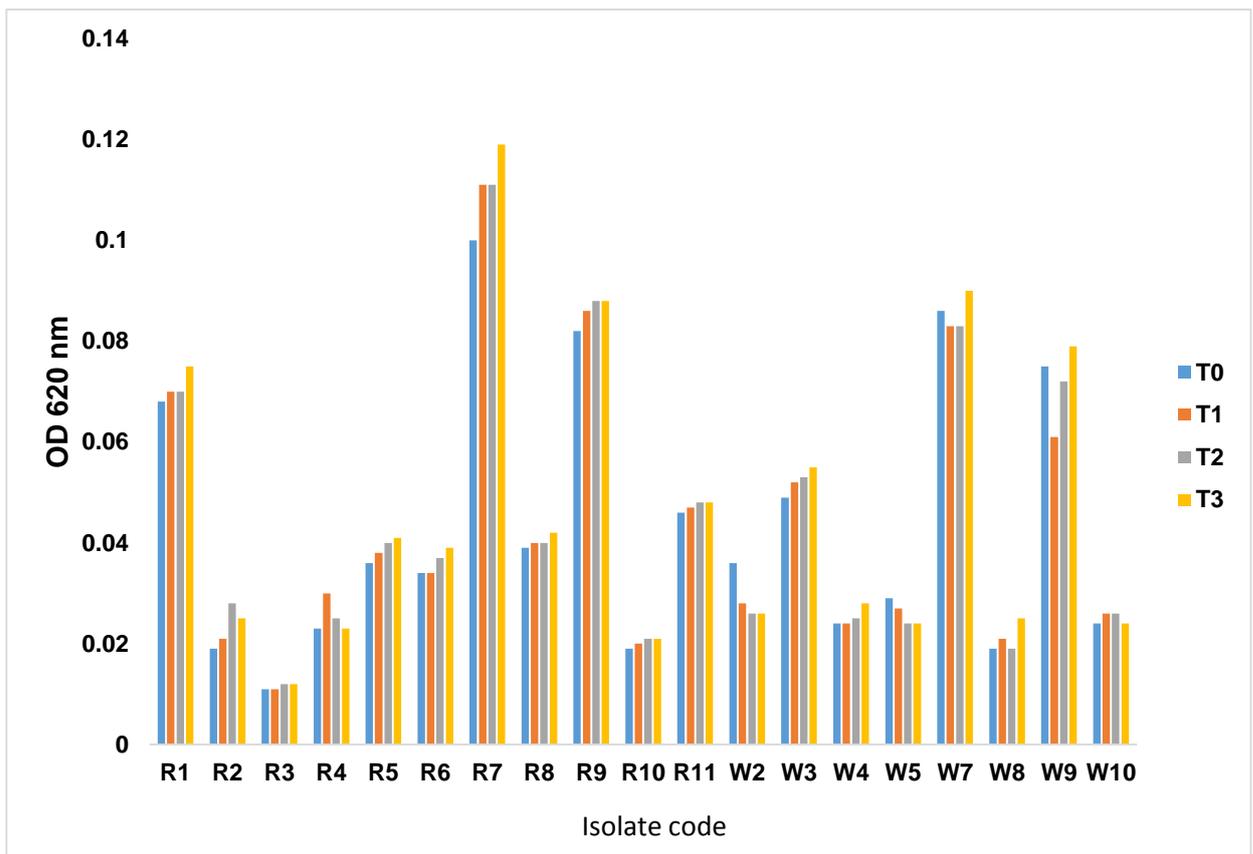


Figure 4.4 OD at 620 nm readings for 19 LAB isolates showing growth in MRS at pH. Tolerance to pH 2 was determined by monitoring growth of the isolates at 37°C for three hours in MRS broth adjusted to pH 2. Monitoring of the growth of the isolates was carried out in duplicate and repeated twice. The graph above shows one data set only.

There was variation in the ability of the isolates to grow in MRS broth adjusted to pH 2. Some of the isolates were able to grow over the three hours, as indicated by increase in OD (R1, R2, R3, R5, R6, R7, R8, R9, R10, R11, W3, W4, W7, W8 and W9). The rest of the isolates were not able to grow at pH 2.

Plates showing change in viable counts after exposure to MRS broth adjusted to pH 2 are shown in Figure 4.5.

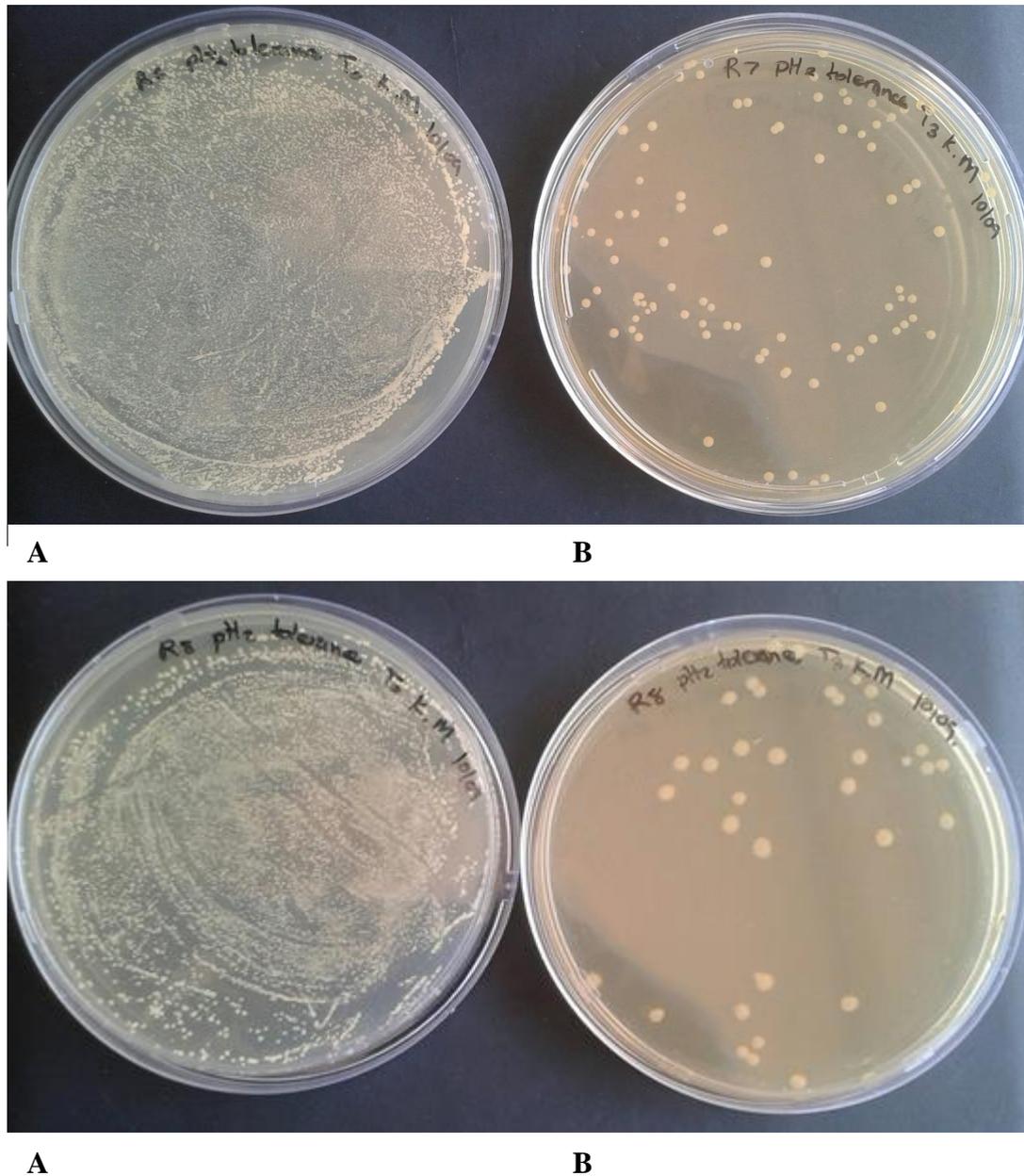


Figure 4.5 MRS agar plates showing LAB counts before incubation (A) and after three hours of incubation (B) in MRS broth adjusted to pH 2. Ten microliter samples were collected before incubation and after three hours and diluted tenfold. The samples were spread onto MRS agar plates and incubated anaerobically for 48 hours. The plates illustrate change in viable counts due to exposure to pH 2. Tolerance was indicated by subsequent growth after exposure to pH 2 for three hours.

Figure 4.5 illustrates a reduction in viable counts of the LAB isolates after exposure to pH 2 for three hours. The results indicate that, although the isolates were able to remain viable at pH 2, they experienced a considerable amount of stress.

4.6.2 Tolerance to pH 3.

OD readings at 620 nm showing growth of the isolates over three hours are shown in Figure 4.6.

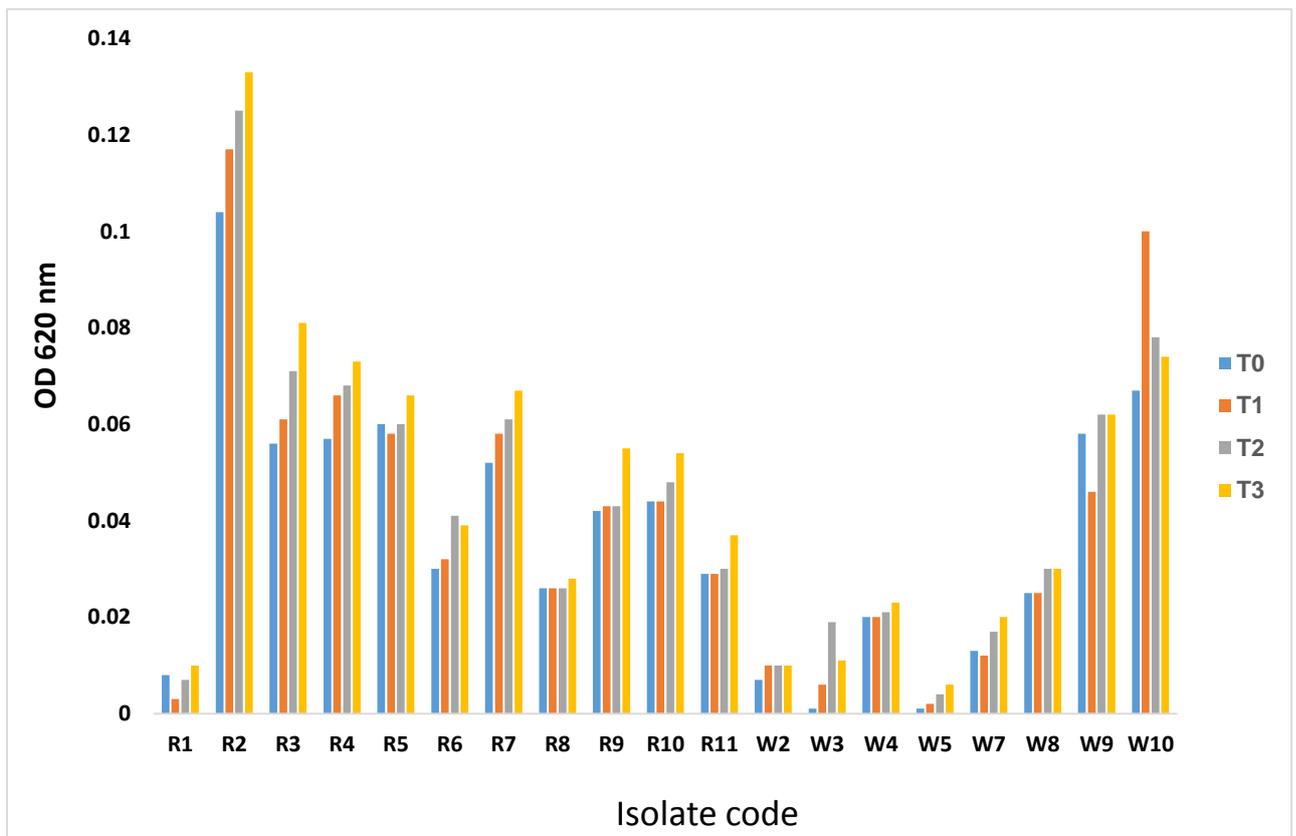


Figure 4.6 OD 620 nm readings for 19 LAB isolates exposed to MRS broth adjusted to pH 3. Tolerance of the isolates was determined by monitoring growth of the isolates for three hours. Monitoring of the growth of the isolates at pH 3 was carried out in duplicate. The graph above shows one data set only.

The graph illustrates that all the isolates were able to grow in MRS broth adjusted to pH 3. Tolerance to exposure to MRS broth adjusted to pH 3 was varied among the isolates, as indicated by differences in change in OD over the three hours.

4.6.3 Growth at pH 6.5 (Control).

OD readings showing growth of isolates over three hours in normal MRS broth (pH 6.5) are shown in Figure 4.7.

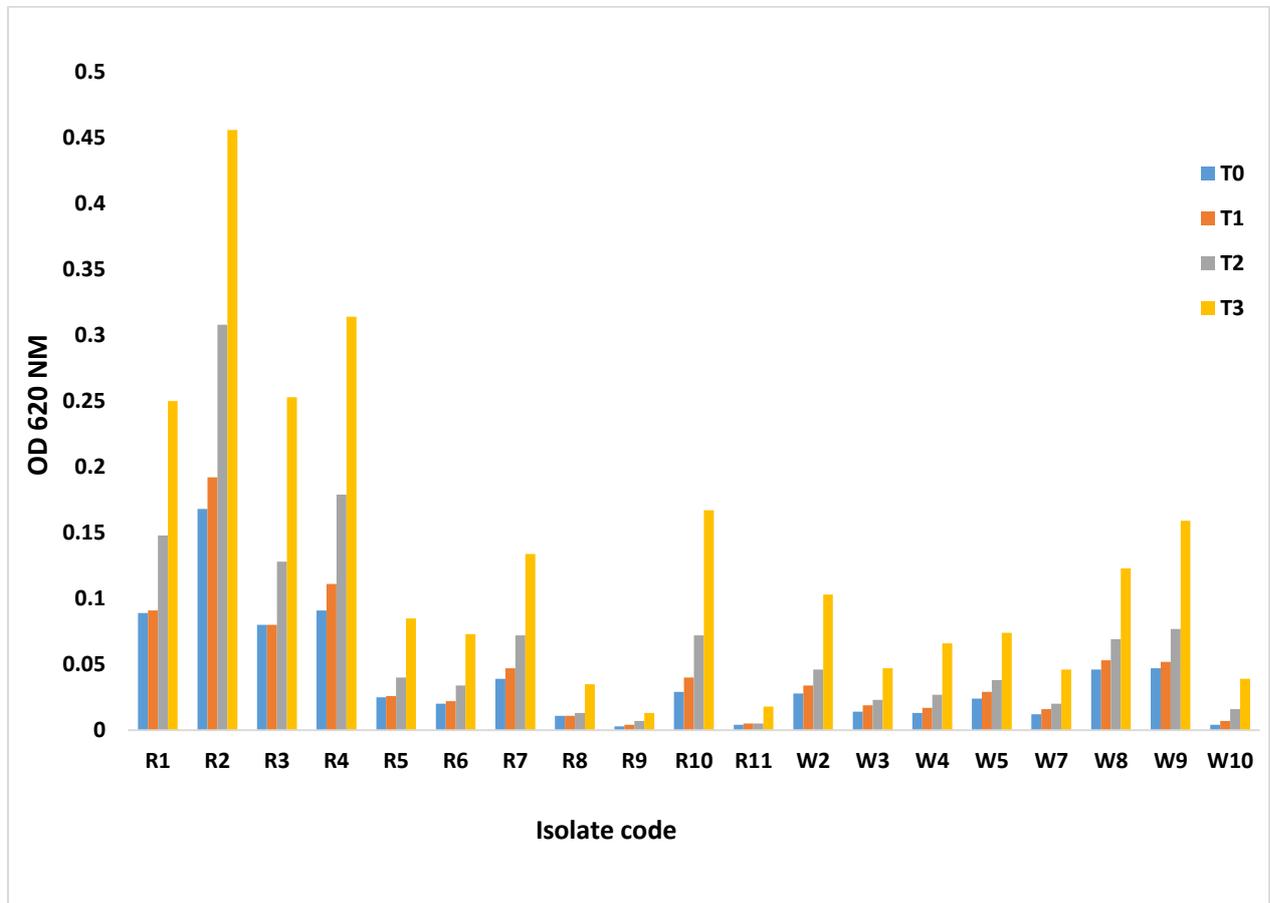


Figure 4.7 Monitoring of the ability of 19 LAB isolates to grow at pH 6.5. Growth in non-acidified MRS broth at pH 6.5 for three hours at 37°C was used as a control for the pH tolerance assay.

The figure illustrates increase in optical density of MRS broth cultures of each isolate at pH 6.5. The graph generally indicates exponential increase in cell numbers over the measured time period.

4.6.4 Percentage growth of the LAB isolates at the different pH values.

The percentage growth of the isolates at pH 6.5, pH 3 and pH 2 is shown in Figure 4.8.

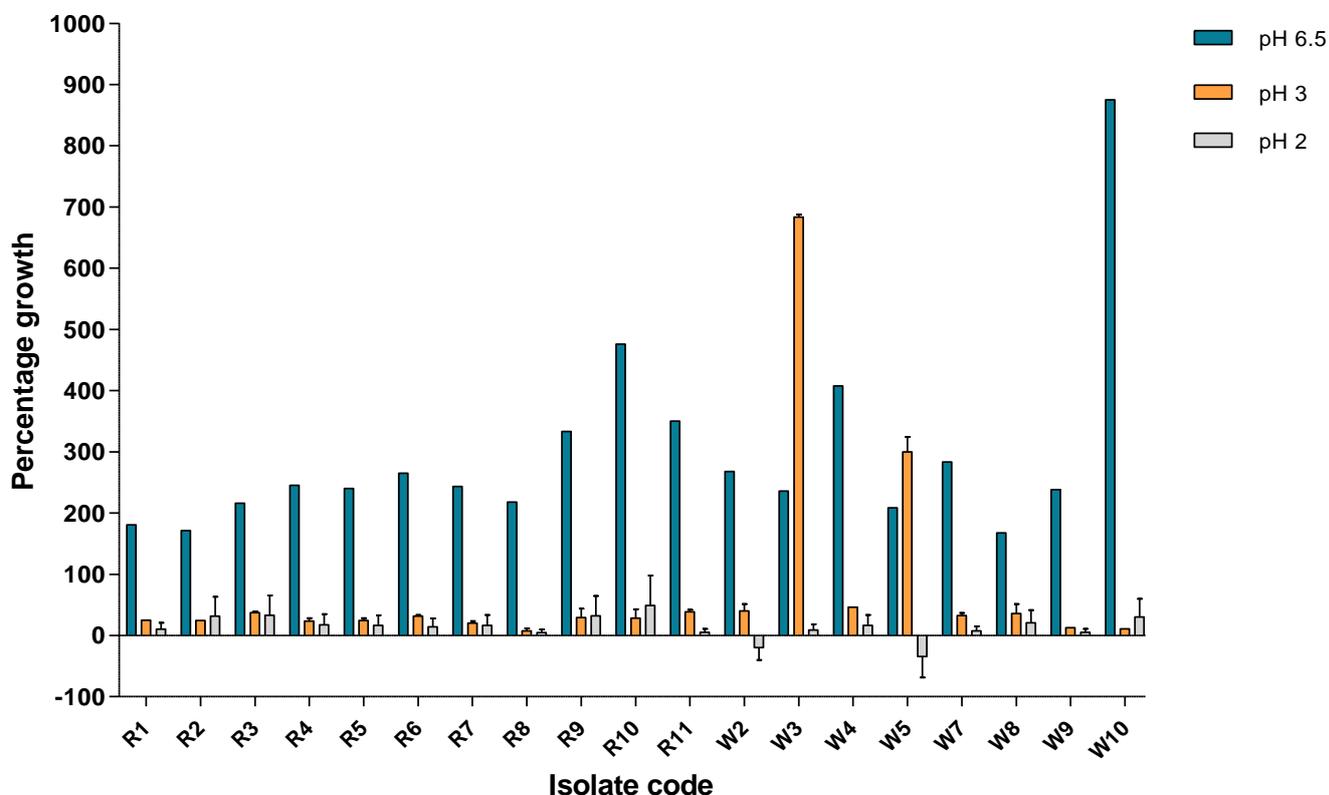


Figure 4.8 Percentage growth of LAB isolates during exposure to MRS broth at pH 2, pH 3 and pH 6.5 for three hours. Percentage growth was calculated by comparing the optical density before incubation at 37°C to the optical density after three hours of incubation. The data were computed as mean \pm standard deviation.

Comparison of percentage growth of the isolates indicated that there was a significant difference between growth of the isolates at pH 6.5 and pH 3 and pH 2. Growth at pH 6.5 was generally significantly higher ($p < 0.05$) than at the lower pH values, except for LAB isolates W3 and W5. There was no significant difference in percentage growth of the isolates at pH 3 compared to percentage growth of pH 2.

4.6.5 Tolerance to simulated gastric juice

Results for evaluation of survival using plate counts showed that all the isolates were able to grow under simulated gastric juice conditions. Sample plates showing growth of isolates after exposure to simulated gastric juice are shown in Figure 4.9.

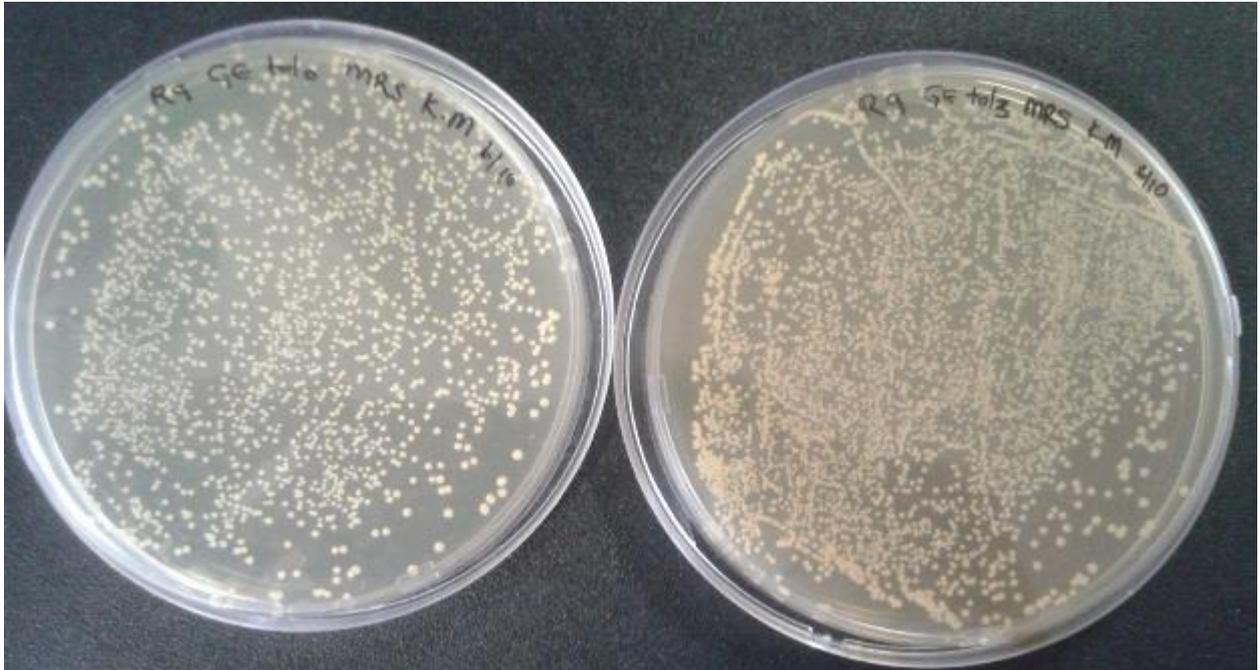


Figure 4.9 MRS agar plate showing change in viable counts in a LAB isolate. A sample was plated before incubation at 37°C in simulated gastric juice (pepsin and 0.5 % NaCl adjusted to pH 2) and after incubation for three hours. The collected samples were diluted tenfold before inoculation on MRS agar plates. Inoculated plates were incubated anaerobically for 48 hours. Tolerance was recorded as subsequent growth on MRS agar after exposure to simulated gastric juice for three hours.

Percentage growth of isolates in simulated gastric juice is shown in Figure 4.10.

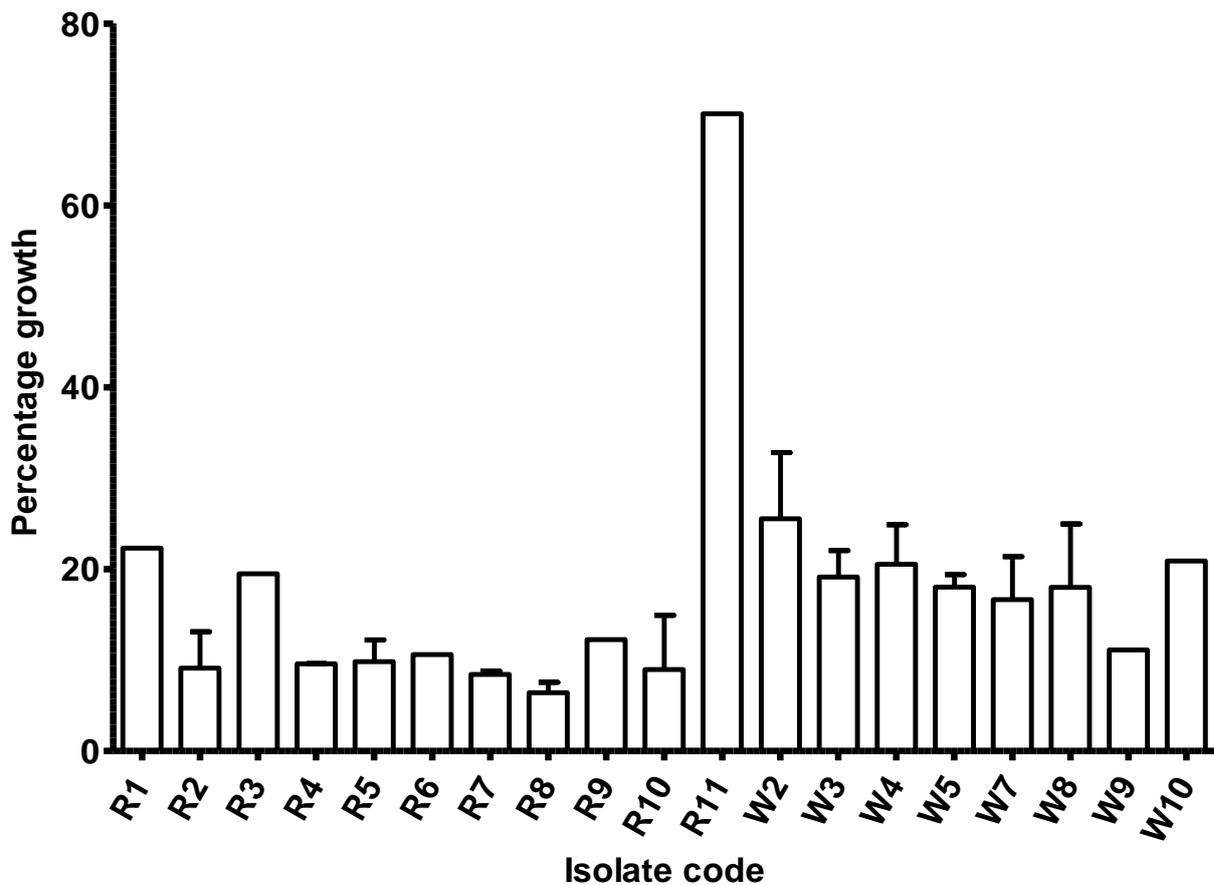


Figure 4.10 Percentage growth of the LAB isolates in simulated gastric juice. Percentage growth of the isolates was determined using OD_{620} values obtained before and after incubation of the isolates in the simulated gastric juice for three hours. The assay was carried out in duplicate. There was a significant difference in the tolerance of the isolates to the simulated gastric juice ($p < 0.05$).

The isolates showed significantly varied ($p < 0.05$) tolerance to simulated gastric juice. LAB isolate R11 showed the highest percentage growth.

4.6.6 Tolerance to porcine bile

OD of isolates exposed to MRS broth containing 0.3 % (w/v) porcine bile are shown in Figure 4:11.

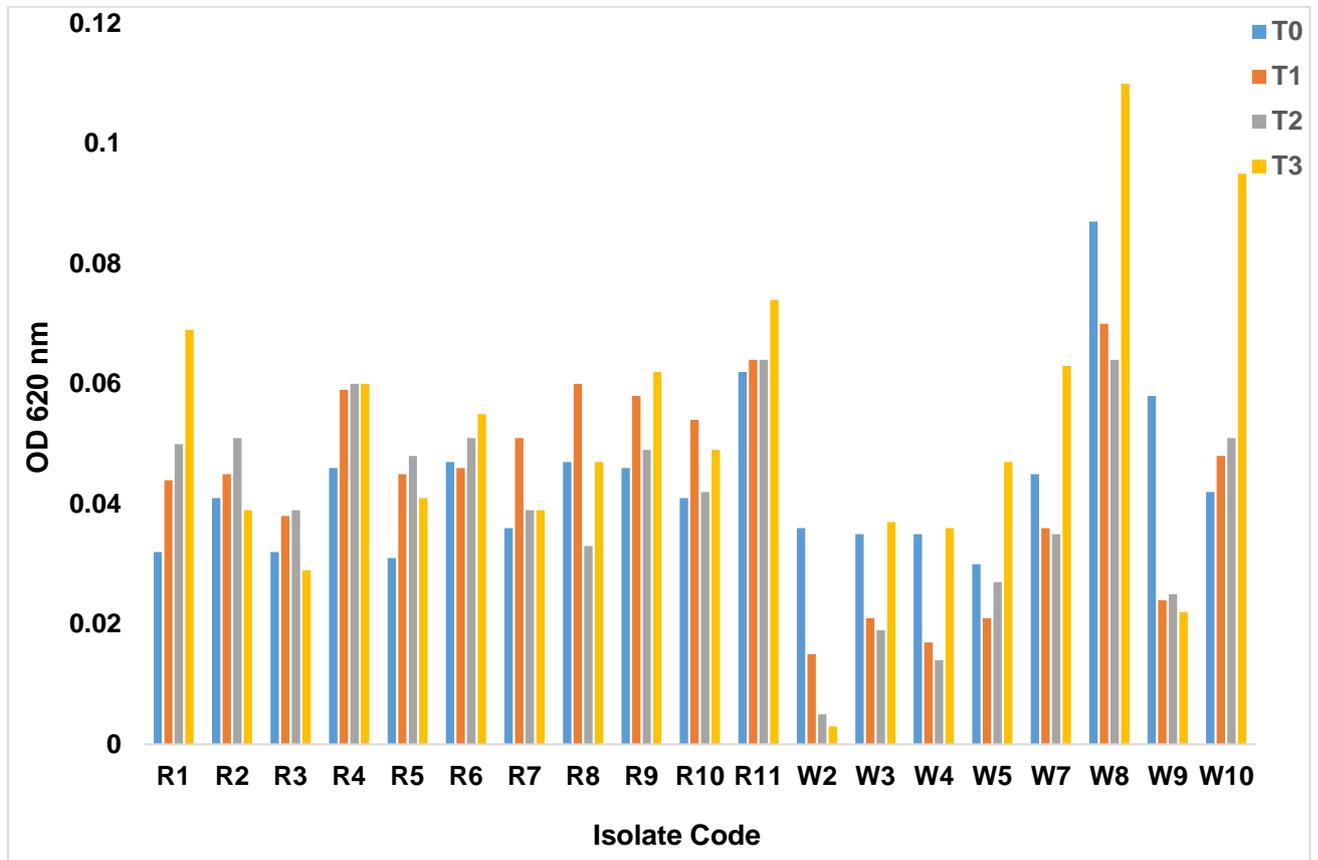


Figure 4.11 OD readings showing growth of 19 LAB isolates exposed to MRS broth with 0.3 % porcine bile over three hours of incubation at 37°C.

Percentage growth of the isolates in MRS broth with 0.3 % porcine bile compared to growth in normal MRS broth with no bile is shown in Figure 4.12.

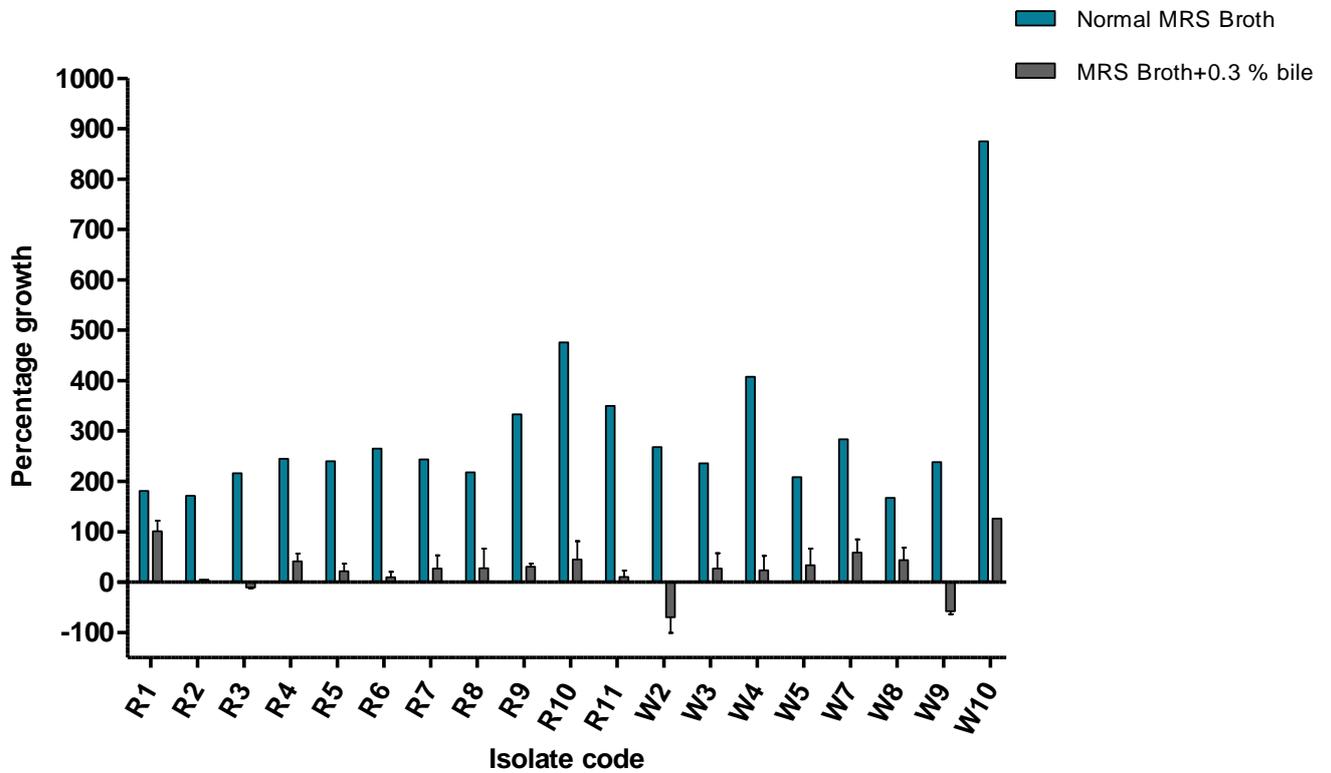


Figure 4.12 Percentage growth of LAB isolates in MRS broth with 0.3 % porcine bile compared to percentage growth in normal MRS broth. The percentage growth was calculated as increment in OD_{620} over three hours of incubation at $37^{\circ}C$ relative to the OD_{620} value before incubation.

The graph illustrates a significant reduction ($p < 0.05$) in percentage growth of isolates in MRS broth containing 0.3 % bile compared to growth in normal MRS broth. Isolates had a varied tolerance to the presence of bile. Some of the isolates were not able to growth in the presence of bile, namely isolates R3, W2, W9.

4.6.7 Antimicrobial activity of the LAB isolates.

The mean sizes of zones of inhibition of each of the culture supernatants of the 19 LAB isolates are shown in Figure 4.13

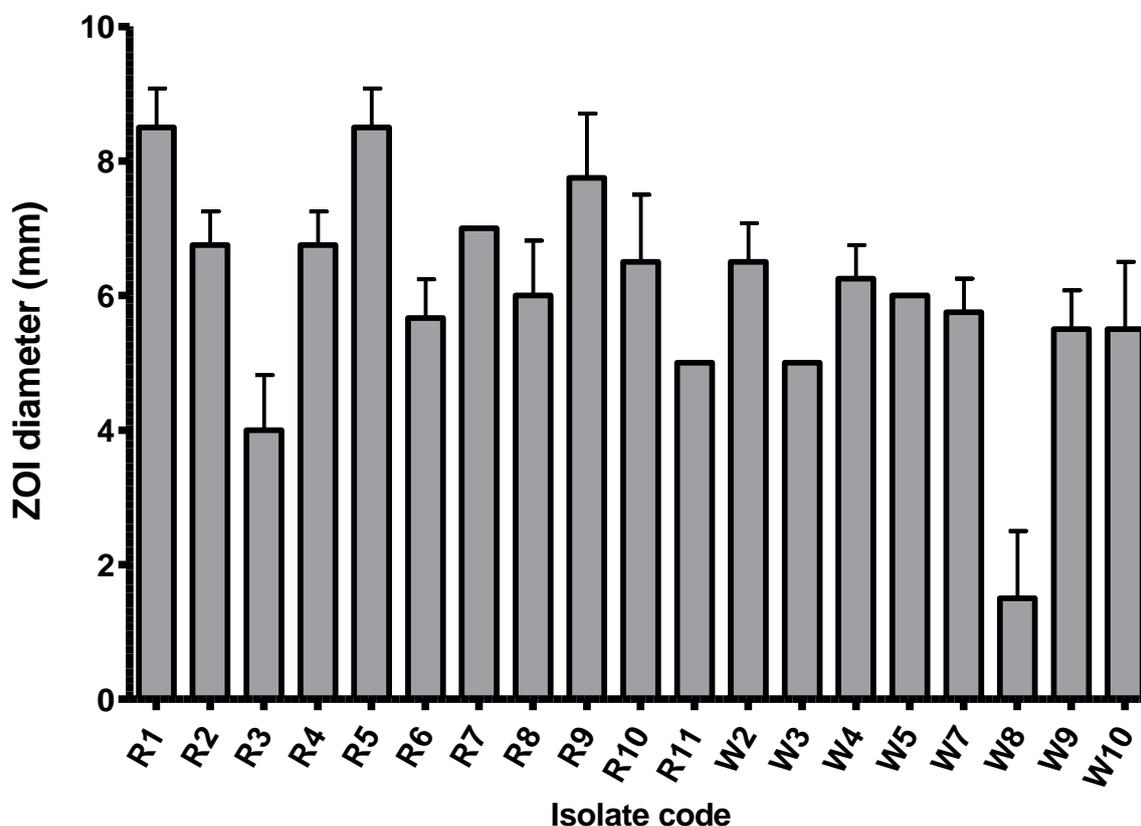


Figure 4.13 Zone of inhibition (ZOI) diameters for culture supernatants of 19 LAB isolates. Activity of the culture supernatants against *E.coli* was determined on Muller Hinton agar using the agar well diffusion technique. The ZOI was measured to the nearest millimetre and the well diameter of 5 mm was subtracted from the obtained value. The ZOI diameters were computed as mean \pm standard deviation of quadruplicate.

There was a significant difference ($p < 0.05$) in the inhibitory effects of the culture supernatants of the LAB isolates against *E.coli*. The isolates with the highest inhibitory activity were R1 and R5 and the lowest inhibitory activity was observed in W8.

4.6.8 Summary of results for evaluation of probiotic properties.

The summary of scores of each isolate for the probiotic attributes evaluated in the study are shown in Table 4.4.

Table 4.4: Evaluation scores of probiotic attributes of the isolates.

Isolate code	Probiotic attribute					Probiotic score ^a
	Tolerance to gastric juice*	pH 3 tolerance**	pH 2 tolerance**	0.3 % bile tolerance**	Antimicrobial activity***	
R1	+	(+)	(+)	++	+++	5
R2	+	(+)	(++)	(+)	++	5
R3	+	(++)	(++)	-	+	4
R4	+	(+)	(+)	(++)	++	5
R5	+	(+)	(+)	(+)	+++	5
R6	+	(++)	(+)	(+)	++	5
R7	+	(+)	(+)	(++)	++	5
R8	+	(+)	(+)	(++)	++	5
R9	+	(++)	(++)	(++)	++	5
R10	+	(++)	(++)	(++)	++	5
R11	+	(++)	(+)	(+)	++	5
W2	+	(++)	-	-	++	3
W3	+	++	(+)	(++)	++	5
W4	+	(++)	(++)	(+)	++	5
W5	+	++	-	(++)	++	4
W7	+	(++)	(+)	+	++	5
W8	+	(++)	(+)	(++)	-	4
W9	+	(+)	(+)	-	++	4
W10	+	(+)	(++)	(++)	++	5

* Criteria for recording tolerance assay in simulated gastric juice: + indicates that the isolate was tolerant, - indicates that the isolate was not tolerant.

** Criteria for recording tolerance assays in modified MRS broth: - indicates that the isolate was not tolerant, (+) indicates that the isolate was weakly tolerant, + indicates that the isolate was tolerant. Isolates with a percentage growth of ≤ 25 % or 26-49 % were considered as weakly tolerant and denoted as (+) and (++) respectively. An isolate with a percentage growth of 50-74 % or ≥ 75 % was considered as tolerant and denoted as + and ++ respectively.

*** Criteria for recording level of inhibition of the culture supernatant of isolates on growth of *E.coli*. The interpretation of the symbols is as follows; (-) no inhibition, (+) weak inhibition, (++) moderate inhibition, (+++) strong inhibition.

^a The probiotic score was determined using a binary system with tolerance being scored as 1 and not tolerant being scored as 0. The probiotic score is the total of the scores of each isolate for the probiotic attributes evaluated in the study.

Chapter Five: Discussion.

In the present study, lactic acid bacteria (LAB) were isolated from sorghum *mahewu* and evaluated for probiotic attributes. The *mahewu* prepared in the study was first analysed for the pH and titratable acid level to determine whether it was of an acceptable level, relative to values reported in other studies (Simango and Rukure, 1992; Bvochora *et al*, 1999). This was done to ensure that the time at which the LAB were isolated coincided pH levels at which *mahewu* is normally consumed.

The results for pH and percentage lactic acid level (Table 4.1) indicated that the properties of the *mahewu* produced in the study were of an acceptable level. The mean pH of red sorghum *mahewu* decreased from 6.342 to 3.245 while that of white sorghum *mahewu* decreased from 6.450 to 3.295 after 48 hours of fermentation. The decrease in pH levels corresponded to increase in percentage lactic acid and number of LAB. Progressive decrease in pH levels and rise in percentage lactic acid is fermentation is characteristic of lactic acid fermented foods. The LAB presumably produced of lactic acid by fermenting carbohydrates in the *mahewu*. This is in agreement with study results by Bvochora *et al.*, (1999), who reported lowering of pH as LAB proliferated.

Characterisation of the sugar fermentation profiles of the LAB isolated from the sorghum *mahewu* indicated that they were able to ferment a wide range of sugars. The fermentation profiles of the sugars were varied. Most of the isolates were able to break down lactose, this might indicate that the LAB isolates are able to produce the enzyme β -galactosidase. Ingestion of such LAB might improve lactose digestion in the host gut and alleviate symptoms of lactose intolerance.

4.7 Evaluation of LAB for probiotic properties.

To assess the potential effectiveness of the isolates in exerting their health promoting benefits to the host, the isolates were assessed for ability to survive simulated conditions encountered during gastro-intestinal passage. The conditions assessed mimicked the acid environment of the stomach and subsequently the bile presence in the small intestine. Assuming these isolates would be able to reach and successfully colonise the large intestine, they were assessed for potential antimicrobial activity against *Escherichia coli*.

4.7.1 Evaluation of tolerance of LAB isolates to low pH.

The tolerance assays indicated that 89.5 % (n =19) of the LAB isolates were able to remain viable after three hours of exposure to pH 2. All the isolates were able to tolerate MRS medium at pH 3 (Figure 4.6). These results are in agreement with studies, where *Lactobacillus* strains of food or animal origin remained viable after exposure to pH values of between 2.5 and 4.0 (Jacobsen *et al.*, 1999; Dunne *et al.*, 2001; Maragkoudakis *et al.*, 2006). There was a significant difference ($p < 0.05$) between ability of the isolates to grow at the control pH of 6.5 and growth at pH 2 and 3. The difference indicated that, although most of the isolates were able to grow at low pH, they experienced a considerable amount of stress. As expected the percentage growth values of the isolates at lower pH values were generally lower. There was no significant difference in the ability of the isolates to grow at pH 2 and pH 3. The isolates however generally showed greater susceptibility to pH 2.0. This was expected as Corconan *et al.*, (2005) reported that some LAB genera generally exhibit increased sensitivity at pH values below 3.0.

4.7.2 Evaluation of tolerance of LAB isolates to simulated gastric juice.

All the LAB isolates showed ability to tolerate simulated gastric juice conditions. The isolates were able to remain viable and grow after exposure to the simulated gastric

juice for three hours. The gastric juice provided a pH and enzymatic barrier to the survival of the isolates. Their ability to survive indicates that these isolates possibly had a mechanism of counteracting the detrimental effects of the pepsin.

4.7.3 Evaluation of tolerance of isolates to 0.3 % bile.

The LAB isolates showed marked differences in percentage growth after exposure to 0.3 % porcine bile (Figure 4.11). Some of the isolates, 15.8 % were not able to tolerate the bile, as indicated by the percentage growth, while the rest of the isolates were able to survive and grow. The isolates that were able to grow had varied percentage growth, indicating differences in bile tolerance. A possible explanation for the differences could be that some strains of LAB secrete bile salt hydrolase (BSH) enzyme, which hydrolyses conjugated bile acids to release de-conjugated bile acids and amino acids (Begley *et al.*, 2006; Franz *et al.*, 2011). Erkkilä and Petäjä (2000) stated that lactobacilli are able to hydrolyse bile salts thereby weakening their detergent. The resistance to bile salts has been reported to vary significantly between the *Lactobacillus* species and even between strains (Xanthopoulos *et al.*, 1997).

The isolates that were able to tolerate bile are good candidates for further research because BSH production allows the bacteria to detoxify bile salts thereby increasing their intestinal survival and persistence. This in turn increases the overall beneficial effects associated with the strain (Begley *et al.*, 2006). The isolates may also have been able to carry out active efflux of bile acids or salts, allowing them to tolerate the bile (Lambert *et al.*, 2008).

4.7.4 Antimicrobial activity of LAB culture supernatants.

The culture supernatants of the 19 LAB isolates each showed antimicrobial activity against *Escherichia coli*. The inhibition of *E. coli* growth possibly indicates that the LAB isolates in the study produced antimicrobial compounds such as the organic acids,

lactic acid and acetic acid, short chain fatty acids and bacteriocins. The sizes of the zones of inhibition (ZOI) diameters were however significantly different ($p < 0.05$). The differences in the degrees of inhibition of different isolates could be an indication that the isolates produced different types and amounts of antimicrobials. It would be useful to characterise the range of antimicrobial compounds produced by each isolate, particularly those that showed strong inhibition of *E. coli* growth, isolates R1 and R9. Antimicrobial activity indicates that the LAB isolates might have a bactericidal or bacteriostatic effect in the host gut and exert probiotic effects (Shah, 2007).

4.7.5 Probiotic scores of the isolates.

After assessment of all the probiotic attributes, each isolate was scored using a binary scoring system (Table 4.4). Most of the isolates (84.2 %) tested positive for all the probiotic attributes tested for in this study. These isolates are good candidates for further studies of probiotic attributes.

In vitro tolerance to simulated gastrointestinal (GI) conditions has been positively correlated to survival in both human and pig GI tracts (Dunne *et al.*, 2001). The results for the tolerance assays could therefore indicate that the isolates would be able to survive transit in the GI tract and possibly exert probiotic effects in the host gut. Levels of tolerance of the LAB isolates to simulated gastro-intestinal conditions was shown to vary according to the isolate. Strain specific tolerance of probiotics to gastro-intestinal conditions is supported by previous research showing that tolerance of probiotic candidates to simulated gastro-intestinal conditions may vary considerably among different genera, different species, or different strains within a species (Masco *et al.*, 2007).

In vitro screening of isolates for tolerance to simulated GIT tract conditions may only be useful in predicting the ability of each isolate to survive *in vivo* if ingested in a non-

protected way (Morelli, 2000). If the LAB are ingested in association with food matrix or consumed in an encapsulated form they may behave differently. Further tests of the efficacy of the isolates using adapted *in vitro* assays and *in vivo* tests may provide a clearer picture of the ability of each isolate to exert probiotic effects.

4.8 Conclusion

The findings of this study indicate that LAB isolated from spontaneously fermented sorghum *mahewu* are tolerant to simulated GIT conditions and may be able to confer health benefits. The isolates which tested positive for all the probiotic attributes evaluated in the studies could be used as starter cultures in production of *mahewu*. However, further studies need to be carried out to assess the performance of these strains *in vivo*.

4.9 Recommendations for future research.

Areas of interest to continue with this research include:

- *In vivo* evaluation of the efficacy of the probiotic strains
- Sequencing of the isolates to accurately identify them and mine for functional genes for production of bacteriocins and bile salt hydrolase enzymes.

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