CHARACTERIZATION OF LACTIC ACID BACTERIA ISOLATED FROM COCONUT WINE (*MNAZI*) FOR PROBIOTIC POTENTIAL

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MASTER OF SCIENCE

(Microbiology)

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY

2015

Characterization of Lactic Acid Bacteria Isolated from Coconut Wine (*Mnazi*) for Probiotic Potential

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A thesis submitted in partial fulfilment for the degree of Master of Science in Microbiology in the Jomo Kenyatta University of Agriculture and Technology

2015

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

This work is dedicated to my beloved dear family; my husband Fredrick Maranga, sons Ignatius Waikwa, Paul Wambugu and daughter Sarah Nungari. Thanks to all of you for the support you have and are still giving me. Without your encouragement and support this journey would be long and tough.

ACKNOWLEDGEMENTS

I thank God Almighty for having brought me this far since I started this long and treacherous journey. I appreciate the support and sacrifice granted by my family. I appreciate Jomo Kenyatta University of Agriculture and Technology (JKUAT) administration for giving me the opportunity to pursue Master's degree in Microbiology in Botany Department, granting me study leave and sponsorship during this period.

I am greatly indebted to my supervisors Prof. Hamadi Boga of Taita Taveta University College, Prof. Philip Kutima, Dr. Julius Maina of JKUAT, and Dr. Titus Tunje Kadere of Technical University of Mombasa (TUM) for the support and guidance they gave me through this research. I specifically thank Dr. Romano Mwirichia for the support I was given in the Institute for Biotechnology Research, JKUAT. I express gratitude to Anne Kelly Kambura of the Institute for Biotechnology Research, JKUAT for the support, guidance and encouragement shown during the period of study and for availing to me the use of the Institution's facilities. Special thanks to Julius Mugweru for assistance in correction of this work.

Lastly, I thank all my colleagues and friends in the Food Science and Technology and other departments for all their assistance, guidance, correction and encouragement. MAY GOD BLESS YOU ALL!

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LIST OF ABBREVIATIONS AND ACRONYMS

BLAST	Basic local alignment search tool
DAP	Diaminopimelic
FAO	Food and agriculture organization
FOS	Frustoologosaccharide
GIT	Gastrointestinal tract
ILRI	International livestock research institute
LAB	Lactic acid bacteria
MEGA	Molecular evolutionary genetic analysis
MRS	de Man Rogosa and Sharpe agar
PCR	Polymerase chain reaction
SSDP	Simulated stomach duodenum passage
WHO	World health organization

ABSTRACT

Lactic acid bacteria (LAB) are useful in human health as probiotics. To achieve this they need to reach the gastro-intestinal tract and remain viable. A total of fifteen strains of lactic acid bacteria isolated from fermented coconut wine (*Mnazi*) were screened *in vitro* for their safety and probiotic properties, acid tolerance, bile tolerance, survival under conditions of simulated gastrointestinal tract (GIT) passage and their antimicrobial activity against indicator organisms using standard techniques. Further they were assessed for production and properties of bacteriocins produced and characterized using sequencing and phylogenetic analysis. The results revealed that the fifteen isolates were safe to use, able to tolerate and grow well in conditions of reduced pH (2 and 2.5) and extremes of bile (up to 2%) that simulate stomach duodenal passage environment. They are also potent against both Gram positive and negative bacteria which they inhibit through production of bacteriocins. Most had β-galactosidase activity which makes them suited for moderation of lactose intolerance. Further, they were able to utilize frustooligosaccharides making them beneficial in regulating the microbiota in the colon. The isolates are phylogenetically distant from other homologous *Lactobacillus spp* though they were similar to *L. paracasei*, *L. casei*, *L. rhamnosus* and *L. zeae*. These findings show that the LAB isolates from *Mnazi* had desirable probiotic properties and were safe to use. This provides an opportunity for further investigation of the isolates for utilisation as starter cultures in fermentation of different food products to increase their value to human or animal health.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Background information

Fermentation occurs as a result of the activities of microorganisms; bacteria, yeast and mould (Steinkraus, 2002). The most important group of microorganisms involved in the spontaneous or natural fermentation of foods are the lactic acid bacteria (Steinkraus, 2002; Jakobsen & Lei, 2004).

Lactic acid bacteria (LAB) are Gram positive, non-spore forming, catalase negative cocci or rods that are anaerobic, microaerophilic or aero-tolerant and they produce lactic acid as a sole, major or an important product from the energy yielding fermentation of sugars (Wood & Holzafel, 1995). They are useful in industry and human health, including preservation of foods and as probiotics (Aysun & Candan, 2003). Lactic acid bacteria normally reside in the mouth and intestinal tract of humans where they enhance immune responses (Nguyen *et al*., 2007). Various culture centres around the world have historically used probiotics for health promotion and preventive purposes. In the past decade, scientific evidence on health benefits of probiotics is quickly emerging. The beneficial bacteria can be incorporated in the diet to have beneficial effects on the gut microflora (Bundesinstitut fur Bgvv working group, 1999).

Lactic acid bacteria are among the most powerful prokaryotes when it comes to antimicrobial potentials as they produce them during carbon source metabolism and they also compete with other species by acidifying their environment and by rapidly depleting the nutrients (Cassandra *et al*., 2004). Among the antimicrobial compounds they produce bacteriocins which include nisin, antibiotics such as reutericyclin and small antibiotic-like molecules such as reuterin (Cassandra *et al.,* 2004). The current need for biopreservation has renewed the interest in the search for food compatible antimicrobials produced by microorganisms. Lactic acid bacteria are also advertised as probiotic microorganisms belonging to the genera *Bifidobacterium, Enterococcus,*

Lactobacillus, *Lactococcus* and *Streptococcus* and commonly used in food products (Wood & Holzafel, 1995; Steinkraus, 2002).).

1.2 Probiotics

Probiotics are live microbial feed/food supplement which beneficially affects the host by improving its intestinal microbial balance. This has become the most widely accepted definition (Fooks *et al*, 1999).

Probiotics represent live microorganisms or microbial preparations or metabolites of stabilized autochtonous microorganisms which assess benefit effect on host organisms and affect microbial composition with stimulating effects on digestion and immunity of microorganisms (Nemcova, 1997). There are many different types of probiotic cultures which provide various benefits. These generally contain genera *Lactobacillus* and *Bifidobacterium* which are predominant members of the intestinal microflora and are classified as probiotics as they exert beneficial effects which include reduction of serum cholesterol, aid in lactose digestion , resistance to enteric pathogens, anticolon cancer effects, small bowel bacterial overgrowth, allergy and mucosal barrier dysfunction including diarrhea, constipation and immune responses and exerting antimutagenic activities (Sanders & Huis In't Veld, 1999; Holzapfel *et al.,* 2001; Ishibashi & Yamazaki, 2001; Aysun & Candan, 2003; Nyuyen *et al.,* 2007).

Certain probiotics have positive effects on intestinal microflora and/or different intestinal functions such as improving intestinal functions, improving intestinal motility and managing diarrhoea. The intestinal tract ecology contains at least 10^{14} bacteria and the probiotic organisms live within this ecology and help maintain health by stimulating immune responses and controlling pathogenic mechanisms (Floch, 2007). The maintenance of health gut microbiota may provide protection against various gastrointestinal disorders including gastrointestinal infections, inflammatory bowel diseases and cancer (Salminen *et al.,* 1998; Fonden *et al.,* 2003).

The human large intestine has a rich and dynamic microbiota consisting of at least 400-500 bacterial species (Berg, 1996). Maintaining a balanced microbiota ecosystem is essential for the normal functions of the gastrointestinal tract, especially in preventing infections and stimulating the host's immune response. Several factors including stress, antibiotic treatments and other medications can alter the gastrointestinal tract microbiota, predisposing the host various diseases (Salminen *et al*., 1995).

The probiotic potential of various *Lactobacillus* and *Bifidobacterium* species include management of intestinal disorders such as lactose intolerance, infant gastroenteritis and rotavirus associated diarrhoea, antibiotic–associated intestinal symptoms (mainly diarrhoea) and food allergy in babies (Salminen *et al.*, 1998; Kaur *et al.*, 2002). These disorders and diseases are associated with intestinal microbiota imbalance and increased gut permeability (Salminen *et al*., 1996b). In addition to these beneficial effects on disturbed intestinal microbiota, probiotics can modulate immune responses, lower biomarkers such as harmful faecal enzyme activities, and show positive effects against superficial bladder cancer and cervical cancer (Mc Farland, 2000). Other potential areas of probiotic nutritional management include alleviation of inflammatory bowel diseases (IBD) and irritable bowel syndrome (IBS) symptoms, mucosal vaccines and immunomodulation, infection control and eradication of multi drug–resistant microbes, treatment of candidal vaginititis, prevention of transmission of AIDS and sexually transmitted diseases, lowering cholesterol and blood, and antimutagenic (anticarcinogenic) activity (Kuar *et al*., 2002; Fonden *et al.*, 2003).

Probiotic viability would be reasonable measure of probiotic activity, but there are situations in which cell viability is not required for probiotic activity such as improved digestion of lactose, some immune system modulation activities and anti-hypertensive effects. (Vinderola,*et al*, 2003). Health beneficial effects have been linked to non-viable cells or to cell components, enzyme activities or fermentation products (Sander & In't Veld, 1999).

When assessing the health promoting effects of probiotics, it is important to keep in mind that all probiotic strains are different. Even strains representing the same species

usually have different properties. Several attributes are characteristics of probiotic bacteria should be considered before clinical trials are performed. *Lactobacillus plantarum* is an important species in the fermentation of various plant products (Ashenafi & Busse, 1991) and it is known to produce antimicrobial substances for example plantaricin that are active against certain pathogens. *Lactobacillius plantarum* is also a most common lactic acid inoculant in silage and as a silage inoculant it enhances dry matter recovery and also animal performance are also enhanced (Aysun & Candan, 2003).

There are some desired characteristics to be accomplished from probiotic bacteria (Salminen *et al.,* 1996; Vaughan & Millet, 1999) to exhibit their beneficial effects, probiotic bacteria need to reach its destination. Thus it is necessary for it to be tolerant to acid and bile salts (Chou & Weimer, 1999). Adherence to gastrointestinal cells is important for successful colonization and therefore beneficial effects will last longer in gastrointestinal tract (Ouwenhand *et al*., 1999). The knowledge of antibiotic susceptibility of potential probiotics strains is necessary in curing, although rare–*Lactobacillus* associated infections (Charteris et *al.,* 1998). Their production of enzyme beta-galactosidase in high amounts aids in lactose digestion (Aysun & Candan, 2003) while strains that are able to ferment fructooligosaccharides (FOS) may be used in symbiotic products (Collins & Gibson, 1999; German *et al.,* 1999).

1.3 Properties of probiotics

1.3.1 Aggregation properties

These can either be in form of autoaggregation, cell-surface hydrophobicity and co-aggregation with pathogen strains. These properties are important as preliminary screening tests for identifying potential probiotic bacteria suitable for human or animal use (Collado *et al.,* 2008).

1.3.1.1 Autoaggregation and cell-surface hydrophobicity

Probiotic bacteria need to achieve an adequate mass through aggregation in order to express beneficial effects. In the GI or vaginal tract it has been suggested that *Lactobacilli* use autoaggregation to promote the colonization of beneficial microorganisms. Thus, the ability of probiotics to aggregate is a desirable property. Bacterial aggregation between microorganisms of the same strain (autoaggregation) and hydrophobicity are related to cell adherence properties (Boris *et al.,* 1997; Del Re *et al.,* 2000; Kos *et al.,* 2003). Adhesion is a complex process involving non-specific (hydrophobicity) and specific ligand-receptor mechanisms. Adherence of bacterial cells is usually related to cell surface characteristics.

1.3.1.2 Co-aggregation

This refers to the aggregation between genetically different strains. Co-aggregation of probiotic strains with pathogens is important in therapeutic manipulation of abnormal GI or vaginal microbiota. Co-aggregation abilities depend on each strain (probiotic and pathogen strains) and time of co-aggregation (Collado *et al.,* 2008). Several studies report that co-aggregation abilities of *Lactobacillus* species enable it to form a barrier thereby preventing colonization by pathogenic bacteria (Pelletier *et al.,* 1997; Bao *et al.,* 2010).

1.3.2 Antimicrobial activity against pathogenic bacteria

Lactobacillus probiotics inhibit pathogens infection by producing antimicrobial substances. It has been noted that organic acids and other potentially antimicrobial metabolites are produced when *Lactobacillus* or *Lactococcus* bacteria are co-cultured with *Bacillus cereus*. A study was done to assess the inhibitory activity of 38 vaginal *Lactobacillus* strains against urogenital pathogens (Juarez *et al.,* 2011). The results showed that the *Lactobacilli* inhibited the growth of various urogenital pathogens except *Candida albicans,* mainly due to the effects of both organic acids and H_2O_2. In another study, 21 *Lactobacilli* strains isolated from infant faeces were analysed for

antimicrobial activities. It was found that some of the isolates had capability of producing broad-spectrum bacteriocin and / or bacteriocin-like substances against some food contaminants and pathogenic bacteria (Arici *et al.,* 2004).

1.3.2.1 Inhibitory substances produced by *Lactobacilli*

a) Fatty acids

Lactobacilli produce different organic acids such as lactic acid, acetic acid, propionic acid, and butyric acid as end products which provide an acidic environment unfavourable for growth of many pathogenic and spoilage microorganisms. These acids act by altering cell membrane potential, inhibiting active transport, reducing intracellular pH and inhibiting a variety of metabolic functions (Ross *et al.,* 2002). They are not only potent against both Gram positive and Gram negative bacteria but also yeast and moulds (Caplice & Fitzgerald, 1999).

b) Hydrogen peroxide

Most *Lactobacilli* are able to produce H_2O_2 which has a toxic potential towards other bacteria but not themselves. *Lactobacilli* and other lactic acid producing bacteria lack heme and thus do not utilize the cytochrome system for terminal oxidation. They utilize flavoproteins which generally convert oxygen to H_2O_2. This mechanism, together with the absence of the heme protein catalase, results in the formation of H_2O_2 in amounts which are in excess of the capacity of the organism to degrade it. The H_2O_2 formed may inhibit or kill other members of the microbiota, especially those with low levels or without H_2O_2-scavenging enzymes such as catalase peroxidase. The microbicidal activity of H_2O_2 is increased by the enzyme peroxidase in the presence of a halide ion (Eschenbach *et al.,* 1989).

c) **Bacteriocins**

These are low molecular mass peptides or proteins (30-60 amino acids) synthesized by bacteria and released to their extracellular environment. They have a bactericidal or bacteriostatic effect on other bacteria such as closely related bacteriocin producing species, food spoilage bacteria and food-borne pathogens (Cheikhyoussef *et al.,* 2009).

Bacteriocins of LAB are classified into four classes based on primary structure, molecular mass, heat stability, and molecular organization (Deraz *et al.,* 2005). Class I bacteriocins (lantibiotics) are small (<5kDa), heat-stable peptides containing thioester amino acids (lanthionine and β-methyl lanthionine). Class II bacteriocins are small, heat-stable, non-lantibiotic peptides (<10kDa). Class III comprises of large (>30kDa) heat labile bacteriocins. Class IV are large, complex bacteriocins containing lipid or carbohydrate groups.

Most bacteriocins are amphiphilic and cationic (Montville *et al*., 1995). Based on their amphiphilic characteristics, there are two mechanisms which explain their membrane-permiabilization action. They contain a region of positively charged amino acids that interact electrostatically with the negatively charged polar head groups of the phospholipids of cell membrane. This contributes to the initial binding with a large membrane. This association dissipates the proton motive force (PMF) of the target cell by forming a pore through the cytoplasmic membrane which causes the rapid efflux of small cytoplasmic compounds e.g. amino acid, potassium, inorganic phosphate, pre-accumulated rubidium and glutamate and flux of essential energy (ATP). Since ATP has no transport system in the sensitive cells and glutamate is not transported by proton motive force driven system, the result induces cell death. Alternatively, bacteriocins may destabilize the integrity of the cytoplasmic membrane in a detergent like fashion (Montville *et al.,* 1995).

1.3.3 Antibiotic resistance

The use of antimicrobial agents (antibiotics) for therapy of bacterial infections has seen the development of resistance in pathogenic bacteria (Mathur & Singh, 2005). Antibiotic resistance genes located on the conjugative or mobilizable plasmids and transposons can be found in species living in habitats e.g. human and LAB (Schwarz & Chaslau-Dancla, 2001; Nordmann & Poirel, 2005; Pruden *et al.,* 2006). Although, LAB may act as reservoirs of antibiotic resistance genes, these can be transferred via the food chain or within the GI tract to pathogenic bacteria (Egervarn, 2009). The

resistances of LAB are often intrinsic and non-transmissible (Curragh & Collins, 1992; Adams & Marteau 1995; Charteris *et al.*, 1998; Salminen *et al.*, 1998). However, some LAB may carry potentially transmissible plasmid-encoded antibiotic resistance genes, as shown for example in certain *L. fermentum*, *L. plantarum* and *L. reuteri* strains (Ishiwa & Iwata, 1980; Ahn *et al.*, 1992; Tannock *et al.*, 1994; Fons *et al.*, 1997). As more new species and strains of probiotic bacteria are identified, it cannot be assumed that they share the historical safety of tested or traditional strains. Therefore, new strains should be carefully assessed and tested for safety.

1.3.4 Adherence to mucus / epithelial cells and ability to reduce pathogen adhesion

It is important to assess a probiotics' ability to adhere to epithelial cells since adherence to mucosal enables colonization (Merk *et al.*, 2005). A number of probiotics have been shown to strongly adhere to human cell lines including *L. rhamnosus* GG, *L. acidophilus* LA-1 and a variety of *Bifidobacteria* (Gopal *et al.*, 2001). *In vitro* models involving human intestinal epithelial cell lines, mostly Caco-2, HT29 and mucus-secreting HT29-MTX cells have been used to assess the adhesion properties of potential probiotic strains (Collado *et al.*, 2010). Adhesion and colonisation properties of three probiotic strains namely, *L. rhamnosus* DR20, *L. acidophilus* HN017, and *B. lactis* DR10, were determined *in vitro* using the differentiated human intestinal cell lines including HT-29, Caco-2 and HT29-MTX, and compared with properties of *L. acidophilus* LA-1 and *L. rhamnosus* GG (two commercial probiotic strains). All three strains showed strong adhesion with the human intestinal cell lines *in vitro*. The adhesion indices of the three strains were not significantly different from the values obtained from the two commercial probiotic strains (Gopal *et al.*, 2001). To evaluate adhesion ability of probiotic strains on vaginal epithelial cells, HeLa cells have been used as *in vitro* model. Examination of the adhesion potential of pre-selected strain *L. plantarrum* AC131 showed that it strongly adhered to HeLa cell lines (Tropcheva *et al.*, 2011).

Lactobacillus strains with high adherence ability can prevent pathogens from adhering and colonizing regions like the GI tract. They are thought to act in a multifactorial process (Collado *et al.*, 2010). A study by Mastromarino *et al.*, (2002) showed co-aggregation of strains of *L. salivarius* and *L. gasseri* with *G. vaginalis* and *C. albicans* and suggested that it is important for healthy urogenital flora. This is because they created a microenvironment around pathogens with consecutive rise of inhibiting substances produced by *Lactobacilli*. The adhesion of pathogens was interfered by probiotic *Lactobacilli* via competition, exclusion and displacement mechanisms (Ren *et al.*, 2012; Dhanani & Bagchi, 2013; Woo & Ahn, 2013). *L. acidophilus* RY2 showed ability to inhibit enteroaggregative *Escherichia coli* adhesion to Caco-2 cells (Lin *et al.*, 2009). In another study, the competition between *Lactobacilli* and *Gonococci* for adherence to human epithelia cervical cells was investigated. The findings showed that *Gonococci* lost when competed against *Lactobacilli* (Vielfort *et al.*, 2008).

1.3.5 Survival in the gastrointestinal tract

The environment of the stomach may highly affect the survival of *Lactobacilli*. The survival of bacteria in gastric juice depends on their ability to tolerate low pH. Though the pH of excreted HCl in stomach is 0.9, .the presence of the food raises the pH value to about 3. After the ingestion of food it takes 2-4 h for the stomach to empty (Goldin & Gorbach, 1992).

In the intestine, bile plays an important role in emulsifying lipids, which enables lipolysis and absorption of lipids (Liong & Shah, 2005). Bile salts are critical to cell membranes of microorganisms since they are composed of lipids and fatty acids. However, some microorganisms are able to reduce this detergent effect by their ability to hydrolyse bile salts by bile salt hydrolase (BSH) and thus to decrease their solubility (Knarreborg *et al.*, 2002).

To reach the intestine, strains must first pass through the stomach which secretes HCl and enzymes. More than two litres of gastric juice is secreted each day, with a pH as low as 1.5 providing a barrier to bacteria from entering the gut (Morelli, 2000). The survival of potential probiotic strains to the gastric juice depends not only on their

intrinsic resistance to the hostile environment but also on the host and the ingestion vector. Foods with a high level of fat and the presence of certain proteins in the food may protect the bacteria from stomach acid and thus increase survival to gastric transit (Zarate *et al.,* 2000). Foods and food ingredients increase the pH of gastric contents hence increase beneficial effects of probiotics (Charteris *et al.,* 1998).

To exert a positive effect on the health of a host, probiotics also need to colonise and survive in the intestine which contains bile salt and enzyme affecting on their viability (Ouwehand, 2002). *Lactobacillus acidophilus* NIT isolated from infant faeces was examined for resistance to pH 2-4. It was observed that *L. acidophilus* NIT had certain resistance ability to acid. Its high survival was shown at pH 4.0 (Pan *et al.,* 2009). Maragkoudakis *et al.,* (2006) examined *in vitro* probiotic potential of 29 *Lactobacillus* stains from dairy origin. Only a few strains were able to survive in the presence of pepsin, while all were unaffected by pancreatin. In addition, the ability to survive the action of bile salts is generally included among the criteria used to select potentially probiotic strains. *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese were tested for their resistance to bile salts. These strains survived in presence of 1%, 1.5% and 2% bile salts (Succi *et al.,* 2005). Although, the bile salt resistant *Lactobacilli* can be selected by testing their survivability in the presence of bile salt and their growth in selected medium containing various levels of bile (Chung *et al.,* 1999). A concentration of 0.15-0.3% of bile salt has been recommended as a suitable concentration for selecting probiotic bacteria for human use (Erkkila & Petaja, 2000).

1.4 Health benefits of probiotics

1.4.1 Alleviation of lactose intolerance

Adults frequently have lactose mal-digestion which presents with the symptoms; loose stools, abdominal bloating, nausea, pain and flatulence. It has however been observed that these individuals can tolerate lactose present in yoghurt better than a similar amount in raw milk (Marteau *et al.,* 2002). It has been shown that yoghurt and probiotic LAB contain high levels of lactase which is released within the intestinal lumen when these bacteria are lysed by bile secretions. The lactase produced acts on

the ingested lactose thereby relieving the symptoms. Also, since yoghurt passes slowly through the intestine, it allows the digestion of lactose thereby reducing symptoms.

1.4.2 Reduction of serum cholesterol level

A high cholesterol level is a risk factor for cardiovascular disease which is a leading cause of death in many countries. Reduction in the level of serum cholesterol has been associated to a reduction in the risk of coronary artery disease. It has been shown that LABs can reduce serum cholesterol levels due in part to the deconjugation of bile salts by producing the enzyme bile salt hydrolase (BSH). The deconjugated bile salts are more readily excreted in faeces than conjugated bile salts. Bacteria with BSH activity may effectively reduce serum cholesterol by enhancing the excretion of bile salts, with a consequent increase in the synthesis of bile salts from serum cholesterol or by decreasing the solubility of cholesterol, and thus reducing its uptake from the gut (Nguyen *et al.,* 2007).

1.4.3 Prevention of pathogens infection in the GI tract and vagina

Several studies have demonstrated that probiotics have protective effects against infections both in the gastrointestinal tract and vagina. In the GI tract, they do so by; production of acids, H_2O_2 or antimicrobial substances, competition for nutrients or adhesion receptors and stimulation of the immune system. It has been shown that *L. rhamnous* had antimicrobial effect against *Salmonella enterica enterica serovar Typhimurium* 1344. Its antagonistic activity involved both lactic acid and secreted non-lactic acid molecules (Marianelli *et al.,* 2010). Also, the LAP5 cell culture showed a higher inhibitory effect on the invasion of *S. choleraesuis* to Caco-2 cells than the spent culture supernatant of LAP5. Also, the pH, organic acids or the bacteriocin may play the roles to reduce invasion of *S. choleraesuis* (Lin *et al.,* 2008).

In the vagina, *Lactobacillus* probiotics are believed to control the microbiota by competing with other microorganisms for adherence to epithelial cells and by producing antimicrobial compounds. These bactericidal compounds include organic acid, which lowers the vaginal pH, H_2O_2, bacteriocin-like substances and possibly

biosurfactants (Boris & Barbes, 2000). A study of the indigenous *Lactobacilli* from the vagina of pregnant women and their antagonistic potential to pathogenic organisms showed that the *Lactobacilli* isolates had antagonistic activity against *Candida albicans*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Xu *et al.,* 2008). The most commonly isolated species from vagina of Chinese pregnant women were *L. acidophilus* and *L. crispatus*. Other studies have reported inhibition of vaginal pathogens by *Lactobacilli* (Juarez Tomas *et al.,* 2003; Saunders *et al.,* 2007)

1.4.4 Modulation of immune system

Probiotics influence the immune system through products like metabolites, cell wall components and DNA. Immune modulatory effects might be even achieved with dead probiotic bacteria or just probiotics-derived components like peptidoglycan fragments or DNA. Probiotic products are recognized by host cells sensitive for these because they are equipped with recognition receptors. The main target cells in that context are therefore gut epithelial and gut-associated immune cells. The interaction of probiotics with host (epithelial) cells by adhesion itself might already trigger a signalling cascade leading to immune modulation. Alternatively release of soluble factors can trigger signalling cascades in immune cells or in epithelial cells which subsequently affect immune cells (Oelschlaeger, 2010).

1.4.5 Reduction of risk of colon cancer

Colon cancer is a multi-factorial and complex neoplasm involving both genetics and environmental factors. There seems to be a strong relationship between colon cancer, diet and internal microflora. The rupture of the intestinal microflora equilibrium due to a bad diet seems to be related to an increase in the risk of developing colon cancer. Probiotics may modulate several major intestinal functions potentially associated with the development of colon cancer preventing the growth of deleterious organisms, producing anti-carcinogenic substances and moving the balance of gut bacteria in favour of the ones beneficial for the organism (Ianniti & Palmieri, 2010)

1.5 Coconut Palm

The coconut palm (*Cocos nucifera*) is the most important palm tree in Kenya. Other palm trees found in Kenya include the wild palm (*Phoenix reclinata*) and the Malaysian palm (*Elaesis guineensis*). Wild palm is commonly found along the Coastal belt of Kenya particularly in Malindi, Kilifi and Kwale districts. Although the Malaysian palm is found scattered along the Kenyan Coastal belt, it has not been produced on a commercial basis (Kadere, 2009).

The coconut palm is a tall and unbranched tree, which can reach a height of 10-30 meters in about 100 years. It has been grown traditionally for its copra –a dried endosperm containing 60-70% oil, of which over 60% is extractable (Ekpa *et al.,* 1994). This oil is widely used in the production of soap, cosmetics, margarine and processed foods. The cake residue is used to make animal feed (Ekpa *et al.,* 1994). In Kenya, the tender coconuts are commonly referred to as "*madafu*". *Madafu* are used as a source of healthy drink, and its gelatinous kernel can be consumed as food. The coconut shell is often used as a source of fuel (Kadere, 2009). It can also be used to make useful products such as beads, bungles, buttons, serving spoons and other decorations. The coconut midribs have been widely used for making brooms and as thatching material "*makuti*" popular in the hotel industry. The coconut tree trunk is useful for construction work and in making furniture and handicrafts (Kahn & de Granville, 1993; Kadere , 2009).

1.5.1 *Mnazi* (coconut wine)

Mnazi is a sugary plant sap which is processed into an alcoholic beverage through fermentation of the sugars present in the sap, yielding alcohol and carbon dioxide. It is sweet, dirty brown in colour, containing 10-20% sugar, mainly sucrose (Okafor, 1975). As fermentation continues, the sap becomes milky-white in appearance due to the presence of large numbers of fermenting bacteria and yeasts (Okafor, 1975). This product is unique in that it can be consumed without pasteurization, hence consumed with live microorganisms in it (Feparus, 1971).

Mnazi is believed to be good for health and eyesight and also serves as a sedative. It is also a mild laxative in relieving constipation (Kadere, 2009). It is prescribed as a tonic for those recovering from diseases such as chicken pox (Okafor, 1977). Previous studies have shown that *Mnazi* is an important source of nicotinic acid and vitamin C and to a lesser extent, proteins, thiamine and riboflavin (Cunningham & Wehmeyer, 1988).

Mnazi is highly perishable. It could start to deteriorate soon as it flows out of the spadix, due to microbial spoilage (yeasts, lactic and acetic acid bacteria), which convert the sucrose into invert sugar and further into ethanol, acetic acid, lactate and other volatile compounds such as acetaldehyde and deacetyl (Kadere, 2009). Commercially bottled *Mnazi* can be preserved best in sodium benzoate at a concentration of 0.15% (m/v). Sodium metabisulphite and propionic acid could also be used as preservatives but they are less effective (Shamala *et al.*, 1988).

1.5.2 Microorganisms in *Mnazi*

a) Acetic acid bacteria

Acetic acid bacteria are associated with wines, beers, fruits, flowers, *Mnazi* and beehives. They are used in industries for manufacture vinegar and other fermentation products such as ketogluconic acid, sorbose and dihydroxyacetone (Swings & De Ley, 1981). However, these bacteria are also associated with spoilage of alcoholic beverages such as beers and wines including *Mnazi*, through the production of acetaldehyde and acetic acid from ethanol.

Spoilage of *Mnazi*, other wines and beers by acetic acid bacteria is as a result of conversion of ethanol to acetic acid in the presence of a small concentration of oxygen (Drysdale & Fleet, 1988; Bartowsky *et al.*, 2003). Acetic acid is the major volatile acid in wine and is considered undesirable at concentrations exceeding 0.4-1.5gl^{-1}, depending on the type of wine (Davis *et al.*, 1985). A study by Kadere (2009) on the acetic acid bacteria in *Mnazi* isolated different *Acetobacter* and *Gluconobacter* strains. These could explain why *Mnazi* readily spoils within 2-5 days of fermentation when

stored in open containers such as bottles, pots, plastic containers and gourds (Kadere, 2009).

b) Yeasts

It has been established that plant materials that contain fermentable sugars provide suitable substrates for yeast species of *Saccharomyces*, *Candida*, *Torula* and *Hansenula* (Campbell-Platt, 1994). Yeasts have for many years provided man with products like leavened bread, beer wine, sake, glycerol, enzymes and vitamins. They have numerous applications in various fields. However, under certain circumstances, yeasts have caused food spoilage especially at below pH 5 and infections to both man and animals. Yeasts such as *Saccharomyces* are typically associated with spontaneous alcoholic fermentations of African opaque beers; *Mnazi* and Asian type of beverages such as rice wine (Campbell-Platt, 1994).

In a study by Kadere (2014) to isolate and identify yeasts in *Mnazi*, a total of twenty-four (24) species were identified as *S. cerevisiae* 1 (68.6%), two (2) species belonged to *S. cerevisiae* 2 (5.7%). Almost 75% of all the isolated strains belonged to the genera *Saccharomyces*. The other isolates were identified as *Candida pelliculosa* (5.7%), Candida utillis (5.7%), *Stephanoascus ciferrii* (2.8%), *Kloeckera spp* (2.8%), *Trichosporon asahii* (2.8%) and *Rhodotorula mucilaginosa* (2.8%).

c) Lactic acid bacteria

Lactic acid bacteria (LAB) are found in foods (dairy products, fermented meat, sour dough, fermented vegetables, silage), beverages (including wine), in plants and in sewage. They can also be found in the genital, intestinal and respiratory tracts of man and animals (Hames *et al.,* 1991). In the food industry LABs act as both beneficial and spoilage organisms. They are used in the production of fermented milk products such as yoghurt, sour milk, cheese and butter, and in the production of sausages, pickles and sauerkraut. The result of these fermentations is more shelf-stable products with characteristic aromas and flavours.

The occurrence of LAB in beers and wines including *Mnazi* is usually associated with spoilage of these products. Bacterial growth in *Mnazi* and beer depends on the ability to scavenge residual nutrients left following yeast growth and tolerance to the relatively hostile environment posed by fermented beverage (Pfenninger *et al.*, 1979). A study to investigate the nutrients that support growth of LABs in wines and beer was done by inoculating them with LABs and following the changes in sugar and amino acid content of media with growth (Pfenninger *et al.*, 1979). It was found that small amounts of sucrose were metabolized along with varying amounts of maltose, maltotriose and maltotetrose. Among the amino acids absorbed by lactobacilli were lysine, tyrosine and arginine. Some of the LAB genera isolated from *Mnazi* include *Leuconostoc* and *Streptococcus* (Okafor, 1972). In a study by Kadere and Kutima (2012) to isolate and identify LAB in *Mnazi*, a total of 15 strains were positively identified after isolation using API 50 CHL kit with the predominant species being; *Lactobacillus paracasei ssp paracasei* 2 (7 strains), *Lactobacillus paracasei ssp paracasei* 1 (4 strains) and *Lactobacillus plantarum* 1 (2 strains).

1.6 Statement of the problem

Many health benefits can be attributed to *Mnazi*. It is believed to be good for health, eyesight and also serves as a sedative. Besides, it is also a mild laxative and has been prescribed as a tonic for those recovering from diseases such as chicken pox (Okafor, 1977). Previous studies have shown that *Mnazi* is an important source of nicotinic acid, vitamin C and to a lesser extent, proteins, thiamine and riboflavin (Cunningham & Wehmeyer, 1988).

The occurrence of LAB in beers and wines including *Mnazi* is usually associated with spoilage of these products. Their growth depends on the ability to scavenge residual nutrients left following yeast growth and tolerance to the relatively hostile environment posed by fermented beverage (Pfenninger *et al.*, 1979). Some of the LAB genera isolated from *Mnazi* include *Leuconostoc* and *Streptococcus* (Okafor, 1972). In a study by Kadere and Kutima (2012) to isolate and identify LAB in *Mnazi*, a total of

15 strains were positively identified with the majority being *Lactobacillus paracasei ssp paracasei* strains (Kadere & Kutima, 2012)

These LABs isolated from *Mnazi* have not been assessed to determine their probiotic potential and whether they can produce antimicrobial substances that can be utilized to produce safe and healthy products. These substances are known to act as natural antibiotics that kill undesirable pathogenic microorganisms.

1.7 Justification

Over prescription and misuse of antibiotics has led to a situation where increasing number of pathogens are becoming resistance to antibiotics. The World Health Organisation (WHO) has indicated that alternative disease control strategies such as use of probiotics may be needed in the future in the prevention and treatment of certain infections.

Probiotics are live microbial preparations with documented health benefits that maintain or improve intestinal microbiota balance. They have become an important part of nutrition because the microbial population in our bodies are altered by the use of antibiotics and other substances that are designed to kill germs and disease. While antibiotics are effective at killing germs they are also effective in killing beneficial bacteria. By introducing friendly microbes to the gut flora, this can strengthen the resident microflora.

This study determined that the LAB isolates from *Mnazi* have probiotic properties and therefore enhance the potential of the wine as a drink. The isolates can thus be adapted and added into foods without losing their viability and functionality, or creating unpleasant flavours or textures. This adds value to foods consumed and thereby bring about health benefits to the consumers.

1.8 Hypothesis

The LABs isolated from *Mnazi* do not have probiotic potential, produce stable antimicrobial substances and bacteriocins which act as natural antibiotic.

1.9 Objectives

1.9.1 General objective

To characterize probiotic potential of *Lactobacillus ssp* isolated from coconut wine (*Mnazi).*

1.9.2 Specific objectives

1. To characterize probiotic properties of the LAB isolates from *Mnazi*
2. To characterize the LAB isolates from *Mnazi* for production of bacteriocins and assess the effect of heat, enzyme and pH on the bacteriocins
3. To characterize and identify the LAB isolates from *Mnazi* using molecular approaches.
4. To assess the safety attributes of the LAB isolates from *Mnazi* including; haemolysis and gelatinase activity

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Samples

Samples of *Mnazi* were obtained from Chonyi and Kikambala areas of the coastal region of Kenya. The samples were collected in sterile sampling tubes. Fifteen (15) strains of Lactic acid bacteria were isolated from coconut wine (*Mnazi)* in Food Microbiology Laboratory in Jomo Kenyatta University of Agriculture and Technology, Kenya. They were identified to species level at Animal Food Functions Laboratory in Okayama University, Japan by use of API 50 CHL system (BIOMERIEUX SA, France) (Kadere & Kutima, 2009). The identified species were used in this study. The cultures were maintained in MRS broth with sterile glycerol (15%) and deep frozen at -18°C in Food Microbiology Laboratory.

2.2 Growth media for *Lactobacillus* isolates

The isolates were sub-cultured by pour plate method on de Man, Rogosa and Sharpe (MRS, Oxoid) agar plates with Bromo-crystal purple and incubated for 2 days. Isolates were picked from plates with less than 30 colonies. Pure colonies of the selected isolates were obtained by transferring three times from the BCP agar into litmus milk medium (skim milk 10%, l-glutamic acid monosodium salt 0.1%) and stored at -20°C with glycerol cryoprotectant, on Microbank vials (pro-lab diagnostics) for long term conservation. A duplicate set was inoculated on litmus milk medium and stored at 4°C and transferred every 2 months.

2.3 Characterization of probiotic potential

2.3.1 Determination of acid tolerance

Acid tolerance was determined according to Jacobsen and Lei (2004). Test culture strains were grown for 16 h in MRS broth at 37°C. An aliquot of 1ml of the 16 h old culture was inoculated into 9ml MRS broth whose pH was adjusted to 2 and 2.5 using

1N HCl. Samples were drawn after 0, 1, 2 and 3 h and immediately diluted ten-fold in Peptone water phosphate-buffered pH 7 to eliminate medium acidity. Decimal dilutions of samples were made using maximum recovery diluent and 0.1 ml was taken from 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} of the samples and pour plated in duplicate in 15ml of MRS agar. Plates were incubated at 37°C, anaerobically using anaerobic jars containing wet anaerocult sachets for 48 h. Viable counts were determined by counting the number of colonies from plates containing 10 to 300 colonies and colony forming units (cfu/ml) determined from the average.

2.3.2 Determination of bile tolerance

Bile tolerance was determined according to modified methods of Jacobsen and Lei (2004). Following 3 h of exposure to pH 2 and pH 2.5, 1 ml of the acid stressed cultures were diluted in peptone water phosphate buffered at pH 7 to eliminate the low pH medium acidity. Aliquots of 1 ml of each of the acid stressed neutralised cultures were then transferred into 9 ml of MRS broth supplemented with 0.3% bile salts and incubated for 48 h at 37°C. Viable cell counts of MRS-bile inoculated acid stressed cultures were determined after 0, 3, 24 and 48 h using pour plate method in MRS agar and incubated at 37°C in anaerobic jars containing wet anaerocult sachets.

2.3.3 Detection of *meso*-diaminopimelic (*meso*-DAP)

Presence of *meso*-DAP in the bacterial cell wall was determined according to modified method described by Schillinger and Lücke (1987). Briefly, isolates were grown in 1 ml MRS broth for 48 h, harvested and washed with 1 ml of distilled water. The sediments were re- suspended in 1 ml 6 N HCl and transferred to a capped test tube. The cells were hydrolysed overnight at 100°C. The content of the tube were dried in an air steam. The sediment were re-suspended in 1 ml of distilled water and dried in the same manner. Finally, the sediments were re-suspended in 0.1 ml distilled water and samples were spotted on thin layer plates [DC Plastic Folien: Cellulose (20×20) Merck 5577]. Ascending one-dimensional chromatography was done in a modified solvent solution containing methanol/pyridine/10 N HCl/water in (32:4:1:7) v/v/v/v.

The solvent was prepared 1 h before use. After drying, the chromatogram was developed with ninhydrin solution and placed for 5 min in a 100°C oven. Spots representing *meso*-diaminopimelic acid appeared as dark green to grey and turned yellow within 24 h in the dark.

2.3.4 Response to simulated stomach duodenum passage (SSDP)

This was determined according to method by Mathara *et al.* (2008) which represent a simplified and standardized test system giving predictive values for the assumed survival of LAB in the human stomach and duodenum under "normal" physiological conditions. The principle involves first a simulation of the stomach containing ingested LAB after a meal under "worst case conditions," that is, without the protective effect of ingested food. After 1 h, bile and artificial duodenum secretions were added in order to simulate the further passage. Sterile MRS broth with pH adjusted to 3.0 with 5 M HCl was used. Synthetic duodenum juice was prepared by completely dissolving $NaHCO_3$ (6.4 g L^{-1}), KCl (0.239 g L^{-1}), and NaCl (1.28 g L^{-1}) in bidest water. The pH was adjusted to 7.4 with 5M HCl before sterilizing at 121°C for 15 minutes. The oxgal solution was prepared by reconstituting 10 g of oxgal in 100 ml bidest water and sterilizing at 121°C for 15 mins. Required volumes of the overnight cultures and MRS broth adjusted to pH 3.0 were aseptically mixed in sterile flasks to give a final concentration of 2×10^8 cfu per 10 ml MRS. After mixing, the initial count was determined by spread plating. The flasks were incubated at 37°C. Samples were withdrawn after 1h and viable counts was determined by spread plating. Four millilitres of oxgal solution was added to the culture in the flasks, followed by 17 ml of duodenum juice. After mixing, the flasks were further incubated at 37°C. Samples were withdrawn after 2 and 3 h, and counts determined as described above.

2.3.5 Production of antimicrobials

Production of antimicrobial products was assayed according to modified methods of Ogunbanwo *et al.*, 2003 and Hernandez *et al.*, 2004. Isolates were screened for production of antimicrobial products by the agar spot method (nutrient agar seeded

with indicator microorganisms, poured into plates then different *Lactobacillus spp* spotted on the plates) using the available Gram positive stains *Staphylococcus aureus* NCTC 6571 as the indicator organism. Isolates showing an inhibition zone were further characterized for antimicrobial activity against *Enterococcus faecalis* NCTC 775 (Gram positive non-lactic acid bacteria strain) and *E.coli* NCTC 10418 (Gram negative bacteria). Strain showing antimicrobial characteristics were further characterized for production of bacteriocins.

2.3.6 Determination of bacteriocin activity

Test isolates were grown anaerobically in MRS broth (to prevent formation of H_20_2) for 24h at 37°C. Cultures were centrifuged at 4193 r.p.m at 4°C for 20 min to obtain a cell free supernatant. Three quarters of the supernatant were neutralized using 1N NaOH to exclude antimicrobial effect of organic acid, then filtered through a 0.2 µm pore size filter.

Nutrient agar seeded with overnight cultures of the indicator strains of *Enterococcus faecalis* NCTC 775, *E.coli* NCTC 10418 and *Staphylococcus aureus* NCTC 6571 each strain separately, was pour plated and 10 mm wells made into the agar. The wells were sealed at the bottom with sterile non-seeded agar. About 100 µl aliquots of sterile neutral supernatant was placed into the agar wells and in duplicates for each test isolate. The plates were kept at 4°C for 2h (to allow for diffusion of antimicrobial substances) then incubated for 48hrs at the optimum temperature for indicator microorganisms (37°C).

As control, wells were made on nutrient agar seeded with overnight cultures of indicator microorganisms (in duplicates for each of the test isolates) into which the following combination of 100µl of test solutions was added; sterile un-inoculated MRS broth; non-neutralised supernatant, neutralised supernatant that had been inoculated for an hour at 37°C with pepsin (proteolytic enzyme); neutralised supernatant that had been incubated for an hour with amylase (non-proteolytic enzyme); neutralised supernatant that had been incubated at 37°C with lipase (non-proteolytic enzyme). The enzymes were prepared in potassium phosphate buffer, pH 7 at a concentration of

1mg/ml. The plates were kept at 4°C for 2hrs followed by incubation at 37°C for 48h, then observed for zone of inhibition.

Following a method suggested by Montville *et al*., (1993) and used by De Martinis *et al.,* (2001) and Torker and Matijasic (2003) for demonstration of proteinaceous nature of inhibition, non-neutralised supernatant was assayed for sensitivity of antimicrobial substance to proteolytic and non-proteolytic enzymes. This was done with a view of eliminating any doubt as to the possibility of production of bacteriocins by assayed strains isolated from *Mnazi.* Two wells were made adjacent to each other in a seeded nutrient agar. For the assay, *E. coli* NCTC 10418 was used as an indicator strain. One well was put non-neutralized supernatant and enzyme put in adjacent well. Phosphate buffer pH 7 used for making enzyme solutions was utilized as control for showing effect of enzymes on inhibition activity.

After 48h incubation at 37°C, the plates were observed for a dimple of growth and reduced inhibition zone onside of well containing supernatant and adjacent to well containing enzyme.

2.3.6.1 Determination of the effect of heat, enzyme and pH on bacteriocin activity

The effect of heat, enzyme and pH on bacteriocin activity was determined as described by Mathara, (2004). Briefly, the effect of heat on cell free neutralized supernatant was assessed at temperature 100°C for 5, 10, 20 and 30 min and 121°C for 15 min. The effect of pH was tested on cell free supernatant fluid adjusted to pH values ranging from 2 to 10 and the effect of enzymes (pepsin, amylase and lipase) on supernatant fluid activity was determined at 1mg ml^{-1} final concentration (Mathara, 2004).

2.3.7 Determination of β-galactosidase activity

ß-galactosidase activity was assayed according to the method of Gilliland and Lara (1988). Assays were performed in duplicates and results represent the means of three independent trials.

Cells were transferred from MRS agar plates/slants to 10% non-fat dried milk, and grown for 24 h at 37°C. The 1:1 dilution of cells were made with 0.05M (pH 7.0) sodium phosphate buffer. To this tube, 0.2ml of lysozyme (50mg/ml) (sigma) was added, the solution mixed, and held in ice-water for 30 min. One millilitre of o-nitrophenol-beta-D-galactopyranoside (Merck) was added to the tube. The mixture was vortexed, and incubated at 37°C for 2 min. To stop the reaction, 2 ml of 0.625M sodium carbonate was added. Centrifugation was done at 3000 r.p.m. for 10 min, to remove turbidity resulting from precipitated milk solids. The absorbance at 420 nm was recorded against a reagent blank, except that lysozyme is replaced by sodium phosphate buffer. Units of enzyme activity is defined as micromoles of 0-nitrophenol released was based on the relationship of the A_{420} to the standard curve. Specific activity is defined as micromoles of 0-nitrophenol released/min/ml/cfu.

2.3.8 Determination of utilization of Fructooligosaccharide

Modified MRS basal agar medium was used (MRS prepared without carbohydrate and lab lemco), 2% of inulin was added to form an MRS-FOS agar medium (Kaplan & Hatkins, 2000). The basal medium containing 0.05% L-cysteine, 1.5% agar and 30 mg of bromocresol purple as indicator. The medium was first be autoclaved then filter sterilised FOS will be added to the liquid agar at 45°C. The MRS-FOS agar medium was then be poured into plates.

Isolates that had been grown for 16 h in MRS broth were streaked onto the MRS-FOS agar plates the incubated anaerobically in anaerobic jars containing anaerocult sachets at 37°C for 48 h .The plates were observed for a yellow hallo around the colonies, which is a positive indication of acid production from FOS fermentation. As control and a way to confirm that the yellow was a result of FOS utilisation or fermentation, a 16hrs culture of isolate strains were streaked onto MRS agar containing 2% glucose and 2% FOS.

Following standard procedures for sugar fermentation profile tests, isolates were harvested from 16 h culture broths by centrifugation at 5000 rev/min for 20 min at 4°C, then washed with modified MRS broth (MRS broth with no carbohydrate and lab

lemco) and inoculated into modified MRS broth containing 1% FOS and andrade's indicator. MRS broth containing glucose as carbon source was used as control. Mineral oil was added to create anaerobic conditions and the tubes were incubated at 37°C for 5 days period during which they were observed daily for red colour. The pH and OD (600 nm) for MRS-FOS as well as MRS-glucose broths was determined to compare the effect on growth and ability to utilize the two types of carbon sources.

2.4 Molecular characterization of LAB isolates from *Mnazi*

2.4.1 Extraction of genomic DNA from LAB isolates from *Mnazi*

Genomic DNA was extracted from the selected fifteen isolates using the chloroform extraction procedure (Sambrook *et al*., 1989). Prior to extraction, bacterial cells were harvested from MRS broth by centrifuging (13000 rpm, 5 mins, at 25°C) (Hettich, Micro 200, Germany). For this case, 1ml of culture was placed in a 1.5ml eppendorf tube and the supernatant poured out after the centrifugation. This was repeated twice to obtain enough cell yields. The bacterial cells were washed by re-suspending in equal volumes of TE buffer (pH 8), centrifuged (Hettich, Micro 200, Germany) for 5 mins at 13000 rpm, at 25°C, and the supernatant discarded. The cells were then re-suspended in 200 µl of solution 1 [50mM Tris (pH 8.5), 50mM EDTA pH (8.0) and 25% sucrose solution], 5 µl of lysozyme (20mg/ml) (Sigma Aldrich, Steinheim, Germany) and 5 µl of RNase A (20mg/ml) (Sigma Aldrich, Steinheim, Germany) then mixed gently.

The mixture was then incubated at 37°C for 1 hour. Then 600 µl of solution 2 [10mM Tris (pH 8.7), 5mM EDTA (pH 8.0) and 1% sodium dodecyl sulphate] and 10 µl of 20mg/ml proteinase K (Sigma Aldrich, Steinheim, Germany) was added and mixed gently. The mixture was then incubated at 50°C for 30 min. Equal volumes of phenol-chloroform were added and spun for 5 minutes at 13000 rpm, at 25°C. The upper aqueous layer was transferred carefully into a separate I.5 ml eppendorf tube. This step was repeated before adding an equal volume of diethyl ether to wash off the phenol. This mixture was then spun at 13000 rpm, at 25°C, for 5 min and the supernatant carefully discarded. The procedure was repeated twice. The DNA was then precipitated by adding an equal volume of ice cold absolute ethanol and 0.1 volumes

of 3M potassium acetate (Sigma Aldrich, Steinheim, Germany) and left overnight at -20°C. The pellet was concentrated by centrifugation at 13000 rpm, at 25°C, for 30 minutes and the supernatant discarded. Equal volumes of 70% ethanol (Scharlab S.L., Spain) were added and centrifuged at 13000 rpm for 5 mins. The supernatant was discarded carefully without disturbing the pellet. This procedure was repeated twice before leaving the pellet on the bench to air dry completely at room temperature in order to eliminate residual ethanol.

The dry DNA pellet obtained was then re-suspended in 45µl of TE buffer and then kept at -20°C for future use. The DNA was separated on a 1% (w/v) agarose (Sigma Aldrich, Steinheim, Germany) gel in 1xTAE buffer and visualized under UV by staining with ethidium bromide (Sambrook *et al*., 1989).

The DNA was then quantified using a spectrophotometer with the absorbance at 260nm and 280nm to determine the purity of the DNA. The ratio 1.8-2.0 was used in the subsequent polymerase chain reaction.

2.4.2 Polymerase chain reaction

For the amplification of the 16S rDNA, 1µl of DNA from each of the fifteen extracts were amplified using Taq polymerase and 10x buffer according to manufacturer's (QIAGEN) instructions. Nearly full-length 16S rDNA gene sequences were PCR-amplified using bacterial primer pair 27F forward 5'-GAGTTTGMTCCTGGCTCA-3' and 1492R reverse, 5'-TACGGYTACCTTACGACT-3' (Bioneer, USA) according to the position in relation to *Escherichia coli* gene sequence (Embley & Stackebrandt, 1994). Amplification was performed using an Eppendorf AG, model 22331 thermal cycler (Hamburg). Amplification was carried out in a 50 µl mixture containing 0.2 Units of Taq polymerase, 20pmol of 27F forward primer, 20pmol of 1492R reverse primer, 1.25mM dNTPs mix (QIAGEN), 10x PCR buffer (QIAGEN), 1 µl of template DNA and 29.8 µl of PCR water. The negative control contained all the above except the DNA template.

Reaction mixtures were subjected to the following temperatures: Initial denaturation of the template at 94ºC for 5 minutes, denaturation at 94°C for 45 seconds, primer annealing at 43ºC for 2 minutes, chain extension at 72ºC for 1.5 minutes and a final extension at 72°C for 5 minutes (Roux, 1995). Denaturation, annealing and extension cycles were repeated for 35 cycles. Amplification products (7.0 µl) were separated on a 1% (w/v) agarose (Sigma Aldrich, Steinheim, Germany) gel in 1X TAE buffer and visualized by ethidium bromide staining (Sambrook *et al*., 1989).

2.4.3 Purification of PCR products

The PCR products were purified using the QIAquick PCR purification Kit protocol according to manufacturer's (Qiagen, Germany) instructions. Five volumes of binding buffer (PB) was added to 1 volume of the PCR sample and thoroughly mixed. The QIAquick spin column was placed in a 2 ml collection tube, the sample applied, and then centrifuged for 60 seconds at 13000 rpm, at 25°C. The flow-through was discarded, and the QIAquick column placed back into the same tubes. To wash the DNA, 0.75 ml washing buffer (PE) was added to the QIAquick column and centrifuged for 1 min at 13000 rpm, at 25°C. The flow-through was discarded and the column centrifuged again for an additional 1 minute at 13000 rpm, at 25°C to remove residual ethanol from buffer PE.

The Qiaquick column was then placed in a 1.5 ml micro centrifuge tube and 30 µl of elution buffer (EB) added to elute DNA. The tubes were then centrifuged for 1-minute at 13000 rpm, 25°C, after which the spin column was removed and DNA stored at -20ºC for further use (Sambrook *et al.*, 1989).

2.4.4 Sequencing and molecular data analysis

Sequencing of purified PCR products was done by a commercial service provider (ILRI) using the dye-terminator sequencing technique. They were compared to those available in the GenBank (Benson *et al.*, 2005) using Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) to determine their sequence similarity value.

Sequences that were > 97% similar to the previously published sequences were used as the criteria to indicate species identity. All the sequences were retrieved and aligned together with those from this study using CLUSTAL W (Thompson *et al.*, 1994). Evolutionary distance matrices were calculated according to the method of Jukes and Cantor (1969) and a phylogenetic tree was constructed based on neighbour-joining algorithm (Saitou & Nei, 1987) using Molecular Evolutionary Genetic Analysis (MEGA) version 5.22 (Kumar *et al.,* 2004). Gaps or missing data were removed from phylogenetic analysis with complete deletion. The robustness of the tree topology was tested by performing bootstrapping for 1000 replicates (Felsenstein, 1985).

2.5 Determination of safety attributes

2.5.1 Determination of haemolysis activity of LAB isolates from *mnazi*

Haemolytic activity was investigated according to modified methods as described in Manual of methods for general bacteriology (Gerhardt *et al*., 1981). A 16h old culture broth was streaked onto sterile blood agar. The blood agar was prepared by adding 7% fresh ox blood, preserved in EDTA, onto sterile blood agar base at 45°C. Plates were incubated anaerobically at 37°C for 48h after which the plates were observed for beta and alpha haemolysis indicated by a clear zone around colonies with a greenish colour for beta haemolysis, but none for alpha haemolysis.

2.5.2 Determination of gelatinase activity of LAB isolates from *Mnazi*

Gelatinase activity was investigated as described in MacCance (1990). A 16h old culture was streaked onto nutrient gelatin agar (Oxoid). The plates were incubated anaerobically in anaerobic jars containing wet anaerucult sachets (Merck) for 48h at 37°C. The plates were then flooded with $HgCl_2$ solution (15% $HgCl_2$ in 20% v/v HCl) and observed for clear zones surrounding colonies (positive reaction for gelatin hydrolysis).

2.6 Data analysis

Preliminary data summaries was done using Microsoft Excel spreadsheets (Microsoft Corp). The results were expressed as means for the different variables investigated. Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 17. These tests were performed to compare the difference between the treatments and a probability level of <0.05 considered significant.

CHAPTER THREE

3.0 RESULTS

3.1 Probiotic properties of LAB isolates from *Mnazi*

3.1.1 Acid tolerance

In general, there was no observed increase in counts for all the isolates under the two pH conditions (2.0 and 2.5) after 3 hours of exposure. Four (4) *Lactobacillus paracasei* strains (CM 203, CM301, CB4041 and CB303) were viable to a level of 50 to 70 % after 3h of exposure to a low pH of 2.0. Four (4) strains of *L. paracasei* (TB402, CM201, CM4091 and TB302) showed a count of 3 log cfu ml $^{-1}$ after 3 hours of exposure at pH 2.0. These had viable counts at a level of 5 to 6 log cfu ml $^{-1}$ after exposure at pH 2.0 for 2 hours, but viable counts reduced thereafter to reach levels of 3 log cfu ml $^{-1}$ (Table 1). Four other strains of *L. paracasei* (TM302, TB405, and CM4081) and *L. plantarum* (CM402) were able to tolerate and survive at pH 2.0 for 2 hours to a level of 3 log to 6 log cfu ml $^{-1}$ but tolerance declined with further exposure to low pH. (Table 1). *L. paracasei* strain CB4041 decreased by only 2 log cycles within the 3 hour period of exposure at pH 2.0. This indicated that the strain was stable at low pH but could not be able to grow.

Table 1: Survival of *Lactobacillus paracasei* and *Lactobacillus plantarum* stains (Iog10 cfu/ml) isolated from *Mnazi* under pH 2.0 and 2.5 for up to 3h in MRS broth.

Strain Code	No.	pH 2.0				pH 2.5			
		0h	1h	2h	3h	0h	1h	2h	3h
***L. paracasei* TB402**	**8**	9.78	3.70 (37)	7.11 (73)	3.69 (38)	10.91	9.65 (88)	9.11 (83)	8.94 (82)
***L. paracasei* CM203**	**4**	9.41	4.04 (43)	5.60 (60)	5.90 (63)	11.70	8.84 (76)	8.68 (74)	8.86 (76)
***L. paracasei* CB204**	**15**	11.74	6.20 (53)	5.72 (49)	___	10.65	9.26 (87)	8.62 (81)	8.41 (79)
***L. paracasei* TD3051**	**2**	9.36	4.38 (47)	3.30 (35)	___	11.20	9.86 (88)	9.78 (87)	8.26 (74)
***L. plantarum* CM402**	**11**	9.74	4.70 (48)	6.40 (66)	___	11.39	8.89 (78)	9.08 (80)	9.45 (83)
***L. paracasei* CM4081**	**13**	8.90	4.32 (49)	3.48 (39)	___	10.08	9.52 (94)	9.97 (99)	9.96 (99)
***L. paracasei* CM301**	**3**	11.43	4.00 (35)	5.32 (47)	6.11 (53)	10.15	9.18 (90)	9.34 (92)	9.04 (89)
***L. paracasei* TB302**	**6**	9.60	6.20 (65)	6.66 (69)	3.60 (37)	11.86	10.04 (85)	8.89 (75)	7.35 (62)
***L. paracasei* TB405**	**7**	10.87	8.00 (74)	7.30 (67)	___	12.30	10.25 (83)	9.90 (80)	9.20 (75)
***L. paracasei* CB3021**	**1**	10.00	4.85 (49)	___	___	12.26	10.04 (82)	9.18 (75)	8.52 (69)
***L. paracasei* CM4091**	**9**	11.70	6.38 (54)	4.30 (37)	3.00 (26)	13.64	9.43 (69)	9.11 (67)	8.57 (63)
***L. paracasei* CB303**	**10**	10.11	3.60 (36)	4.71 (47)	6.28 (62)	12.28	10.00 (81)	9.49 (77)	9.43 (77)
***L. paracasei* CB4041**	**5**	8.30	4.32 (52)	5.78 (70)	6.15 (74)	11.70	10.30 (88)	10.20 (87)	10.20 (87)
***L. paracasei* TM302**	**12**	10.62	3.00 (28)	6.80 (64)	___	12.04	9.52 (79)	9.30 (77)	9.30 (77)
***L. paracasei* CM201**	**14**	9.81	7.54 (77)	5.60 (57)	3.60 (37)	12.26	10.44 (85)	10.32 (84)	7.35 (59)

Data represents the mean of duplicate experiments. Figures in brackets represent the percentage survival rate of each strain

All *Lactobacillus* strains assayed by exposure at pH 2.5 for 3 hours were able to survive within a range of 7–9 log cfu counts throughout the period. *L. paracasei* (TB402, CM203, CB204, TD3051, CM 301, TB302, TB405, CB302, CM4091, CB303, CB4041, TM302) and *L. plantrum* (CM402) survival decreased by two log cycles, showing a survival level of 60–90%. The isolated strains of *L. paracasei* (CB204, TD3051, CM4081, TB405, CB3021, TM302) and *L.plantarum* (CM402) were not tolerant to pH 2.0 but were stable in pH 2.5 showing a viable count of 7–9 log cfu ml^{-1} by the end of 3 hour exposure to pH 2.5 as compared to no viable counts when exposed to pH 2.0.

All the isolated strains did not show any observed change in viable count at pH 2.5 with exception of *L. plantarum* (CM 402) which showed an increase from 8 log cycle to 9 log cycle from 1 hour exposure in viable count for the 3 hour exposure while *L. paracasei* CM 4081 showed an increase after the first hour of exposure at pH 2.5 indicating that these isolates were able to tolerate and survive in a low pH of 2.5 but they were not able to grow. The strains *L. paracasei* (CB4041, CM4081 and TM302) showed constant growth. Strains *L. paracasei* (CB204, TD3051, CM4081, TB405, CB3021, TM302) and *L. plantarum* (CM402) were not able to tolerate a pH of 2 but were able to tolerate a higher pH of 2.5 (Table 1).

3.1.2 Bile tolerance

There was better survival of the isolates after exposure to bile when grown at pH 2.5 than pH 2.0. At pH 2.5, thirteen (13) of the 15 isolates showed viable counts of between 6 and 7 log cfu ml^{-1}. Only *L .paracasei* (TD3051) showed a lower viable count of 5 log cfu ml^{-1} and *L. paracasei* CM 4091 showed the highest viable count of 9 log cfu ml^{-1}. Among all the isolates, only *L. paracasei* (CB303) and *L .paracasei* (CB4041) showed a viable counts ranging from 3.0-6.7 log cfu ml^{-1} when grown for up to 48h at both pH 2 and 2.5 after exposure to 0.3% bile. Their viable counts increased from 4 log cfu ml^{-1} to 4 and 6 log cfu ml^{-1} after 24 and 48h respectively showing a 165% and 190% survival after 48h of incubation at pH 2.0. This suggests that these stains are able to grow in the bile supplemented conditions.

Table 2: **Survival of *Lactobacillus paracasei* and *Lactobacillus plantarum* stains (Iog_{10} cfu/ml) isolated from *Mnazi* in MRS broth supplemented with 0.3% bile salts, following a 3h to 48h exposure to pH 2 and 2.5.**

Strain Code	No.	pH 2.0				pH 2.5			
		0h	3h	24h	48h	0h	3h	24h	48h
***L. paracasei* TB402**	**8**	___	___	___	___	7.70	6.88 (89)	8.79 (114)	9.04 (117)
***L. paracasei* CM203**	**4**	3.30	___	___	___	7.65	5.78 (76)	4.91 (64)	4.60 (60)
***L. paracasei* CB204**	**15**	___	___	___	___	7.58	___	___	___
***L. paracasei* TD3051**	**2**	3.48	___	___	___	5.57	5.85 (105)	6.00 (108)	6.32 (113)
***L. plantarum* CM402**	**11**	___	___	___	___	7.67	7.32 (95)	7.18 (94)	7.30 (95)
***L. paracasei* CM4081**	**13**	___	___	___	___	7.65	3.00 (39)	3.78 (49)	6.49 (85)
***L. paracasei* CM301**	**3**	___	___	___	___	7.68	3.85 (50)	4.30 (56)	6.51 (85)
***L. paracasei* TB302**	**6**	___	___	___	___	7.89	4.73 (60)	5.15 (65)	6.59 (84)
***L. paracasei* TB405**	**7**	___	___	___	___	6.83	___	___	___
***L. paracasei* CB3021**	**1**	4.00	3.00 (75)	___	___	6.62	5.48 (83)	4.79 (72)	4.51 (68)
***L.paracasei* CM4091**	**9**	3.60	___	___	___	9.45	6.62 (70)	5.95 (63)	5.38 (57)
***L.paracasei* CB303**	**10**	3.00	3.00 (100)	4.69 (156)	4.96 (165)	7.73	3.85 (50)	3.30 (43)	6.52 (84)
***L.paracasei* CB4041**	**5**	3.48	4.55 (131)	4.60 (153)	6.60 (190)	7.62	3.34 (44)	4.60 (60)	6.70 (88)
***L.paracasei* TM302**	**12**	3.00	___	___	___	7.66	4.70 (61)	3.30 (43)	3.48 (45)
***L.paracasei* CM201**	**14**	4.48	___	___	___	7.92	3.79 (48)	4.73 (60)	9.15 (116)

Data represents the mean of duplicate experiments. Figures in brackets represent the percentage survival rate of each strain

The strain *L. paracasei* (CB3021) showed a 10% decrease in viable count from 4 to 3 log cfu ml^{-1} after 3h of growth at pH 2.0 but thereafter died off. The other strains *L. paracasei* (TB402, CM203, CB204, TD3051, CM4081, CM301, TB302, TB405, CM4091, TM302, and CM201) and *L. plantarum* (CM402) showed no viable count after 3h of exposure (Table 2). These results suggest that these strains are not able to tolerate and grow in conditions containing bile salts and at pH 2.0.

At pH 2.5, all the isolates except *L. paracasei* (CB204 and TB405) were able to grow for up to 48h after exposure to 0.3% bile. The two isolates initially had a viable count of 7.58 and 6.83 log cfu ml^{-1} respectively at 0h after bile exposure but died thereafter. The best growth was registered by the isolates *L. paracasei* (TB402, TD3051 and CM201) had viable counts of 9.04, 6.32 and 9.15 log cfu ml^{-1} (survival rates of 117%, 113% and 116%) respectively after 48h of growth. Despite an initial dip in growth from 0 to 3h, the three strains increased in growth at 24 and 48h respectively (Table 2). All the other strains generally had diminishing growth and survival rates with continued exposure to bile and pH 2.5 growth conditions.

Notably, isolate *L. plantarum* (CM402) had the highest viable count of 7 log cfu ml^{-1} after 3h of growth while the rest had counts ranging from 3 and 6 log cfu ml^{-1}. Its growth remained stable at 24 and 48h respectively. Four (4) isolates *L. paracasei* (CM4081, CM301, CB303 and CB4041) showed survival rates of >80% when grown up to 48h. After an initial drop in their viable counts at 3h (3.0, 3.85, 3.85 and 3.34 log cfu ml^{-1} respectively), their growth increased at 24h and 48h (6.49, 6.51, 6.52 and 6.70 log cfu ml^{-1} respectively) (Table 2). The isolates *L. paracasei* (CM203, TM302 and CM4091) showed the least survival rates of 50–60% after 48 hours of incubation. These results suggest that the isolates *L. paracasei* (TB402, TD3051 and CM201) from *Mnazi* can tolerate conditions of 0.3% bile and pH 2.5 to grow exponentially.

3.1.3 Survival under conditions of simulated stomach duodenum passage

The 15 isolates were tested under conditions of simulated stomach duodenum passage to determined how they survive. All the isolates showed survival rates of 33-56% after 3h despite the high bile concentration of more than 2% (Table 3). The strains *L. paracasei* (TB402, CM203, TB302, CB3021 and CM201) had survival rates of more than 50% while the rest; *L. paracasei* (CB204, TD3051, CM4081, CM301, TB405, CM4091, CB303, CB4041, TM302) and *L. plantarum* (CM402) had survival rates of less than 50%.

Table 3: Survival rates of *L paracasei* and *L plantarum* strains (Log CFU/ml) isolated from *Mnazi* in response to simulated stomach duodenum passage, following 3hr exposure at 37°C.

Strain Code	No.	0h	1h	2h	3h
***L. paracasei* TB402**	**8**	10.6	5.56 (52)	5.66 (53)	5.7 (54)
***L. paracasei* CM203**	**4**	11.3	4.53 (40)	6.23 (55)	6.34 (56)
***L. paracasei* CB204**	**15**	11.8	4.72 (40)	5.88 (50)	5.75 (49)
***L. paracasei* TD3051**	**2**	12.1	4.46 (37)	4.95 (41)	5 (41)
***L. plantarum* CM402**	**11**	12.5	5.7 (46)	5.66 (45)	5.6 (45)
***L. paracasei* CM4081**	**13**	11.3	4.3 (38)	4.6 (41)	4.48 (40)
***L. paracasei* CM301**	**3**	11.9	4.28 (36)	4.36 (37)	4.43 (37)
***L. paracasei* TB302**	**6**	12.4	7.48 (60)	6.66 (54)	6.85 (55)
***L. paracasei* TB405**	**7**	11.9	4.85 (41)	4.46 (37)	4.41 (37)
***L. paracasei* CB3021**	**1**	12	7 (58)	6.3 (53)	6.18 (52)
***L. paracasei* CM4091**	**9**	11.4	5.3 (46)	4.79 (42)	4.79 (42)
***L. paracasei* CB303**	**10**	11.9	6.52 (55)	5.3 (45)	5.52 (46)
***L. paracasei* CB4041**	**5**	12.7	5 (39)	4.23 (33)	4.23 (33)
***L. paracasei* TM302**	**12**	9.78	6.81 (70)	5.93 (61)	3.82 (39)
***L. paracasei* CM201**	**14**	12.1	7.36 (61)	6.81 (56)	6.72 (56)

Data represents the mean of duplicate experiments. Figures in brackets represent the survival rate of each strain

3.1.4 *meso*–diaminopimelic acid activity (*meso*–DAP)

Detection of *meso*-DAP acid in the cell wall was demonstrated by appearance of dark green to grey and turning to yellow of thin layer chromatography plates of eleven isolates after they were left in the dark for 24h (Plate 1). The yellow spots developed after 24h represent presence of *meso*-diaminopimelic acid in the cell wall of the LAB isolates (Plate 2).

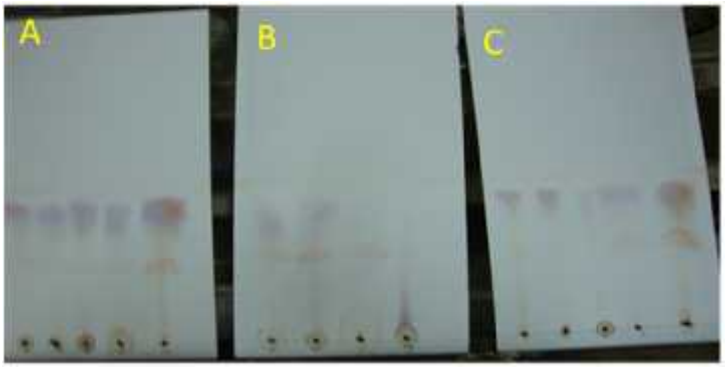

Plate 1: Thin layer chromatography plates showing ninhydrin development pattern of LAB isolates from *Mnazi*

The TLC plates were developed with ninhydrin after drying in 1000°C oven for 5min, the strains spotted on the plates and developed in a modified solvent of Methanol/Pyridine/10NHcl/water in (32:4:1:7) v/v/v/v. The panel consists of: (A) *L. paracasei* (CB3021, TD3051, CM301, CM203, and CB4041); (B) *L. plantarum* (CM402), *L.paracasei* (TM302, CM4081, and CM201), (C) *L.paracasei* (TB302, TB405, TB402, CM4091 and CB303).

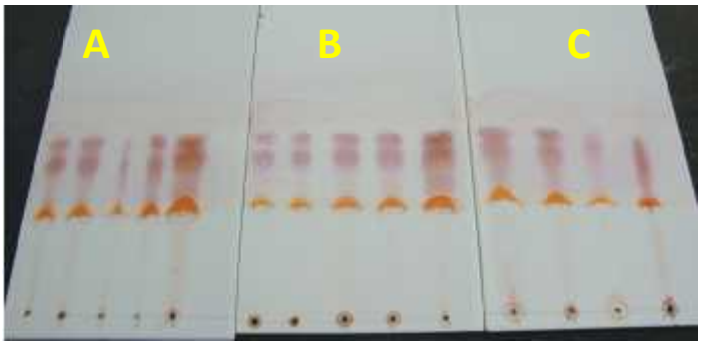

Plate 2: Thin layer chromatography plates showing *meso*-DAP activity of LAB isolates from *Mnazi*

The TLC plates developed with ninhydrin after 24 h incubation in the dark of the strains spotted on the plates and developed in a modified solvent of Methanol/Pyridine/10NHcl/water in (32:4:1:7) v/v/v/v; (A) *L. paracasei* (CB3021 TD3051, CM301, CM203, CB4041); (B) *L. paracasei* (TB302, TB405, TB402, CM4091, CB303) and (C) *L. plantarum* (CM402), *L. paracasei* (TM302, CM4081, CM201).

3.1.5 ß-galactosidase activity

Beta-galactosidase activity was highest in isolates *L. paracasei* (CB4041 and TD3051), moderate in isolates *L. plantarum* (CM402) and *L. paracasei* (CB204, TB302, CM203, TM302, TB402, CM4081 and CM301) and lowest in isolates *L. paracasei* (CB3021, TB405, and CB303). There was no activity of the enzyme observed in isolates *L. paracasei* (CM201 and CM4091) indicating loss of function. These results suggest that majority of the LAB isolates tested showed ß-galactosidase activity and can therefore be important in breakdown of lactose in the upper regions of the small intestine if used as probiotics.

Table 4: ß-galactosidase activity of LAB isolates from *Mnazi*

Strain Code	No.	A_{420}
***L. paracasei* CB204**	**15**	1.127
***L. plantarum* CM402**	**11**	1.161
***L. paracasei* CM301**	**3**	1.026
***L. paracasei* CM203**	**4**	1.065
***L. paracasei* CM201**	**14**	0.919
***L. paracasei* TB302**	**6**	1.1175
***L. paracasei* TD3051**	**2**	1.321
***L. paracasei* CB4041**	**5**	1.439
***L. paracasei* CM4091**	**9**	0.857
***L. paracasei* TM302**	**12**	1.109
***L. paracasei* TB402**	**8**	1.069
***L. paracasei* CB303**	**10**	0.994
***L. paracasei* TB405**	**7**	0.984
***L. paracasei* CB3021**	**1**	0.952
***L. paracasei* CM4081**	**13**	1.042
Blank (Control)		0.942

Data represents the mean of duplicate experiments.

3.1.6 Antimicrobial activity

The LAB isolates from *Mnazi* were tested for antimicrobial activity against indicator organisms (Gram negative; *Escherichia coli*, *Enterococcus feacalis* and Gram positive; *Staphylococcus aureus*, *Bacillus subtilis*). The cell-free supernatants from the different *Lactobacillus* strains inhibited the growth of indicator organisms as shown

by inhibition zone results (Table 5 and Plate 3). Among the test strains isolate *L. paracasei* (CB4041) showed the highest antibacterial activity against *E. coli* (18mm), *B. subtilis* (17mm) and *E. feacalis* (18mm) compared to *L.casei* (20mm) a probiotic bacteria used as a control while the least activity was observed on isolate *L. paracasei* CM301 against *B. subtilis* (12mm).

Four (4) strains *L. paracasei* (CB4041, CM4091, CM4081 TB302 and CM203) exhibited moderate potency against Gram positive bacteria *S. aureus and B. subtilis.* The strains *L. paracasei* (CB4041, TB302, CM203, and CB303) showed higher potency against Gram negative than the Gram positive indicator organisms. A lower activity was detected against *E.coli, B. subtilis, and S. aureus* in *L. paracasei* CB3021, TD3051, CM301, TB402, TM302, CM4081, and CM201) demonstrating low antimicrobial activity against the 4 indicator organisms while strain *L. paracasei* (CM203) showed high resistance to *E. fecalis*. These results suggest that the LABs isolated from *Mnazi* can be potent antimicrobial agents against both Gram positive and negative bacteria.

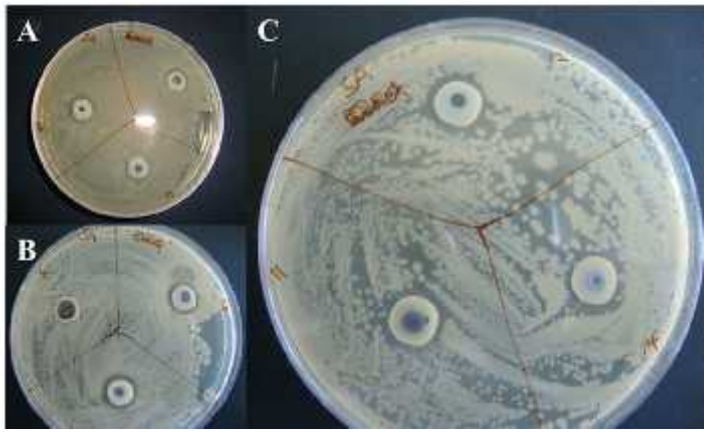

Plate 3: **Antimicrobial activity against *S. aureus* of *Mnazi* LAB isolates 8 (TB402), 9 (CM4091), and 10 (CB303) (panel A); 4 (CM203), 6 (TB302) (panel B); 11 (CM402), 12 (TM302), and 14 (CM201) (panel C)**

Table 5: Antimicrobial effects of the *Mnazi* bacterial strains on against selected indicator common pathogens (baseline=12mm diameter of the well)

Test Strains	Diameter of the inhibition zones (mm)			
	E. coli	***B. subtilis***	***S. aureus***	***E. feacalis***
L. paracasei CB3021	14	13	13	14
L. paracasei TD3051	14	14	14	13
L. paracasei CM301	13	12	13	13
L. paracasei CM203	13	14	14	17
L. paracasei CB4041	18	17	15	18
L. paracasei TB302	15	14	15	16
L. paracasei TB405	13	15	14	14
L. paracasei TB402	16	14	13	13
L. paracasei CM4091	14	20	14	15
L. paracasei CB303	15	14	14	16
L. plantarum CM402	13	14	13	14
L. paracasei TM302	16	13	13	13
L. paracasei CM4081	13	14	15	14
L. paracasei CM201	15	16	13	14
L. casei (Probiotic)/ Control	20	19	18	18

Data represents the mean of duplicate experiments.

3.1.7 Fructooligosaccharide (FOS) utilization

All the *Lactobacillus* isolates from *Mnazi* were positive for FOS utilization. The colour of agar zone around the colonies changed from purple to yellow and of broth from pink to red (Plate 4). The colour change was observed to begin after 24h and became intense after 36h. When glucose was used as the carbohydrate source the colour changed to yellow and red for broth. These results show that all the *Lactobacillus* stains from *mnazi* were able to utilize inulin (FOS) and produce acid.

Plate 4: Fructooligosaccharide utilization assay.

Positive inulin utilization showing yellow zones (A), positive glucose utilization and acid production by the test isolates (red tubes) and blank remained pink (B)

The LAB isolates were tested for their utilization of either inulin or glucose by measuring their population density (OD 600nm) and pH of culture broth. In general, the population density was higher when isolates were grown in inulin than in glucose (mean= 0.38, 0.33 respectively). Strain *L. paracasei* (CB4041) had the highest density with inulin (0.68) while the least was *L. paracasei* (CM203) (0.15). In glucose utilization, strain *L. paracasei* (CB303) had the highest density (1.16) while the least was *L. paracasei* (CM203) (0.12). The pH of culture broth was more acidic for glucose at pH 3.7 than for inulin at pH 5.0 (Table 6).

Table 6: Effect of utilization of inulin and glucose on growth of LAB isolates from *Mnazi*

Strain code		Population density as OD (600nm)		pH of culture broth	
	No.	Inulin	Glucose	Inulin	Glucose
***L. paracasei* CB3021**	**1**	0.19	0.21	5.06	3.73
***L. paracasei* TD3051**	**2**	0.44	0.24	5	3.7
***L. paracasei* CM301**	**3**	0.38	0.15	5.01	3.73
***L. paracasei* CM203**	**4**	0.15	0.12	5.02	3.73
***L. paracasei* CB4041**	**5**	0.68	0.37	5.06	3.71
***L. paracasei* TB302**	**6**	0.17	0.23	5.08	3.73
***L. paracasei* TB405**	**7**	0.2	0.19	5.08	3.7
***L. paracasei* TB402**	**8**	0.66	0.42	4.95	3.7
***L. paracasei* CM4091**	**9**	0.39	0.24	5.01	3.73
***L. paracasei* CB303**	**10**	0.42	1.16	5.03	3.69
***L. plantarum* CM402**	**11**	0.56	0.36	5.07	3.72
***L. paracasei* TM302**	**12**	0.34	0.25	5	3.7
***L. paracasei* CM201**	**14**	0.32	0.38	5.06	3.7

Data represents the mean of duplicate experiments.

3.2 Production of bacteriocins by LAB isolates from *Mnazi*

All the isolates produced bacteriocins except isolates *L. paracasei* (CM4091 and CM4081). They were able to inhibit the growth of indicator organism *Staphylococcus aureus* NCTC 6571 (Plate 5).

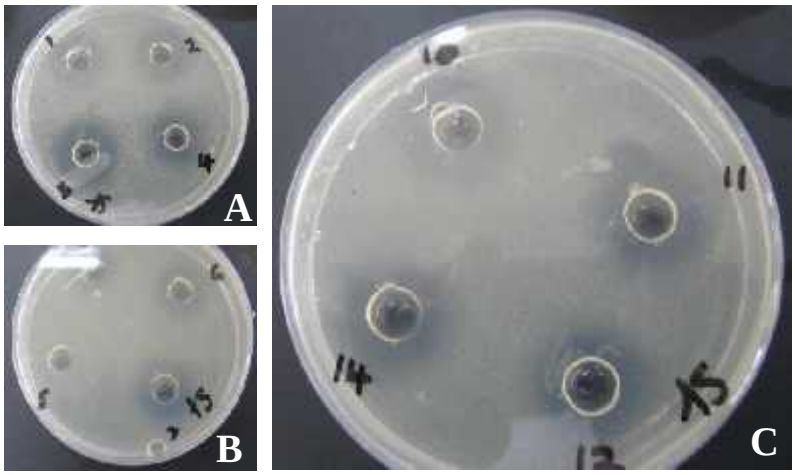

Plate 5: Inhibition of indicator organism *Staphylococcus aureus* NCTC 6571 by bacteriocin produced by LAB isolates from *Mnazi*.

Key: Panel A: *L. paracasei* (CB3021=1, TD3051=2, CM301=3 and CM203=4); B: *L. paracasei* (CB4041=5, TB302=6, TB405=7 and TB402=8); C: *L. paracasei* (CB303=10, TM302=12, CM201=14 and *L. plantarum* CM402=11)

3.2.1 Effect of heat, enzyme and pH on bacteriocins

It was observed that the isolates *L. paracasei* (CB303 and CB4041) tested showed no inhibition (Plate 6A and B). Their activity was inhibited at the temperatures and pH tested. This suggests that activity of the bacteriocins produced by the two isolates is affected both by heat and pH treatment.

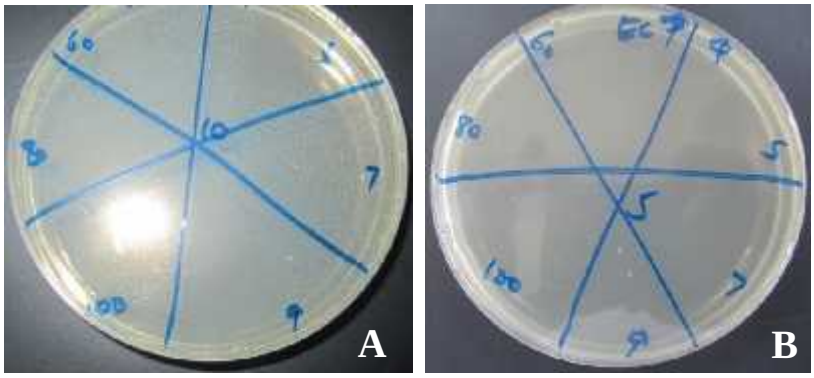

Plate 6: **Effect of heat and pH on activity of bacteriocins produced by selected LAB isolates from *Mnazi***

Key: Panel A: *L. paracasei* (CB303=10); B: *L. paracasei* (CB4041=5); heat treatments (60, 80 and 100°C), pH treatments (pH 5, 7 and 9)

Supernatants from selected LAB isolates from *Mnazi* were assayed to determine their activity against indicator organism *Staphylococcus aureus* NCTC 6571 when treated with the enzymes pepsin, lipase and amylase. It was observed that the supernatants from isolates *L. paracasei* (TB405 and TD3051) were inactivated by the enzymes compared to the control that inhibited growth of indicator organism (Plate 7A and C). This indicates that the two isolates therefore produced bacteriocins that are either protein, lipid or carbohydrate in nature. In contrast, in the experimental set up with isolate *L. paracasei* (TB402), inhibition was observed in wells seeded with supernatant treated with enzymes and no inhibition in the controls (Plate 7B). This suggests that there was activity against the indicator organism that would be attributed to the enzymes themselves and not the supernatant from the isolate.

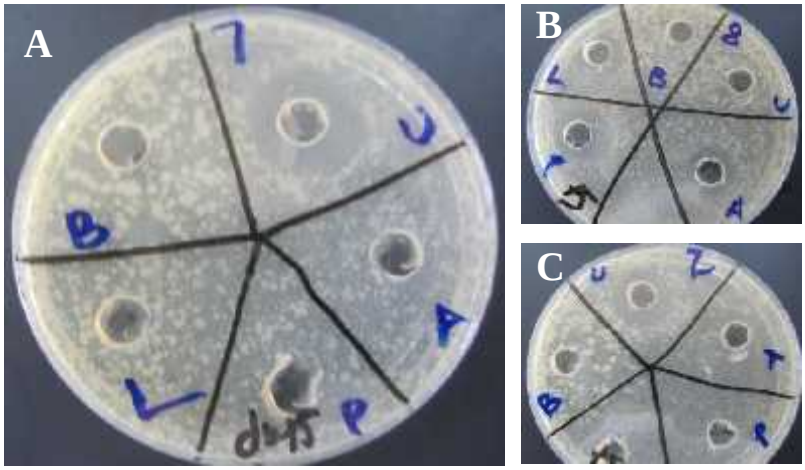

Plate 7: **Effect of enzymes on activity of bacteriocins produced by selected LAB isolates from *Mnazi***

Key: Enzymes (P=pepsin, L=lipase and A=amylase), controls (U=without enzyme, B=un-inoculated broth). Panel A: *L. paracasei* (TB405=7); B: *L. paracasei* (TB402=8); C: *L. paracasei* (TD3051=2)

3.3 Molecular characterization of LAB isolates from *Mnazi*

3.3.1 PCR amplification of 16S rRNA genes from isolates

Amplification of 16S rRNA gene yielded fragments of approximately 1500bp corresponding to the almost full length of 16S rRNA obtained in different *Lactobacilli* strains as expected (Figure 1).

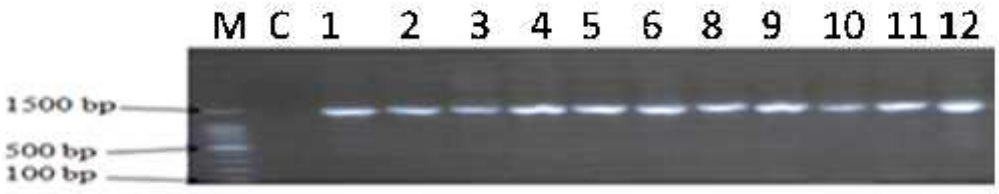

Figure 1: **PCR amplified 16S rRNA gene products of the bacterial isolates using universal primers bac 27F and bac 1492R.**

Lanes 1(CB3021), 2(TD3051), 3(CM301), 4(CM203), 5(CB4041), 6(TB302) 8(TB402), 9(CM4091), 10(CB303), 11(CM402), 12(TM302) *L. paracasei* strains, (C*) negative control, M-1500 bp molecular marker size

3.3.2 Phylogenetic analysis of sequences of LAB isolates from *Mnazi*

Sequence similarity search using the sequences of LAB isolates from *Mnazi* revealed significant similarity (97-99%) with the 16SrRNA gene sequences of known *Lactobacillus* species (Table 7). The *Lactobacillus* strains identified were; *L. paracasei*, *L. casei*, *L. rhamnosus*, *L. zeae* and *L. plantarum*.

To determine phylogenetic relationship of the LAB isolates from *Mnazi*, molecular phylogeny analysis was conducted and a phylogenetic tree was constructed (Figure. 2). The tree revealed that the isolates from *Mnazi* clustered together and were far removed from the other *Lactobacillus* sequences. This suggests that despite showing high percentage similarity to the *Lactobacillus* strains *zeae*, *rhamnosus*, *casei* and *paracasei*, their evolutionary distance from these is great. Hence they do not cluster together. These findings concur with those of identification by the API 50 CHL system that classified the isolates from *Mnazi* to be predominantly of *L. paracasei spp* with one of them belonging to *L. planturum spp*.

Table 7: *Lactobacillus* strains identified 16S rRNA gene sequences of LAB isolates from *Mnazi*

Query	Next Neighbour	Accession No.	E value	Identity
Seq 1(844bp)	*Lactobacillus paracasei* strain KLDS1.0655	FJ607297.1	0	99%
	Lactobacillus sp. SCA34	AB602936.1	0	99%
	Lactobacillus casei strain NM60-1	HM218266.1	0	99%
Seq 2 (1234bp)	*Lactobacillus casei* strain LvK2	KM350168.1	0	98%
	Lactobacillus zeae strain LB	KM350162.1	0	98%
	Lactobacillus paracasei strain IMAU32644	KF149312.1	0	98%
Seq 3 (1029bp)	*Lactobacillus paracasei* strain 37131	KC755091.1	0	99%
	Lactobacillus casei strain LvK2	KM350168.1	0	99%
	Lactobacillus zeae strain LB	KM350162.1	0	99%
Seq 4 (1243bp)	*Lactobacillus casei* strain NM58-3	HM218255.1	0	97%
Seq 5 (1160bp)	*Lactobacillus casei*	AB969779.1	0	97%
	Lactobacillus paracasei subsp. tolerans	JN415190.1	0	97%
Seq 6 (600bp)	*Lactobacillus paracasei* strain E10-20	KF030777.1	0	99%
	Lactobacillus casei strain NM30-1	HM218157.1	0	99%
	Lactobacillus rhamnosus strain LS8	KJ152776.1	0	99%
Seq 8 (833bp)	*Lactobacillus paracasei*	AB795644.1	0	99%
	Lactobacillus casei strain JNLAB-2	KC336483.1	0	99%
	Lactobacillus rhamnosus	JQ621982.1	0	99%
Seq 9 (801bp)	*Lactobacillus rhamnosus* strain Z5	KM350174.1	0	99%
	Lactobacillus casei strain LvK2	KM350168.1	0	99%
	Lactobacillus zeae strain LB	KM350162.1	0	99%
Seq 10 (681bp)	*Lactobacillus rhamnosus* strain Z5	KM350174.1	0	99%
	Lactobacillus casei strain LvK2	KM350168.1	0	99%
	Lactobacillus zeae strain LB	KM350162.1	0	99%
Seq 11 (843bp)	*Lactobacillus plantarum* strain C88	GU290217.1	0	99%
Seq 12 (1168bp)	*Lactobacillus casei*	AB969779.1	0	97%
	Lactobacillus casei strain SK04B5	KJ764643.1	0	97%

Source (GenBank release 205, Dec 2014 at NCBI). **Key**: Identity (Percentage similarity of query and subject), E-value (Expectation value),

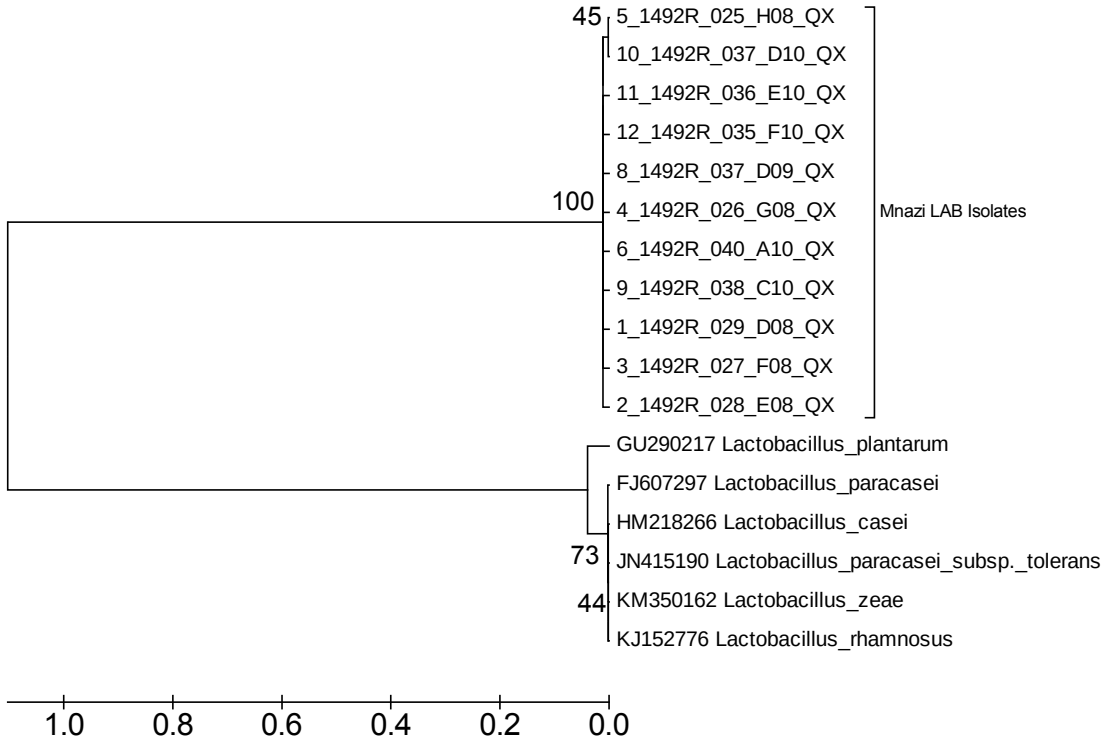

Figure 2: Phylogenetic relationship of LAB isolates from *Mnazi* and other homologous *Lactobacillus* species

The evolutionary history of 17 nucleotide sequences was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were edited.

3.4 Safety attributes of LAB isolates from *Mnazi*

The isolates from *Mnazi* were tested for haemolytic and gelatinase activity to determine their safety attributes. None of the isolates showed haemolytic or gelatinase activity.

CHAPTER FOUR

4.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 Discussion

The LAB isolates were tested for tolerance to acid at pH 2 and 2.5. All the tested isolates had residual microbial counts greater than 10^7 CFU/ml after 3h of incubation under pH 2.5. Majority of them had a survival rate of 77-90% suggesting they are able to tolerate well stomach conditions. However, only 8 strains (*L. paracasei* TB402, CM203, CM301, TB302, CM4091, CB303, CB4041 and CM201) were able to survive at pH 2 with viable counts of between 10^3-10^6 CFU/ml, the rest had a reduction in their count for the first 1-2h but had died by the 3h period. These results indicate that in general the strains were stable at pH 2.5 but could not be able to grow. They agree with those of Wang *et al.*, (2010), who found that *Lactobacilli* strains remained viable after exposure to pH of 2.5-4.0. This can be attributed to the ability of *Lactobacilli* strains to withstand stressful conditions and survive for longer periods in highly acidic environments. It is desirable that probiotic microorganisms are able to reach the GIT and remain viable there for 4 hours or more. A similar study by Lei and Jakobsen (2004) reported stability but no growth of strains of *Lactobacillus* isolated from *koko* (a fermented millet porridge) where the isolates were capable of surviving at a level of 10^5 CFU/ml.

When tested for tolerance to 0.3% bile, the LAB isolates survived better when grown at pH 2.5 than pH 2. At pH 2.5, thirteen (13) of the 15 isolates showed viable counts of 10^6-10^7 CFU/ml, only *L. paracasei* (CB303 and CB4041) tolerated growth at pH 2 and exposure to bile. In fact, their viable counts increased by 165% and 190% respectively after the 48h period showing they could tolerate and grow well at 0.3% bile salt. On the other hand, the isolates *L. paracasei* (TB402, TD3051 and CM201) from *Mnazi* were able to tolerate conditions of 0.3% bile and pH 2.5 to grow exponentially.

These findings agree with those from similar studies where *Lactobacilli* strains were viable even after being exposed to bile ranges of 0.3-0.5% but showed diminished viability at higher bile concentrations (Wang *et al.,* 2010; Pan *et al.,* 2009; Alp & Aslim, 2010). Tolerance of LABs to bile exposure is important since for them to act as probiotics they need to survive its effect in the human GIT (Begley *et al.,* 2006). In other studies, *L. plantarum* strains from cheeses displayed good resistance to bile salts which is similar to the current study (Zago *et al.,* 2011; Pisano *et al.,* 2008).

The LAB isolates from *Mnazi* were tested under conditions of simulated stomach duodenum passage to determine their survival. All the isolates showed survival rates of 33-55% after 3h despite the high bile concentration of more than 2%. The strains *L. paracasei* (TB402, CM203, TB302, CB3021 and CM201) had survival rates of more than 50% while the rest had survival rates of less than 50%. This suggests that they can tolerate the conditions of the stomach and can therefore be potent probiotics. Survival at pH 3.0 is significant as ingestion with food raises the pH in stomach to 3.0 or higher (Martini *et al.* 1987).

In the current study the antibacterial activity of LAB isolates from *Mnazi* was determined. The results indicate that the isolates were potent against both Gram positive and negative bacteria used as indicator organisms. The inhibition activity of these isolates can be attributed to their production of bio-substances with bactericidal or bacteriostatic activities, such as bacteriocin, organic acids, and low molecular weight peptides that are inhibitory to the pathogens (Savadogo *et al.,* 2004; Lefteris *et al.,* 2006). The Gram positive pathogenic bacteria were the most sensitive to the bacteriocin produced by the lactic acid bacteria. The resistance of Gram negative bacteria can be attributed to the particular nature of their cellular envelop.

Several studies have documented the use of LAB as natural bio-protective agents due to their effectiveness in treating various food borne pathogens, especially *Staphylococcus aureus, E. coli,* and *Listeria monocytogenes*. (Vesterlund *et al*., 2005; Vinderola & Reinheimer, 2003). Further, Mathara *et al.,* (2008) working on *Lactobacillus spp.* showed that they were effective in eliminating pathogenic bacteria in fermented dairy products. This suggests that the LAB isolates from *Mnazi* can be

potent antimicrobial agents against Gram positive and Gram negative bacteria which possess strongly defended structure of cell membrane impermeable to several antimicrobial agents resulting to less sensitive to many drugs.

The LAB isolates from *Mnazi* were tested for ß-galactosidase activity. The results showed that ß-galactosidase activity was present in all but two of the isolates tested. Majority had moderate activity with two isolates *L. paracasei* (CB4041 and TD3051) showing the highest activity and two isolates *L. paracasei* (CM201 and CM4091) showing no activity. Beta-galactosidase activity is an essential feature in probiotic strains. Its deficiency (lactose intolerance) is linked to the inability to breakdown lactose in the duodenum thereby making it available for use by indigenous microbiota (Moser & Savage, 2001). The very high ß-galactosidase activity detected in the two isolates of LAB might be used as a dietary adjunct to moderate lactose intolerance in the gut (Belicova *et al.,* 2013).

All the LAB isolates from *Mnazi* tested positive for fructooligosaccharide (FOS) utilization. They were able to use either inulin or glucose as evident in the formation of yellow zones on agar and acid production in broth. The population density of the isolates when grown in inulin was higher compared to glucose. The pH of broth was more acidic (pH 3.7) when glucose was used as substrate compared to inulin (pH 5). These findings are similar to those of Kalui *et al.,* (2008) who tested two strains of *L. plantarum* isolated from *ikii* (fermented porridge). This attribute is important when selecting probiotics (Kaplan & Hutkins, 2000; Bengmark, 2000; Desai *et al.,* 2004) since they can utilize carbohydrates found in the colon thereby influencing the microbiota. These carbohydrates usually end up in the colon when they escape absorption in the small intestine. They have the potential of being prebiotics that can be selective for probiotic organisms present in the colon.

Since the LAB isolates could utilize FOS they would therefore be beneficial in the colon. Studies have shown that probiotics may modulate several major intestinal functions potentially associated with the development of colon cancer like preventing the growth of deleterious organisms, producing anti-carcinogenic substances and

moving the balance of gut bacteria in favour of the ones beneficial for the organism (Ianniti & Palmieri, 2010).

The LAB isolates from *Mnazi* were assayed for production of bacteriocins and thereafter the effect of heat, enzymes and pH on the bacteriocins. All the isolates produced bacteriocins except two. When subjected to different pH (5, 7 and 9) and heat (60, 80 and 100°C) treatments, the isolates *L. paracasei* (CB303 and CB4041) tested showed no inhibition showing that their activity was inhibited. This suggests that activity of the bacteriocins produced by the two isolates was affected both by heat and pH treatment. When treated with the enzymes pepsin, lipase and amylase, the supernatants from isolates *L. paracasei* (TB405 and TD3051) were inactivated by the enzymes compared to the control. This suggests that the bateriocins produced by the isolates were not only proteinaceous in nature but also had active lipid and carbohydrate compounds.

Studies have shown that LABs display a wide range of antimicrobial activities. Amongst these activities, the production of lactic acid and acetic acid is the most important. However, certain strains of LAB are further known to produce bioactive molecules such as ethanol, formic acid, fatty acids, hydrogen peroxide, diacetyl, reuterin, and reutericyclin. Many strains also produce bacteriocins and bacteriocin-like molecules that display antibacterial activity (De Vuyst & Vandamme, 1994).

Besides the production of bacteriocins, some LAB are able to synthesize other antimicrobial peptides that may also contribute to food preservation and safety (De Vuyst & Leroy, 2007). In a study by Mensah (1990), he reported the presence of an antimicrobial substance other than organic acids produced by isolates from fermented maize dough that was optimally active at a pH of 3.

Sequence similarity search using the sequences of LAB isolates from *Mnazi* revealed significant similarity (97-99%) with the 16SrRNA gene sequences of known *Lactobacillus* species: *L. paracasei*, *L. casei*, *L. rhamnosus*, *L. zeae* and *L. plantarum*. *Lactobacillus rhamnosus* is a bacterium that was originally considered to be a subspecies of *L. casei*. It is sometimes used in yogurt and dairy products such as

fermented and pasteurized milk and semi-hard cheese. While frequently considered a beneficial organism, *L. rhamnosus* has been discovered to be pathogenic in rare circumstances, primarily involving those with a weakened immune system or infants (Avlami *et al.,* 2001). In particular, *Lactobacillus rhamnosus* GG (ATCC 53103) is able to survive the acidic and bile conditions of the stomach and intestine (Conway *et al.,* 1987) thereby colonizing the digestive tract and balances intestinal microflora.

Lactobacillus paracasei is a Gram-positive, facultatively heterofermentative, non-spore forming and non-motile microorganism. It exist as a common inhabitant of the human gastrointestinal tract as part of the normal flora. Naturally fermented vegetables, milk, and meat may also contain strains of *L. paracasei* (Hessle *et al.,* 1999, Rogan *et al.,* 1988). Its cells are typically rod shaped, ranging from 2.0µm to 4.0µm in width, and 0.8 to 1.0µm in length (Collins *et al.,* 1989). *Lactobacillus casei* is a species of genus *Lactobacillus* found in the human intestine and mouth. It is considered a probiotic safe for consumption and is applied industrially for dairy production. *L. casei* is typically the dominant species of nonstarter lactic acid bacteria present in ripening cheddar cheese, and, in naturally fermented Sicilian green olives (Randazzo *et al.,* 2004).

The phylogenetic relationship revealed that the isolates from *Mnazi* clustered together and were far removed from the other *Lactobacillus* sequences. This suggests that despite showing high percentage similarity to the *Lactobacillus* strains *zeae, rhamnosus, casei* and *paracasei,* their evolutionary distance from these is great.

Following testing of the LAB isolates for safety, none of them showed haemolytic or gelatinase activity. This suggests that the LAB isolates are safe to use as probiotics since they would not interfere with the normal functioning of GIT by breaking down the epithelial layer and deranging the mucoid lining. These linings are important for the exchange of substances and their destruction would cause pathways for infections (Bengmark, 2003). It is therefore desirable that a probiotic strain should not have haemolytic and gelatinase activity.

According to FAO/WHO Guidelines on evaluation of probiotics, absence of haemolytic or gelatinase activity is one of the recommended attributes for probiotics (FAO/WHO, 2002). These results concur with those of Kalui *et al.,* (2009) who tested *L. plantarum* and *L. rhamnosus* strains isolated from *ikii* (Kenyan traditional fermented maize porridge) and found them to be haemolytic and gelatinase negative. Further, isolates tested for gelatinase activity produced a mucoid substance around colonies that would be beneficial in enhancing the mucosal lining of the GIT. (Kalui *et al.,* 2009)

4.2 Conclusions

The results of this study show that the LAB isolates from *Mnazi* have probiotic potential, produce stable antimicrobial substances and bacteriocins. In summary, the study:

i. Has characterized the probiotic properties of LAB isolates from *Mnazi*. The isolates can tolerate low acid levels of pH 2 and 2.5, bile of up to 2% and can survive under conditions of simulated Stomach Duodenum Passage (SSDP). Apart from being able to utilize fructooligosaccharides, they have *meso*-diaminopimelic acid and β-galactosidase activity.

ii. Has shown that LAB isolates from *Mnazi* produce antimicrobials in form of bacteriocins which cause their antimicrobial activity against both Gram positive and negative bacteria. The activity of the bacteriocins produced was affected by heat, pH and enzymes

iii. Has shown that the LAB isolates from *Mnazi* are evolutionary distant from their homologous *Lactobacillus* spp. Despite this, they clustered closer to *L. zeae, L. paracasei, L. casei* and *L. rhamnosus.*

iv. Has demonstrated that the LAB isolates from *Mnazi* don't show any haemolytic or gelatinase activity.

4.3 Recommendations

Based on this study it is recommended that:

i. It would be necessary for further assays to be done for the *Lactobacillus* isolates from *Mnazi* in vivo for safety and viability.

ii. The *Lactobacillus* isolates from *Mnazi* should be assayed whether they can be able to utilize nutrients and substrates in normal diet in the gastrointestinal tract.

iii. The crude bacteriocin of the *Lactobacillus* isolates should be identified.

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APPENDICES

Appendix 1: Publication